THE EFFECT OF SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN
ON THE CONCENTRATION OF SERUM CHOLESTEROL AND THE LIPOPROTEIN COMPOSITION
IN RABBITS

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STELLINGEN

1. Bij studies in proefdieren is tot nu toe onvoldoende rekening gehouden met het feit, dat de dichtheidsgrenzen van de verschillende serumlipoproteinen-klassen zoals die bij de mens worden gehanteerd, niet zonder meer toepasbaar zijn op de serumlipoproteinen van de verschillende diersoorten.

    Dit proefschrift.

2. Een indeling van de high density serumlipoproteinen van de mens in twee sub-frakties met als dichtsheidsgrenzen 1.063 < d < 1.10 en 1.10 < d < 1.21 g/ml lijkt juister dan een indeling in 3 subfrakties zoals beschreven door Anderson *et al.*

    Dit proefschrift.

    Anderson, D.W.; Nichols, A.V.; Forte, T.M., and Lindgren, F.T.: Particle distribution of human serum high density lipoproteins. Biochim. Biophys. Acta 493: 55-68 (1977).

3. In de literatuur wordt te weinig nadruk gelegd op het feit dat het verschil in effect van caseine en soja eiwit in het rantsoen op het serumcholesterol-gehalte bij proefdieren, met uitzondering van het konijn, pas duidelijk naar voren komt als atherogene, cholesterolrijke rantsoenen worden gebruikt.

4. "There is much left to be clarified in the area of diet and its effect on serum lipids and atherosclerosis. Research findings to date should be used as clues to further inquiry rather than as the basis for dietary dicta".

    David Kritchevsky, in: Nutrition, Lipids and Coronary Heart Disease, blz. 240, Raven Press, New York (1979).

5. Het behandelen van hyperlipoproteinemie door middel van een dieet is te prefereren boven het toedienen van medicijnen.

    Descovich, G.C.; Gaddi, A.; Mannino, G.; Cattin, L.; Senin, U.; Caruzzo, C.; Fragiacomo, C.; Sirtori, M.; Ceredi, C.; Benassi, M.S.; Colombo, L.; Fontana, G.; Mannarino, E.; Bertelli, E.; Noseda, G., and Sirtori, C.R.: Multicentre study of soybean protein diet for outpatient hypercholeste-rolaemic patients. Lancet ii: 709-712 (1980).

6. Bij het behandelen van hyperlipemische patienten m.b.v. een dieet zou meer
aandacht moeten worden geschonken aan voedingseiwitten die betere sensorische
eigenschappen bezitten dan soja eiwit en bij dierproeven eveneens een serum-
cholesterol verlagend effect te zien geven.

Descovich, G.C.; Gaddi, A.; Mannino, G.; Cattin, L.; Senin, U.; Caruzzo,
C.; Fragiacomo, C.; Sirtori, M.; Ceredi, C.; Benassi, M.S.; Colombo, L.;
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Multicentre study of soybean protein diet for outpatient hypercholeste-
rolaemic patients. Lancet ii: 709-712 (1980).

Carroll, K.K., and Hamilton, R.M.G.: Effects of dietary protein and
carbohydrate on plasma cholesterol levels in relation to atherosclerosis.
J. Food Sci. 40: 18-23 (1975).

7. Belangrijke vindingen worden vaak eerder door toeval gedaan dan door systema-
tisch onderzoek.

8. Onder het motto: "Quot linguas quis callet, tot homines valet" (zoveel malen
is men man, als men talen spreken kan), moet het terug brengen van het aantal
verplichte talen op de middelbare school als een verarming van het onderwijs
worden beschouwd.

9. Een promotie-assistent zou bij zijn of haar aanstelling als welkomstgeschenk
een exemplaar moeten worden aangeboden van het boek: "How to write and to
publish a scientific paper".

Day, R.A.: How to write and to publish a scientific paper.
ISI Press, Philadelphia (1979).

10. Het poneren van stellingen bij een proefschrift is eigenlijk in strijd met
het dialektisch denken, dat een wetenschapper eigen zou moeten zijn.

Foar Heit en Mem

CONTENTS

VOORWOORD

Het onderzoek, dat in dit proefschrift is beschreven, werd uitgevoerd op de Vakgroep Humane Voeding van de Landbouwhogeschool in het kader van een promotie assistentschap. Bij het tot stand komen van het proefschrift hebben vele mensen mij terzijde gestaan.

Op de eerste plaats zou ik mijn promotor, Prof.dr. J.G.A.J. Hautvast willen bedanken dat hij mij de mogelijkheid heeft gegeven dit onderzoek uit te voeren. Verder ben ik dank verschuldigd aan Ruud Hermus en Martijn Katan voor het opstellen van het onderzoeksprotocol en voor hun vele ideeën. Erkentelijk ben ik eveneens Clive West voor zijn hulp bij het schrijven van dit proefschrift.

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Verder gaat mijn dank uit naar de medewerkers van het ILOB (instituut voor landbouwkundig onderzoek van biochemische produkten), Ben Schutte en Kaspar Deuring voor het huisvesten van de konijnen en Pieter Roeleveld en Mient Bouius voor het samenstellen van de rantsoenen en naar Jo Haas en Willem Capelleveen van het centrum kleine proefdieren voor het verzorgen van de konijnen aldaar.

Ook zou ik Riet Hoogkamer willen bedanken voor het typen van het proefschrift en Alfred van Baaren voor het fotografisch werk.

Tenslotte ook mijn dank aan iedereen van het laboratorium, met name Frans Schouten en alle anderen die mij tijdens het onderzoek met raad en daad hebben bijgestaan.

En last but not least wil ik in het bijzonder Ypie bedanken voor de morele steun.

INTRODUCTION

The role of diet and nutrition in the etiology of hypercholesterolaemia and atherosclerosis was recognized as far back as 1909 by Ignatowski. Serendipitously, he found that the feeding of animal products to rabbits resulted in arterial lesions similar to those found in humans. Although he ascribed the deleterious effect to the protein source in the diet, the focus of attention was mainly directed on dietary cholesterol in further studies by other investigators. However more recently, the role of dietary protein in the etiology of hypercholesterolaemia and atherosclerosis has again become a subject of investigation.

In the present study the effect of dietary protein on the serum cholesterol concentration and lipoprotein composition has been studied in an experimental animal, the rabbit. Since the rabbit is highly susceptible to the induction of hypercholesterolaemia and atherosclerosis, this animal can be considered as very suitable for these studies.

In all the experiments described, use was made of two different types of dietary protein, i.e. casein and soybean protein. These proteins have been chosen, since they vary largely in their cholesterolaemic effect. Rabbits fed a diet containing casein very soon develop hypercholesterolaemia and atherosclerosis, whereas rabbits on a diet containing soybean protein do not.

The effect of dietary protein on serum cholesterol concentrations and the genesis of atherosclerosis has been extensively studied both in man and experimental animals. In the following chapter these studies will be summarized and discussed.

Before starting the experiments on dietary protein, a method was developed for the separation of serum lipoproteins, the carriers of serum cholesterol. With this technique, alterations in serum lipoproteins due to dietary protein could be easily studied.

Subsequently, the time course of changes in serum cholesterol concentration and lipoprotein composition when rabbits were transferred from a commercial diet to a semipurified diet containing either casein or soybean protein was studied. This was followed by an investigation into whether the hypercholesterolaemic effect of casein could be enhanced by the incorporation of a higher proportion of this protein in the diet. Finally the progression and regression

of hypercholesterolaemia was studied when rabbits fed a diet containing soybean protein were transferred to a casein diet and *vice versa*. In order to accentuate the effect, high protein diets were used in this final study.

1. THE ROLE OF DIETARY PROTEIN IN CHOLESTEROL METABOLISM

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CONTENTS

I INTRODUCTION

The impact of diet and nutrition on the etiology of hypercholesterolaemia and atherosclerosis has been well established (114). Since it was observed in the first decade of this century that atherosclerosis could be produced by dietary means, the effect of various food components on serum cholesterol concentration and atherosclerosis has been studied. Initially the focus of attention was mainly directed towards dietary cholesterol; later to dietary fat, carbohydrate and fibre and more recently dietary protein has become the subject of investigation. In this review studies on dietary protein will be scrutinized. The effect of both the nature and the amount of dietary protein on cholesterol metabolism and atherogenesis in man as well as in experimental animals will be discussed.

II HISTORICAL BACKGROUND

In order to study atherosclerosis and to elucidate the etiology of this disease, much effort has been directed towards the production of arterial lesions in experimental animals. Various methods were tried, such as mechanical means, injury of nerves or injection with alcohol, lead or epinephrine. However, the arterial lesions produced by all these methods were more akin to the Mönckeberg type of arteriosclerosis characterized by necrosis and calcification of the media (133) rather than to the type of human atherosclerosis recognized as a pathogenic accumulation of material consisting mainly of lipid in the intima (126) (atherosclerosis is derived from the Greek athere meaning gruel or mush). Therefore, Saltykow (170) concluded in his review of the literature in 1908 that all attempts up to that time to produce arterial lesions experimentally in animals resembling those in humans were unsuccessful.

A great breakthrough was achieved by Ignatowski (82) who studied the effect of dietary protein derived from animal sources on the structure and function of parenchymous organs in rabbits. He discovered fortuitously that rabbits fed meat, milk and eggs developed arterial lesions; the resemblance of these lesions to human atherosclerosis was immediately recognized. Great interest was aroused by the findings of Ignatowski (82) since now on experimental model for studying human atherosclerosis was provided. Furthermore, he had demonstrated that atherosclerosis could be produced by dietary means.

The changes in the vessels were initially ascribed to the injurious effects of animal protein (82). However, Stuckey (191) tested different food-

stuffs of animal origin and found that the feeding of egg yolk to rabbits resulted in atheromatous changes in the intima, whereas other animal products, such as milk, egg white and meat juice had no effect. These findings together with the observation that feeding egg yolk resulted in deposition of large amounts of fatty substances in the liver and aorta, prompted the hypothesis that the lipid in the egg yolk was responsible and that the protein in the diet had little to do with atherosclerosis. Therefore, feeding trials were carried out using rabbits fed various types of fat, such as tallow, cod-liver oil, sunflower oil and ox brain (190). Only ox brain produced atheromatous changes in the aorta identical to those in rabbits fed egg yolk. Therefore, neutral fat was no longer considered to be an atherogenic agent. The livers of the rabbits used in Stuckey's experiment were studied by Chalatow (23) and microscopic examination of the livers of rabbits fed egg yolk revealed the presence of small birefringent crystals in the form of needles and plates. However, the identification of these crystals was rather difficult and Chalatow suggested that they were composed mainly of lecithin contaminated with cholesterol. Since egg yolk contains large amounts of lecithin, Wesselkin (203) proposed that this lipid might be the atherogenic factor, but feeding lecithin to rabbits produced negative results. Further studies on rabbits fed egg yolk by Wesselkin (203) provided evidence that the fatty substance deposited in the liver and aorta contained mainly cholesteryl esters. This prompted the idea that in the diet the cholesterol from the egg yolk was the culprit. This early work was rounded off by Anitschkow and Chalatow (3) and Wacker and Hueck (200) who demonstrated independently that feeding to rabbits crystalline cholesterol dissolved in sunflower oil resulted in similar arterial lesions as did the feeding of egg yolk.

Therefore, the view of Ignatowski (82) that the arterial lesions in rabbits fed animal products could be attributed to animal protein, was largely abandoned. From then on the inclusion of cholesterol in the diet was generally considered as a prerequisite for the production of experimental atherosclerosis in rabbits. Duff (36,37) stated in 1935 in his comprehensive review of the literature on experimental atherosclerosis that no data were available to show that a sterol-free diet could produce arterial lesions similar to those found in experimental arteriosclerosis produced by including cholesterol in the diet. This view has been expressed more recently by Armstrong (5) who wrote in 1976 that dietary cholesterol is a *sine qua non* for the induction of atherosclerosis in experimental animals.

Nevertheless, a number of investigators over the years have still adhered to the idea that dietary protein might play an important role in the etiology

of atherosclerosis. Newburgh (141) observed in 1919 that high casein diets were
able to produce atherosclerosis whereas the feeding of soybeans could not.
Similar results were reported by Meeker and Kesten (129) several years later
and more recently it has been clearly demonstrated that in rabbits hyperchole-
sterolaemia can be produced by feeding cholesterol-free semipurified diets
containing casein (106, 205). The effect of both the nature and the amount of
dietary protein on cholesterol metabolism has been studied extensively in man
and various experimental animal species and this will be the subject of this
article.

III   STUDIES IN EXPERIMENTAL ANIMALS

A. The Role of the Nature of Dietary Protein

1. Rabbits

     As mentioned above, the role of dietary protein in the etiology of athero-
sclerosis was recognized as far back as 1909 by Ignatowski (82). Serendipitous-
ly, he found that rabbits fed animal protein very soon developed arterial
lesions. Later, Newburgh (141,143) observed while studying Bright's disease,
that rabbits fed diets high in casein (30 g/day), developed arterial lesions,
whereas no such changes were detectable in rabbits consuming soybeans. He
ascribed these changes, as did Ignatowski (82) to the protein in the diet;
furthermore he found that diets containing lean beef muscle were also able to
produce atherosclerosis (142). In addition, Clarkson and Newburgh (27) pointed
out that the amount of cholesterol in the meat was insufficient to produce the
lesions. Unfortunately, the cholesterol in the meat-free control diet was
added as a powder, so it was probably not absorbed from the gut as completely
as the cholesterol in the meat.

     Nuzum and coworkers (148) observed that rabbits fed a high protein diet
containing liver, casein and cod-liver oil developed atherosclerosis; those
upon a diet composed of oats, tomatoes, alfalfa and cod-liver oil also
exhibited arterial lesions, albeit to a lesser extent. However, a diet contain-
ing soybeans instead of oats failed to produce arterial lesions, which
suggests an inhibitory effect of soybeans on atherogenesis. Freyberg (47) also
did not observe atherosclerosis in rabbits fed high protein diets (33-38%)
containing soybeans, gluten flour and alfalfa meal.

     Impressed by the results in these studies, Meeker and Kesten (129) reinvest-
igated the effect in rabbits of diets high in either soybean protein or casein.
When rabbits were fed cholesterol-supplemented diets, soybean protein exhibited

a protective action, while casein tended to increase the degree of athero-
sclerosis and hypercholesterolaemia. Furthermore, high casein diets, essential
ly free of cholesterol, were also able to induce atheromatous lesions indis-
tinguishable from those induced by dietary cholesterol (Table 1).

Table I

INCIDENCE OF AORTIC SCLEROSIS IN RABBITS FED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN, ACCORDING TO Meeker and Kesten (129)[1]

Diet	Dietary cholesterol (mg/day)	Rabbits			Degree of Sclerosis of Aorta[2]		
		Number Used	Number Sclerotic	Percentage Sclerotic	+	++	+++
Basic	none	8	0	0			
Casein	none	12	6	50	4	1	1
Soybean	none	8	0	0			
Basic	60-250	21	15	70	6	7	2
Casein	250	13	10	75		3	7
Soybean	60-250	16	6	35	5	1	

[1] The diets were fed for 6 months.

[2] + , Presence of lesion was doubtful on gross examination, but one became evident on microscopic examination.

++ , One to several small atheromatous plaques were visible on gross examination.

+++, Numerous plaques were present.

The hypercholesterolaemic effects in rabbits of cholesterol-free diets
containing casein as the protein source were confirmed later by Wigand (205)
and Lambert et al. (106), using the semipurified diets described by Wooley
(207) and Thacker (195), respectively. Howard et al. (78) varied the composi-
tion of these diets and found that the replacement of casein by soybean protein
was effective in preventing hypercholesterolaemia.

Much research on the effect of dietary protein on hypercholesterolaemia in
rabbits has been done by Carroll and coworkers. They have provided strong
evidence that the hypercholesterolaemic properties of semipurified diets con-
taining casein were attributable to the protein source. The addition of 25%
casein to a diet consisting of powdered commercial pellets resulted in a mode-
rate hypercholesterolaemia (19,20). Furthermore, they published the results of
a series of feeding trials designed to test the effect of different proteins on
plasma cholesterol levels (22,60). Some protein sources, such as extracted
whole egg, skim milk powder and casein produced rather high concentrations of

17

Table II

CONCENTRATION OF PLASMA CHOLESTEROL IN RABBITS FED SEMIPURIFIED DIETS CONTAINING VARIOUS ANIMAL AND PLANT PROTEINS, ACCORDING TO Carroll and Hamilton (22, 60)[1]

Dietary protein	Number of rabbits	Plasma cholesterol (mg/100 ml, mean ± S.E.M.)	Dietary protein	Number of rabbits	Plasma cholestero (mg/100 ml, mean ± S.E.M.
Animal protein			Plant protein		
Extracted whole egg	4	235 ± 89	Wheat gluten	6	80 ± 21
Skim milk	6	230 ± 40	Peanut protein concentrate	6	80 ± 10
Lactalbumin	5	215 ± 69	Peanut meal	4	75 ± 27
Casein	20	200 ± 22	Soybean protein concentrate	6	25 ± 5
Beef protein concentrate	5	160 ± 60	Soybean protein isolate	6	15 ± 5
Pork protein concentrate	6	110 ± 17			
Raw egg white	6	105 ± 28			

[1] The diets were fed for 28 days.

serum cholesterol, whereas other sources of protein, such as pea, soybean and fababean protein were able to maintain low levels of serum cholesterol (Table II).

The involvement of dietary protein in cholesterol metabolism has been reported also by Hermus (63). In search of an improved semipurified diet for
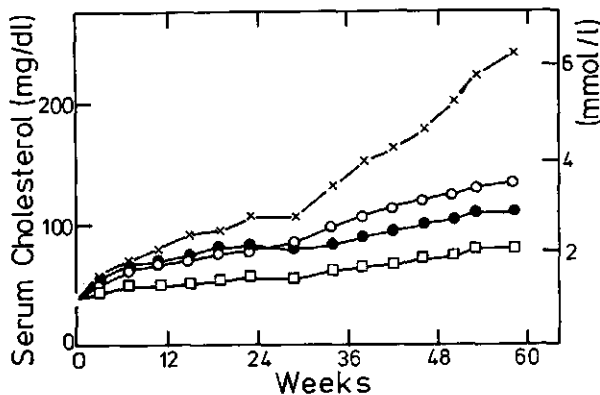

Fig. 1. Concentration of serum cholesterol in rabbits fed semipurified diets containing different proteins.
×————×, 20% casein (6 rabbits); ○————○ , 12% casein + 8% gelatin (8 rabbits); ●————● , 7.5% casein, 7.5% fish protein and 5% gelatin (9 rabbits); □————□ , 6.2% casein, 6.2% fish protein, 4.3% gelatin and 4.1% soybean protein (9 rabbits).
Reproduced from Hermus et al. (63,67).

rabbits, the casein in the diet was replaced by a protein mixture composed of fish protein, casein, gelatin and soybean protein which resembled more or less the amino acid composition of a commercial diet. This mixture turned out to be less hypercholesterolaemic than casein which indicates a role for dietary protein in the genesis of hypercholesterolaemia (Fig. 1).

Similarly, Kritchevsky (102) observed that rabbits fed lactalbumin exhibited levels of serum cholesterol twice as high as animals fed corn protein or wheat gluten; the degree of aortic lesions paralleled the levels of serum cholesterol.

Hypercholesterolaemia, produced in rabbits by feeding semipurified diets containing casein has been found to be accompanied by increased levels of cholesterol in low density and very low density lipoprotein particles (16,165, 193,194,105). The increase in the level of total serum cholesterol is reflected initially in an increased concentration of low density lipoprotein cholesterol. However, when the level of serum cholesterol increases to a greater extent there follows an increase in the level of cholesterol in the very low density lipoproteins (Table III). It is noteworthy that feeding semipurified diets to

Table III

CONCENTRATION OF CHOLESTEROL IN SERUM LIPOPROTEINS OF RABBITS WITH DIFFERENT CONCENTRATIONS OF SERUM CHOLESTEROL PRODUCED BY FEEDING SEMIPURIFIED DIETS CONTAINING DIFFERENT PROPORTIONS OF CASEIN, ACCORDING TO Terpstra *et al.* (193)[1]

	Cholesterol concentration (mg/100 ml, mean ± S.E.M.)			
	initial	semipurified diets		
		10% casein	20% casein	40% casein
VLDL[2]	17.8 ± 3.4	40.7 ± 5.4[x]	119.1 ± 33.7	542.2 ± 197.1[x]
LDL	14.0 ± 2.0	30.9 ± 13.7[xx]	146.8 ± 15.3	210.0 ± 57.4
HDL	18.4 ± 1.2	15.1 ± 2.8[xxx]	60.2 ± 5.5	44.7 ± 11.5
Total	50.2 ± 4.4	86.8 ± 14.6[xx]	326.1 ± 45.3	796.9 ± 235.8[x]

[1] The diets were fed for 28 days; each group comprised 6 animals, the initial values represent the average values of all the 18 rabbits immediately before the change to the semipurified diets. Comparison of the 20% casein group with the 10% and 40% casein group:
[x] $P < 0.05$; [xx] $P < 0.01$; [xxx] $P < 0.001$

[2] VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

rabbits produces marked variations between individual rabbits in the density profile of the lipoproteins in the serum (194).

Much evidence is available that the role of dietary protein in the regulation of cholesterol metabolism in rabbits might be attributable to the amino acid composition of the proteins. Huff *et al.* (79,81) fed rabbits semipurified diets containing an amino acid mixture resembling the composition of either casein or soybean protein. Enzymic hydrolysates of both proteins were also used. The hydrolysates produced slightly lower serum cholesterol values than the intact proteins, but the difference between the two proteins was still present. The amino acid mixture equivalent in amino acid composition to casein produced concentrations of serum cholesterol similar to those obtained with casein, whereas the mixture imitating soybean protein induced somewhat higher levels of serum cholesterol than the intact soybean protein isolate. Addition of amino acids to casein and soybean protein in order to give an amino acid composition equivalent to soybean protein and casein, respectively, revealed that the intact protein component of each mixture had an overriding effect. (Table IV).

Table IV

CONCENTRATION OF SERUM CHOLESTEROL IN RABBITS FED SEMIPURIFIED DIETS CONTAINING VARIOUS SOURCES OF PROTEIN OR AMINO ACID MIXTURES, ACCORDING TO Huff and Carroll (79)[1]

Protein and amino acid source	Amino acid composition equivalent to:	Number of animals	Plasma cholesterol (mg/100 ml, mean ± S.E.M.)
Casein	Casein	6	221 ± 38
Amino acids	Casein	9	213 ± 42
Soybean protein (46%) + amino acids (54%)	Casein	6	94 ± 23
Soybean protein	Soybean protein	6	68 ± 14
Amino acids	Soybean protein	10	124 ± 30
Casein (53%) + amino acids (47%)	Soybean protein		185 ± 50
Sunflower protein	Sunflower protein	6	53 ± 12
Amino acids	Sunflower protein	6	140 ± 28
Egg yolk protein	Egg yolk protein	5	286 ± 35
Amino acids	Egg yolk protein	6	225 ± 39

[1] The diets were fed for 28 days.

Similar results were reported by Hermus *et al.* (66,67).In these studies a number of diets were prepared. In one diet casein was supplemented with amino acids to give an amino acid composition identical to a protein mixture composed of casein, gelatin and fish protein. In another diet casein was supplemented with amino acids resembling casein to provide similar experimental conditions. The imitation proteins induced higher levels of serum cholesterol than did the intact proteins. Nevertheless, the diet containing protein resembling casein gave higher serum cholesterol levels than did the diet resembling the protein mixture (Fig. 2).
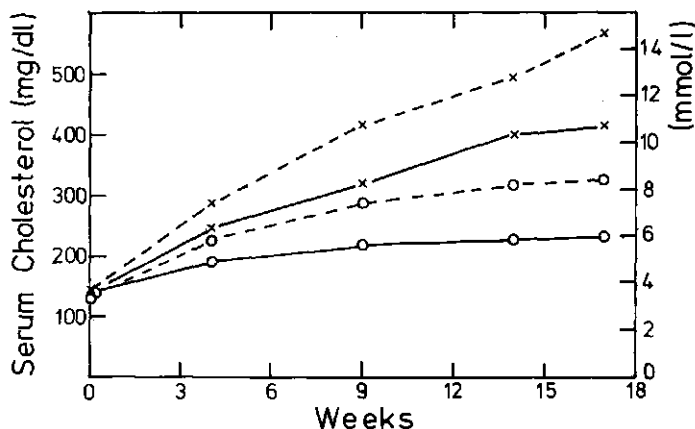
Fig. 2. Concentration of serum cholesterol in rabbits fed semipurified diets containing different proteins and amino acid supplementations. x———x , 20% casein (22 rabbits); ○———○ , 7.5% casein, 7.5% fish protein and 5% gelatin (14 rabbits);○-----○ , 14% casein and 6% amino acids to give the diet an amino acid composition identical to the diet containing 7.5% casein, 7.5% fish protein and 5% gelatin (14 rabbits); x------x , 14% casein + 6% amino acids to give the diet an amino acid composition identical to the 20% casein diet (14 rabbits). Reproduced from Hermus et al. (66,67).

Hermus (63) indicated that the amino acids present in a semipurified diet containing 20% casein, meet or exceed the tentative requirements of the rabbit. Therefore, the hypercholesterolaemic action of dietary casein might be ascribed to an imbalance of amino acids rather than to an absolute deficiency of some particular amino acids.

From the studies of Hermus *et al.* (66,67) and Huff *et al.* (79,81) it can be concluded that the amino acid composition of the protein under investigation plays an important role in the regulation of serum cholesterol levels. However,

the observed effects can not be entirely ascribed to the amino acid composition. Therefore, it is possible that other factors such as the rate and extent of digestion of the proteins and the sequence of absorption of the amino acids may also be determinants in the etiology of hypercholesterolaemia (79). Furthermore, it has been suggested that some secondary components present in plant protein preparations might play an important role in maintaining low levels of serum cholesterol; for example, saponins commonly found in plant products, have been suggested to exert a hypocholesterolaemic action (149,158). On the other hand, evidence has been provided that the hypocholesterolaemic effect of soybean protein can not be readily explained by the saponin content (155).

Evaluating these studies in rabbits, it should be taken into account that all the experiments have been done in young, growing animals. As far back as 1909, Ignatowski (82) reported that young rabbits were more susceptible to diet-induced atherosclerosis than their adult counterparts. Similarly, more recently it has been found that in rabbits the differential cholesterolaemic effect of casein and soybean protein is largely reduced when adult rabbits are used (Table V).

Table V

EFFECT OF AGE ON THE LEVELS OF SERUM CHOLESTEROL IN RABBITS FED SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN, ACCORDING TO West and Terpstra (204)

Diet	Serum cholesterol (mg/100 ml, mean ± S.E.M.)					
	Young rabbits[1]			Old rabbits[1]		
	initial	6 weeks	12 weeks	initial	6 weeks	12 weeks
Casein						
ad libitum feeding	41 ± 4	158 ± 17	186 ± 43	20 ± 1	55 ± 10	43 ± 7
restricted feeding	41 ± 4	127 ± 18	148 ± 27	20 ± 4	49 ± 10	38 ± 12
Soybean protein						
ad libitum feeding	41 ± 5	51 ± 5	42 ± 5	20 ± 2	31 ± 5	34 ± 6
restricted feeding	41 ± 5	68 ± 12	66 ± 10	20 ± 2	31 ± 5	17 ± 3

[1] Age at the beginning of the experiment of the young and old rabbits was 13 weeks and 10 months, respectively.
Thirteen animals per group.

Furthermore, various dietary components might modulate the cholesterolaemic properties of the protein source in the diet. Kritchevsky *et al.* (104) reported that in rabbits the differential effect of casein and soybean protein on serum cholesterol concentration disappeared when wheat straw or cellulose in the diet was replaced by alfalfa. Likewise, the hypercholesterolaemic response of casein diets in rabbits could be erased by the inclusion of considerable amounts of polyunsaturated fat in the diet (106,205).

2. Rats

The influence of dietary protein on cholesterol metabolism and atherogenesis has also been extensively studied in rats. The source of dietary protein markedly affects the concentration of serum cholesterol in rats fed atherogenic diets. The differential effect on the serum cholesterol concentration of soybean protein and casein in rats was reported over 25 years ago by Moyer *et al.* (135). At about the same time, more extensive studies were carried out by Nath and coworkers (138). They observed that the ingestion of diets containing 40% of a protein such as casein, fibrin, pork protein or zein produced elevated levels of serum cholesterol, whereas wheat gluten and purified soybean protein resulted in reduced serum cholesterol concentrations. However, quite the opposite result was reported by McGregor (128) who found that wheat gluten induced higher serum cholesterol levels than casein. Similarly, Fillios and Mann (43) observed that casein diets were less hypercholesterolaemic than diets containing soybean protein. A satisfactory explanation for these contrasting results is not readily available.

De Groot (56) studied the effect of the addition of various proteins to a basal diet containing 15% casein. Each of the supplements which provided an additional 5% of protein in the diet, caused a decrease in the serum cholesterol level. The lowest level was obtained with dried whole egg, wheat gluten, fish and meat meal whereas supplementation of the basal diet with casein resulted in the highest serum cholesterol concentration. Furthermore, it was shown that a mixture of amino acids identical to wheat gluten had an effect similar to intact wheat gluten.

The role of the amino acid composition of dietary protein in determining serum cholesterol levels in rats was confirmed by the studies of Yadav and Liener (Table VI).
Rats fed atherogenic diets containing soybean flour, soybean protein isolate and navy bean flour exhibited lower levels of serum cholesterol than rats on diets containing casein as the protein source. Likewise, an amino acid mixture

TABLE VI

CONCENTRATION OF SERUM CHOLESTEROL IN RATS FED SEMIPURIFIED DIETS CONTAINING VARIOUS SOURCES OF PROTEIN OR AMINO ACID MIXTURES, ACCORDING TO Yadav and Liener (208)[1]

Protein and amino acid source	Number of animals	Plasma cholesterol (mg/100 ml, mean ± S.E.M.)
Casein	6	363 ± 60
Soybean flour	6	172 ± 28
Soybean isolate	6	208 ± 22
Navy bean flour	6	100 ± 3
Amino acids (resembling casein)	6	347 ± 37
Amino acids (resembling soybean protein)	6	236 ± 12

[1] The diets were fed for 4 weeks.

formulated to simulate the amino acid pattern of soybean protein induced levels of serum cholesterol which were lower than those obtained with an amino acid mixture corresponding to that of casein.

The differential effect of casein and a protein mixture consisting of gelatin, fish protein and casein on the level of serum cholesterol observed in rabbits by Hermus (63) has been reproduced succesfully in rats. The replacement of casein by the protein mixture resulted in a reduction in the serum cholesterol concentrations in both lean and genetically obese Zucker rats (Table VII). The increased serum cholesterol concentration was mainly reflected in elevated cholesterol concentrations in the low density and very low density lipoproteins (58).

In all of the studies reviewed above, use was made of atherogenic diets, i.e. diets containing considerable amounts of cholesterol. However, if no cholesterol was incorporated in the diets, generally less striking or even no effects at all were found. Hevia and Visek (72) studied the effects of soybean protein and casein on serum cholesterol in the presence and absence of dietary cholesterol. In diets both with and without cholesterol, higher serum cholesterol levels were observed in the rats fed casein but only when very high levels of dietary protein (45%) were used. When the diets contained 30 or 15%

Table VII

CONCENTRATION OF SERUM CHOLESTEROL IN LEAN AND GENETICALLY OBESE
ZUCKER RATS FED SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR A
PROTEIN MIXTURE, ACCORDING TO Guigoz et al. (58)

Diet	Number of animals	Serum cholesterol (mg/100 ml, mean ± S.E.M.)			
		lean rats		obese rats	
		initial	12 weeks	initial	12 weeks
Casein	10	107 ± 3	155 ± 19	134 ± 4	343 ± 17
Protein mixture[1]	10	108 ± 2	110 ± 14	137 ± 4	292 ± 17
Commercial diet	5	105 ± 5	65 ± 4	139 ± 5	115 ± 20

[1]The protein mixture consisted of casein, fish protein and gelatin
(8 : 8 : 5.5 by weight).

protein no differential effect was detectable. It should be mentioned that in
the experiments of Hevia and Visek (72) the animals were fed for only two weeks,
which might explain why effects were only observed at high levels of dietary
protein. A hypocholesterolaemic action of soybean protein compared to casein was
also observed by Olson et al. (151) in rats fed cholesterol-free, choline-
deficient diets. However, the protein effect was eliminated when the diets were
supplemented with choline. Similarly, Nagata et al. (137) using rats on a
cholesterol-free low-fat diet observed lower serum cholesterol levels with
soybean protein than with casein.

A large series of dietary proteins was studied by Neves et al. (140) using
cholesterol-free diets. Although total plasma cholesterol varied between the
rats fed the various proteins, the changes were not associated with any parti-
cular protein source and plant proteins were not found to have a hypocholesterol-
aemic effect compared with animal proteins. Similar findings have been reported
by Eklund and Sjöblom (38). Finally, Sautier et al. (171) and Pathirana et al.
(155) reported no differential effects at all in rats fed cholesterol-free
diets containing either casein or soybean protein.

In most of these studies, male rats have been used. However, it is interest-
ing to note that there is evidence for a difference in susceptibility to hyper-
cholesterolaemia between male and female rats. Fillios et al. (44) observed
that female rats exhibited more pronounced changes in the levels of serum
cholesterol produced by dietary protein than their male counterparts.

Table VIII

CONCENTRATION OF SERUM CHOLESTEROL IN CONVENTIONAL AND GERM-FREE CHICKENS
FED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN, ACCORDING TO
Kritchevsky *et al.* (103)[1]

Group	Diet	Number of chickens	Serum cholesterol (mg/100 ml)	
			mean	range
Experiment I				
Conventional	Soybean protein	10	365	245-587
Conventional	Casein	10	539	300-1062
Germ-free	Soybean protein	10	521	233-1328
Germ-free	Casein	10	627	262-1054
Experiment II				
Conventional	Soybean protein	11	286	138-418
Conventional	Casein	10	713	372-1157
Germ-free	Soybean protein	11	565	240-920
Germ-free	Casein	10	819	396-1092

[1] The diets, which also contained 3% cholesterol, were fed for 28 days.

3. Birds

In chickens, several studies have been carried out to examine the involve-
ment of dietary protein in cholesterol metabolism and atherogenesis. Stamler
et al. (186) produced data showing that semipurified diets containing chole-
sterol tended to be less hypercholesterolaemic and atherogenic when soybean
protein was included rather than casein and gelatin. Similarly, Kritchevsky
et al. (103) reported that replacement of casein by soybean protein in chole-
sterol-enriched diets lowered the levels of serum cholesterol (Table VIII).
The results of the experiments described by Kenney and Fisher (90) are in
agreement with these findings. However, when no cholesterol was incorporated
in the diet, the effect of the dietary protein source on the serum cholesterol
level has been found to be less pronounced or absent in chickens (85,71) and
pigeons (118).

4. Pigs

The effect of dietary casein and soybean protein on cholesterol metabolism in pigs has been extensively studied by Kim and coworkers (94). Pigs were maintained on semipurified diets high and low in fat and cholesterol. When pigs were fed high-cholesterol, high-fat diets containing either casein or soybean protein a clear difference in cholesterol response was observed (Table IX). The animals fed casein exhibited elevated levels of serum cholesterol, whereas the concentration in the pigs kept on the soybean protein diet did not significantly differ from the group fed a commercial diet. When a cholesterol-free casein diet was consumed, no changes in serum cholesterol levels were observed compared with those animals fed a commercial diet (Table IX). Nevertheless, the feeding of the semipurified diet produced a decrease in the hepatic microsomal HMG-CoA reductase activity together with a reduced excretion of bile acids. Neutral steroid excretion did not alter significantly.

Consistent with these findings of Kim *et al.* (94) are the results of Forsythe *et al.* (46). They reported that in pigs fed diets high in both fat and cholesterol significantly higher serum cholesterol levels were found when an

Table IX

EFFECT OF SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN ON SERUM CHOLESTEROL LEVELS IN SWINE, ACCORDING TO Kim *et al.* (94,95)

Diet	Proportion of energy derived from fat (%)	Dietary cholesterol (mg/day)	Number of swine	Serum cholesterol (mg/100 ml ± S.E.M.)	
				initial	final
Experiment A[1]					
Commercial	10	0	5	85 ± 8	95 ± 4
Casein	42	1055	5	97 ± 3	219 ± 33
Soybean protein	43	1055	5	85 ± 5	107 ± 3
Experiment B[2]					
Commercial	10	0	4	100 ± 8	101 ± 12
Casein	9	0	4	96 ± 4	88 ± 7
Casein	40	0	4	91 ± 4	118 ± 12

[1] The diets were fed for 6 weeks.

[2] The diets were fed for 2 weeks.

animal protein mixture (casein and lactalbumin) was replaced by a plant protein mixture (soybean, corn and wheat protein). These effects occurred regardless of whether polyunsaturated or saturated fats were included in the diet.

Similarly, Julius and Wiggers (88) observed that there was a general trend toward higher plasma cholesterol concentrations in pigs fed casein than soybean protein isolate.

B. The Role of Amino Acids

As indicated above, the amino acid composition has been found to be an important factor in determining the effects of dietary protein on serum cholesterol levels and atherogenesis. Therefore, several investigations have been carried out in order to elucidate the role of particular amino acids in experimental animals such as chickens (96,98,85), rats (55,56,57,69,70) and rabbits (79). Some of the amino acids that have been extensily studied will be discussed.

1. Sulphur-containing amino acids

Much attention has been focused upon the sulphur-containing amino acids such as methionine, cysteine, cystine and also taurine. Mann *et al.* (121,123) observed that hypercholesterolaemia and atherosclerosis in monkeys fed diets containing soybean protein and high in cholesterol could be prevented by supplementation with methionine. Similar results have been obtained in rats (154) and mice (43). Soybean protein is relatively poor in methionine, and Leveille *et al.* (113) reported that the addition of methionine to a diet containing marginal levels of this amino acid resulted in a reduction of the hypercholesterolaemia in chicks. However, there is evidence that supplementation of diets with methionine has also a hypocholesterolaemic effect in rats whether or not the diet is limiting in this amino acid (57). The cholesterol-lowering effect of methionine has also been reported by other authors using rats (139,173,174,175,176) and chicks (73). On the other hand, there are also reports showing that when rats were fed diets supplemented with methionine the serum cholesterol level was increased (7,167) or did not change (87). Similarly, in rabbits (60) and pigs (94) no effect of methionine was observed when diets containing soybean protein were given. Addition of methionine to a casein diet has been found to increase the serum cholesterol level (Table X).

Cystine has also been shown to provoke a hypocholesterolaemia in rats (41, 43,175), mice (43,110) and monkeys (121,123). Furthermore, the concentration of

Table X

EFFECT OF SUPPLEMENTING CASEIN DIETS WITH VARIOUS AMINO ACIDS ON THE
CONCENTRATION OF SERUM CHOLESTEROL IN RABBITS, ACCORDING TO Hermus (64,65)

Diet	Number of animals	Serum cholesterol concentr (mg/100 ml, mean ± S.E.M.
Experiment A[1]		
Casein (20%)	22	477 ± 59
Casein (20%) + methionine (0.20%)	14	710 ± 88
Casein (20%) + arginine (0.80%)	14	548 ± 78
Casein (20%) + methionine (0.20%) + arginine (0.80%)	13	800 ± 117
Experiment B[2]		
Casein (20%)	14	244 ± 55
Casein (20%) + glycine (1.38%)	10	106 ± 15
Casein (20%) + glycine (1.38%) + arginine (0.35%)	14	171 ± 33

[1] The diets were fed for 17 weeks.

[2] The diets were fed for 13 weeks.

cholesterol in serum has been reported to be reduced by taurine in rats (68,175
and monkeys (123) and by ethionine in rats (173,174) and dogs (40,50).
Itokawa et al. (83) described the anti-hypercholesterolaemic effects of
S-methylcystine sulphoxide and S-allylcysteine sulphoxide which are commonly
found in plants of the families *Liliaceae* and *Cruciferae* and may be one of the
largest sources of non-essential sulphur-containing amino acids in the diet of
the Japanese. They even suggest that consumption of these amino acids may play
a role in delaying cholesterol-related diseases in the Japanese population.

2. Other amino acids

Olson et al. (51,150,152) observed that in man glutamic acid was able to
reduce the concentration of serum cholesterol. In order to find an experimental
model for studying this phenomenon, experiments were carried out in gerbils,
rats, rabbits and chicks (11). When glutamic acid was included in the diet as a
source of non-essential amino acids, serum cholesterol levels were significant-

ly lowered in chicks and gerbils, whereas they were unchanged in the rabbit or increased in the rat. Further studies in gerbils suggested that α-ketoglutarate which can be readily produced from glutamic acid, was responsible for the hypo-cholesterolaemic effect rather than glutamic acid *per se* (12).

The feeding of an excess of histidine to infant monkeys has been found to result in an elevation of the total serum lipids (91). Similar results were found in rats (160,181,182) and rabbits (52) where the addition of 5-8% histidine to the diets was associated with an increase in serum cholesterol levels.

It has been suggested that the low content of lysine in plant proteins

Table XI

EFFECT OF THE ADDITION OF LYSINE AND ARGININE TO SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN ON THE CONCENTRATION OF SERUM CHOLESTEROL AND EXPERIMENTAL ATHEROSCLEROSIS IN RABBITS, ACCORDING TO Kritchevsky (102)[1]

Dietary protein	Number of animals	Serum cholesterol (mg/100 ml, mean ± S.E.M.)	Mean atheromata grading ± S.E.M.	
			arch	thoracic
Casein	7	174 ± 30	2.2 ± 0.5	1.5 ± 0.4
Soybean protein	6	59 ± 14	0.8 ± 0.4	0.5 ± 0.2
Caseine + arginine[2]	6	129 ± 12	1.4 ± 0.4	0.8 ± 0.3
Soybean protein + lysine[3]	6	106 ± 29	1.6 ± 0.4	1.1 ± 0.2

[1] The diets were fed for 8 months.

[2] Arginine was added to give the diet an arginine/lysine ratio equal to that of soybean protein.

[3] Lysine was added to give the diet an arginine/lysine ratio equal to that of casein.

might be responsible for their hypocholesterolaemic properties (69,202). In rats supplementation of wheat gluten, a protein known to be limiting in lysine, with lysine did not alter the serum cholesterol level (128). Likewise, when this amino acid was added to diets containing casein, no significant changes in the serum lipids were observed (69,70). On the other hand, addition of lysine to a commercial diet resulted in a decrease in the serum cholesterol (84). In rabbits, however, a diet containing 1% cholesterol and deficient in lysine brought about lower serum lipid levels together with less aortic atherosclerosis than a diet with sufficient lysine (202).

Kritchevsky has provided data suggesting that the ratio of arginine to lysine might play an important role in the development of hypercholesterolaemia and atherosclerosis (102). The addition of arginine to casein in order to

secure a ratio of arginine to lysine in the diet equal to that provided by
soybean protein resulted in a decrease of the serum cholesterol levels together
with a lower degree of atherosclerosis. Conversely, the supplementation of
soybean protein with lysine to give the diet a ratio of arginine to lysine
equal to that of casein enhanced atherogenicity and resulted in an elevation of
the serum cholesterol levels (Tabel XI). However, contrasting results have been
reported by Hermus *et al.* (65,67). The addition of lysine to a protein mixture
composed of casein, fish protein and gelatin in order to secure a ratio of
arginine to lysine in the diet equal to that of casein, did not affect the
hypocholesterolaemic property of this protein mixture (Fig. 3).
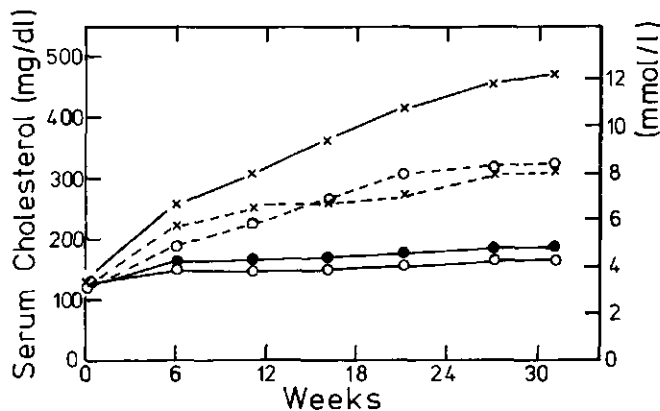
Fig. 3. Concentration of serum cholesterol in rabbits fed semipurified diets
        containing different proteins and amino acid supplementations.
        x———x , 20% casein (15 rabbits); o———o , 7.5% casein, 7.5% fish
        protein and 5% gelatin; •———• , 7.5% casein, 7.5% fish protein and
        5% gelatin supplemented with 1% lysine to give diet a lysine/arginine
        ratio equal to that in casein (17 rabbits); x------x , 7.5% casein,
        7.5% fish protein and 5% gelatin supplemented with amino acids present in
        casein in relative large amounts (methionine, lysine, tryptophane, leucin
        isoleucine, tyrosine, threonine, valine, histidine, proline, serine,
        glutamine and phenylalanine) (15 rabbits); o------o , 20% casein
        supplemented with amino acids present in casein in relatively low
        amounts (arginine, alanine and glycine) (15 rabbits).
        Reproduced from Hermus et al. (65,67).

Hermus *et al.*(67) also studied the effect of various other amino acids in
rabbits. On one hand casein was supplemented with the amino acids which are
relatively scarce in casein. On the other the amino acids abundantly present in
casein were added to a protein mixture composed of casein, fish protein and
gelatin and known to be hypocholesterolaemic compared to casein. From the
results (Fig. 3) it could be concluded that the hypercholesterolaemic effect of
casein could be ascribed either to a relative deficiency of some amino acids in
casein or to a relative surplus of other amino acids. Further studies revealed
that when glycine was added to a casein diet, it was able to reduce the serum
cholesterol levels, whereas the addition of arginine and methionine tended to
increase the level of serum cholesterol (Table X). However in rats, Aust *et al.*
(6) observed that semipurified diets rich in glycine reduced the levels of tri-
glycerides but did not affect the serum cholesterol levels. In addition, Katan
et al.(89) found that increased or decreased levels of serum cholesterol pro-
duced by modifications in the amino acid composition of the diets, paralleled
the proportion of the aortic surface covered by atheromatous plaques (Fig. 4).
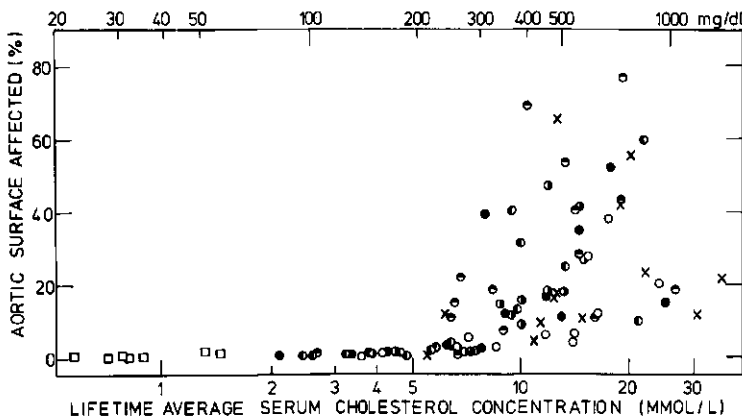
Fig. 4. Proportion of aortic surface covered by plaques as a function of serum
cholesterol concentration in rabbits fed semipurified diets containing
different proteins and supplementations of amino acids.
x , 20% casein; ● , 7.5% casein, 7.5% fish protein and 5% gelatin;
○, 20% casein supplemented with 0.35% arginine and 0.54% alanine;
◐, 20% casein with 0.35% arginine, 0.54% alanine and 0.65% glycine;
◑, 20% casein with 0.35% arginine, 0.54% alanine and 1.40% glycine;
◓, 20% casein with 0.35% arginine, 0.54% alanine and 3.15% glycine;
□, commercial diet. The diets were fed for 49 weeks.
Reproduced from Katan et al. (89).

C. The Role of the Amount of Dietary Protein

1. Rabbits

As discussed earlier, Newburgh and Clarkson (142) demonstrated that the atherogenicity of lean beef muscle in rabbits was also determined by the proportion in the diet of this protein source. Rabbits consuming a diet with 36% protein derived from lean beef muscle developed atherosclerosis sooner than those receiving a diet with 27% of this protein.

A similar experiment was carried out by Freyberg (47) using vegetable proteins instead of meat. Rabbits were fed diets containing increasing proportions (13,32 and 38%) protein derived from gluten flour, ground soybeans and alfalfa meal. Regardless of the proportion of protein in the diets no hypercholesterolaemia or atherosclerosis was observed.

Polcâk *et al.* (157) reported when rabbits were fed an atherogenic diet containing 10% casein and 1% cholesterol, that the addition of meat to a final protein concentration of 15% had a protective effect against hypercholesterolaemia. Supplementation with meat also tended to reduce atherosclerotic changes in the aorta. Although the authors suggested that the favourable effects might be attributable to the meat, it should be borne in mind that the increase in the protein content of the diet *per se* could also be responsible.

Munro *et al.* (136) demonstrated that in rabbits fed commercial diets containing casein and 1% cholesterol the accumulation of cholesterol in the liver and other organs could be enhanced by diets high in protein (30% vs 8%). No differential effects were observed in the levels of serum cholesterol and *degree of atheromatous degeneration in the aorta.*

The effects of doubling the proportions of casein and soybean protein in the diet were examined by Huff *et al.* (81). When the level of soybean protein was increased from 27% to 54 % no significant differences in serum cholesterol were found. At both levels of protein, low serum cholesterol levels were maintained. However doubling the proportion of casein, an atherogenic protein, resulted in even more elevated cholesterol levels. Similar results have been obtained by Terpstra *et al.* (193). Rabbits were fed semipurified diets containing 10, 20 or 40% casein; increasing proportions of casein produced higher serum cholesterol levels (Fig. 5).

From the results of Newburgh and Clarkson (142), Freyberg (47), Huff *et al.* (81) and Terpstra *et al.* (193), it might be concluded that in rabbits the hypercholesterolaemic and atherogenic effects of a protein can be enhanced by increasing the proportion of the protein in the diet. On the other hand, there

is no effect when the proportion of a protein without such hypercholesterol-
aemic properties is changed.
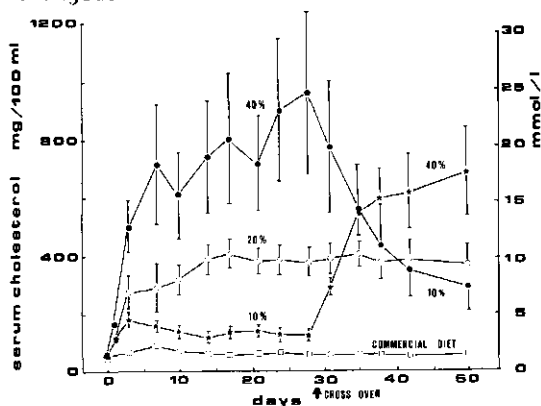

Fig. 5. Concentration of serum cholesterol in rabbits fed semipurified diets
containing different proportions of casein.
✱——✱ , 10% casein before the cross-over and 40% casein after the
cross-over (6 rabbits); ●——● , 40% and 10%, respectively (6 rabbits);
○——○ , 20% casein throughout the experiment (6 rabbits);
□——□ , commercial diet (3 rabbits). The vertical bars represent the
S.E.M. at each time point.
Reproduced from Terpstra et al. (193).

2. Rats

In rats, several experiments have been done to study effect of different pro-
portions of dietary protein on cholesterol metabolism. Baghi *et al.*(7) and Hevia *et
al.* (69,70) using cholesterol-free diets reported that increasing proportions
of casein in the diet were associated with increasing levels of serum choleste-
rol. No differences in serum cholesterol concentrations were found by Chang and
Johnson (25) in rats fed cholesterol-free diets containing either 12% or 27%
casein. In contrast, other studies using either cholesterol-supplemented or
cholesterol-free diets have shown that serum cholesterol levels were reduced by
increasing the proportion of casein in the diet (26,42,55,135,175). Likewise,
high protein diets have been found to be associated with a decrease in serum
cholesterol levels when diets containing wheat gluten (138), soybean protein
(135,138), fibrin or pork (138) were used.

Jones and Huffman (86) described a more complex relationship between the
proportion of protein in the diet and serum cholesterol levels. The lowest
serum cholesterol levels were achieved with diets containing 12 to 18% casein;
a moderate elevation occurred when 7.5% casein diets were fed; and the highest
increase was found on a 40% casein diet. A biphasic response was also observed
by Nath *et al.* (138).

In the experiments of Nath *et al.* (138) with rats increased levels of serum cholesterol were produced on a 10% casein diet. There was a reduction on a diet containing 30% casein and again an increase when a 69% casein diet was fed. In addition, the study of Fillios *et al.* (44) likewise showed that diets containing a low (5%) or high (40 and 60%) proportion of casein resulted in more severe aortic sudanophilia than diets containing an intermediate proportion of casein (10% or 20%).

Thus, the results in rats are rather inconsistent. An explanation for these differences is not readily available, but it is possible that factors such as differences in strain or age of the rats or the duration of the experiments have affected the results.

3. *Birds*

Abundant literature is available describing the effects of different levels of dietary protein on cholesterolaemia and atherogenesis in birds. In most studies an ameliorating effect of high protein diets on hypercholesterolaemia and atherosclerosis has been found. Various experiments were carried out by Leveille and coworkers (107,108,109,112,113) with chickens fed soybean protein or sesame meal as the dietary protein source. The effects of different levels of dietary protein were examined in the presence and absence of dietary cholesterol. In all cases diets low in protein were associated with elevated levels of serum cholesterol. Similar results have also been reported by various authors using cholesterol-free or cholesterol-supplemented diets with soybean protein (85,90,97,100,125,145,166,186,187, Table XII). In several other studies it was found that a cholesterol-lowering effect of diets high in plant protein could be achieved only when cholesterol was included in the diet (144, 127,166). Diets containing increasing proportions of casein have also been reported to be effective in reducing serum cholesterol levels in chickens (45, 85,90,99,188).

Likewise, a number of authors have reported that high protein diets have a suppressive effect on atherogenesis (45,144,166,186,187,188) and are effective in producing regression. Pick *et al.* (156) studied the effect of different levels of dietary protein on the regression of hypercholesterolaemia and coronary atherosclerosis. Cockerels were first made hypercholesterolaemic by feeding cholesterol and subsequently studied on cholesterol-free diets containing different proportions of soybean protein. High proportions of dietary protein proved to be more effective in reducing both the serum cholesterol

concentration and atherosclerosis than low protein diets.

Table XII

EFFECT OF THE PROPORTION OF SOYBEAN PROTEIN IN THE DIET ON THE CONCENTRATION
OF PLASMA CHOLESTEROL IN YOUNG CHICKENS FED EITHER CHOLESTEROL-FREE OR
CHOLESTEROL-SUPPLEMENTED DIETS, ACCORDING TO Johnson *et al.* (85)[1]

Proportion of dietary protein (%)	Proportion of cholesterol (%, w/w)	Plasma cholesterol (mg/100 ml, mean $\pm$ S.E.M.)
7	-	253 $\pm$ 14
13	-	199 $\pm$ 10
19	-	156 $\pm$ 4
25	-	139 $\pm$ 3
31	-	143 $\pm$ 3
7	2	611 $\pm$ 14
13	2	342 $\pm$ 29
19	2	185 $\pm$ 10
25	2	169 $\pm$ 5
31	2	165 $\pm$ 11

[1] The diets were fed for 3 weeks; 10 animals per group.

Studies on the cholesterolaemic effect of different levels of dietary
protein have also been carried out in atherosclerosis-susceptible pigeons.
Lofland and coworkers (28,117) maintained White Carneau pigeons on diets with
three levels of protein (5, 15 and 30% casein) and two types of dietary fat
and also with or without cholesterol supplementation (0.25%). When cholesterol
was included in the diet, the pigeons on the high casein diets developed
higher levels of cholesterol in the serum and aorta together with a higher
degree of aortic and coronary atherosclerosis. Consistent with these findings
are the data of Little and Angell (116) from the same laboratory. Pigeons fed
20 or 40% casein diets with 0.25% cholesterol exhibited higher serum chole-
sterol levels and a higher degree of atherosclerosis than their counterparts
on a 10% casein diet. Similar results were reported by Subbiah (192). A
commercial diet containing 7.5 or 15% protein and not supplemented with
cholesterol was fed to pigeons for a period of six months. The low protein
diet resulted in a slight but significant reduction of serum cholesterol level

together with a decrease in the total sterol content in the aorta.

An explanation for these contrasting results obtained in chickens and pigeons on the effects of the proportion of dietary protein on serum cholesterol level and atherosclerosis is not readily available. It is possible that there is a genuine difference between the species but it can not be ruled out that other factors such as the overall composition of the diet might play a role in determining the levels of serum cholesterol.

4. Monkeys

The data obtained from studies on the role of the proportion of dietary protein on cholesterol metabolism and atherogenesis in monkeys are rather inconsistent and even contradictory. Strong and McGill (189) investigated baboons fed high (25%) and low (10%) casein diets in combination with high and low dietary cholesterol levels and also with saturated and unsaturated fats. Only with diets high in cholesterol and containing saturated fat did a low proportion of protein induce a higher level of serum cholesterol and more sudanophilia in the aorta than did the high protein diet. However in all other cases, the high casein diets were more hypercholesterolaemic and atherogenic.

In squirrel monkeys fed commercial diets, the proportion of dietary protein (either 9 or 25%) exerted little effect on serum cholesterol concentrations (130). Such findings were observed regardless of whether cholesterol-free or cholesterol-supplemented diets were used. However, aortic atherosclerosis was more extensive in monkeys fed a high proportion of dietary protein when cholesterol was added to the diet.

Srinivasan (183,184) maintained spider monkeys upon cholesterol-free and cholesterol-supplemented (0,5%) diets containing 4, 8 or 25% protein. The rations were prepared from commercial feed to which casein and other components were added in various proportions. In the absence of exogenous cholesterol a lower protein intake induced a lower concentration of total cholesterol and also of β plus pre-β-lipoprotein cholesterol, though not significantly. When the diets were supplemented with cholesterol, a reduction in the proportion of protein in the diet from 25 to 8% was accompanied by a fall in the serum total cholesterol concentration. In contrast, a further reduction of dietary protein to the 4% level markedly increased the level of total cholesterol in the serum. Interesting enough both in the presence and absence of exogenous cholesterol, the very low protein diet (4%) produced a highly significant elevation of the α-lipoprotein cholesterol concentration.

5. Pigs

Various studies have been done in pigs. Barnes *et al.* (9) reported that adult pigs fed diets containing 4.9 and 13.7% soybean protein and different types of fat exhibited similar levels of serum cholesterol. In another experiment these authors (10) fed weanling pigs diets containing either 9 or 16% soybean protein and also either high or low proportions of fat. In both the high and low protein group a steep increase in the concentrations of serum cholesterol occurred, but this elevation was less in the animals fed the diet high in protein and low in fat. After reaching a maximum, the levels of serum cholesterol declined toward the levels found in adult pigs with the exception of the low-protein, high-fat group. In these pigs the levels remained high. These findings suggest that the effect of dietary protein may be strongly influenced by other food components such as fat.

In the study of Gupta *et al.* (59) pigs were kept for a period of 18 months on a protein-deficient diet (5% protein) with a high content of both fat and cholesterol. The animals developed all the characteristics of protein deficiency. Though no consistent differences in serum cholesterol levels were observed compared with animals fed a 25% protein diet, the atherosclerotic lesions in the low protein group were more extensive and severe. In contrast, Greer *et al.* (54) reported no consistent effect of the proportion of dietary protein on the incidence of atherosclerotic lesions, whereas increasing the proportion of dietary protein tended to elevate the serum cholesterol levels. However, it should be taken into account that in this study the two levels of dietary protein derived from soybeans were not that different (12 vs 18%).

In studying the effects of the nature of dietary protein on the serum cholesterol levels in pigs, Kim *et al.* (94) observed a hypercholesterolaemic effect of casein compared to soybean protein. Subsequently a mixture of casein and soybean protein containing equal amounts of these two proteins was fed. Doubling the proportion of this mixture in the diet did not have any further effect. One explanation might be that upon increasing the proportion in the diet of this protein mixture (composed of 50% casein and 50% soybean protein) the hypercholesterolaemic action of casein and the hypocholesterolaemic action of soybean protein were both enhanced to the same extent and therefore balanced each other.

6. Other animals

In dogs, protein depletion has been found to cause lipaemia and hypercholesterolaemia (115). When dogs were fed a diet practically devoid of protein,

elevated levels of serum cholesterol were produced compared with dogs receiving 4.4 g casein daily per kg of body weight. When cholesterol was included in the diets, an elevation in the serum cholesterol levels occurred but the differential effect between the protein-depleted and control animals was maintained.

An inverse relationship between serum cholesterol concentrations and dietary protein level has also been found in calves. A diet containing 9% protein derived mainly from soybean meal brought about significantly higher serum cholesterol levels than did a 25% soybean protein diet (29). When diets containing different levels of soybean protein in the range 0-28% were used similar effects were observed (24). Consistent with these findings are the data of Bohman *et al.* (15). Calves fed a high-fat diet exhibited lower serum cholesterol levels when the diets were supplemented with alfalfa pellets which contained 15% protein. The authors ascribed these changes in serum cholesterol to differences in the proportion of dietary protein. However, the high fibre content (36%) of the alfalfa may be responsible since such fibres have been shown to lower serum cholesterol levels in rabbits (104).

IV   STUDIES IN HUMANS

A.   The Epidemiological Approach

Epidemiological studies comparing data in different countries have provided strong evidence for a link between the intake of protein, particularly of animal protein, with the incidence of coronary heart disease. As early as 1957 relationships between the coronary mortality and the intake of animal proteins were described by Yudkin (213) and by Yerushalmy and Hilleboe (74,212). The relationships of mortality from coronary heart disease with the intake of animal protein were even better than with the intake of either total or saturated fat. A similar relationship was also reported by Olson *et al.* (151) in 1958 and has been confirmed by other authors (Table XIII). On the other hand, a negative correlation has been found with vegetable protein (30). It is interesting to note that comparisons between countries reveal relatively minor differences in the intake of total protein compared with the ratio of the intake of animal protein to the intake of vegetable protein (Table XIV). Similar information have been compiled by Kritchevsky (101) derived from time trend studies within the United States. He showed that the total protein availability in the United States did not essentially change during the period from 1909 to 1972. Nevertheless, the ratio of animal to vegetable protein did increase considerably and this increase paralleled the increase in mortality from cardiovascular disease.

Table XIII

RELATIONSHIP BETWEEN THE MORTALITY RATE FROM CORONARY HEART DISEASE AND THE INTAKE (g) OF CERTAIN NUTRIENTS IN THE DIET AS CALCULATED BY VARIOUS AUTHORS[1]

	Correlation coefficients								
Author	Yerushalmy and Hilleboe (212)	Connor and Connor (30)	Armstrong et al. (4)		Stamler (185)				
Number of countries	22	30	30		20				
Sex	Men	Both	Men	Women	Men	Women	Both		
Dietary component									
Total protein	0.694	-	0.49	0.45	0.670	0.562	0.657		
Animal protein	0.695	0.782	0.75	0.58	0.690	0.498	0.641		
Vegetable protein	-0.153	-0.403	-	-	-	-	-		
Total fat	0.470	0.676	0.59	0.39	0.603	0.373	0.537		
Saturated or animal fat	0.562	0.632	0.71	0.58	0.681	0.473	0.625		
Vegetable fat	-0.282	0.011	-	-	-	-	-		
Cholesterol	-	0.762	-	-	0.656	0.581	0.657		

[1] For 20 countries, an r of 0.444 is significant at P = 0.05; an r of 0.561 is significant at P = 0.01

For 22 countries, r of 0.423, P = 0.05; r of 0.537, P = 0.01

For 30 countries, r of 0.361, P = 0.05; r of 0.463, P = 0.01

et al. (153) the low protein diet contained vegetable protein and in the high protein diet mainly animal protein was used. This may mean that the low protein diet had a higher P/S ratio and less cholesterol and this may also have contributed to the marked decrease in serum cholesterol.

Furthermore, in the study of Olson et al. (153) the rather high initial

Table XIV
DATA ON MORTALITY FROM CORONARY HEART DISEASE (CHD) BY SEX AND COUNTRY (1973) AND NUTRIENT AVAILABILITY PER CAPITA (1954-1965), ACCORDING TO Stamler (185)

Table XV
EFFECT OF DIFFERENT AMOUNTS OF DIETARY PROTEIN ON SERUM CHOLESTEROL CONCENTRATION IN MAN

Subjects	Number of subjects	Protein intake (g/day)	Protein source	Composition of diets protein (energy%)	fat (energy%)	cholesterol (mg/day)	Duration of test periods (days)	Serum cholesterol (mg/100 ml, mean) initial	effect of low protein diet [1]	Authors
Physically healthy schizophrene men (32 - 54 yr)	25[2]	69	41% animal	8.6	19.5	1379	28	207	+0.42[NS]	Keys and Anderson (92)
		132	62% animal	17.7	19.4	1427				
Hypercholesterolaemic(5) and normocholesterolaemic (4) patients	9[2]	25	vegetable	4.2	30	-	7	311	-44[xxx]	Olson et al. (153)
		100	mainly animal	16.7	30	-				
-	5[2]	-	formula diets with skim milk protein	0	40	-	-	-	reduction	Furman et al. (49)
				14	40					
Adult men	5	60	-	8.5	37	-	42	-	no effect	Lutz et al. (120)
	5	160	-	23	37					
Convalescent adult patients	16[2]	-	self-selected	13-18	30-40	-	7-35	221	-19.4[x]	Albanese et al. (1)
			self-selected + 30 g milk protein	17-19	28-38					
Healthy men (19 - 23 yr)	7[2]	33	mixed	4.6	33.2	192	28	201	+4.5[NS]	Leveille et al. (111)
		106	mixed + 100 g egg white	15.1	33.5	180				
Male students	6	-	formula diets with calcium caseinate	5	30	-	8	158	+22.4[x]	Beveridge et al. (13)
	7	-		25	30	-				
University atletes (av. 22 yr)	24[2]	-	mixed	10	35	945-1332	17	181	-5[NS]	Wilcox et al. (206)
		-	mixed	16.8	35					
Young healthy women (18-21 yr)	6[2]	55	mixed	10.8	36.4	310	28	197	-51[x]	Prather (159)
		91	mixed + beef, gelatin and dairy products	17.5	35.9	416				
Healthy young men (18 - 20 yr)	6[2]	48	46% animal	6.4	40	< 200	45	218-330	-44[xx]	Elson et al. (39)
		141	60% animal	18.8	35	< 200				

[1] Level of statistical significance: NS, not significant; x P < 0.05; xx P < 0.01; xxx P < 0.001

[2] In this study a cross-over design was employed.

Table XVI

EFFECT OF THE TYPE OF DIETARY PROTEIN ON SERUM CHOLESTEROL CONCENTRATION IN MAN

Subjects	Number of subjects	Protein source	Composition of diets protein (energy%)	fat (energy%)	P/S	cholesterol (mg/day)	Duration of test periods (days)	Serum cholesterol (mg/100 ml, mean) initial	effect of plant protein[1]	Authors
Healthy women (17 - 22 yr)	6	animal	8	36	-	-	42	189	-13NS	Walker et al. (20)
	6	vegetable	8	36	-	-		182		
Healthy men (53 - 70 yr)	6[5]	casein-lactalbumin2(74%)[3]	7	40	low[4]	-	25	258	-18NS	Campbell et al. (18)
		wheat gluten (74%)	7	40	low	-				
		casein-lactalbumin (74%)	7	40	high[4]	*			+9 NS	
		wheat gluten (74%)	7	40	high	-				
Healthy men (33 - 46 yr)	6[5]	mixed	12	45	0.78	420	14	295	-100xxx	Hodges et al. (75)
		vegetable (mainly soybean protein)	13-15	15	1.00	0	28			
Healthy men (21 -26 yr)	11[5]	egg white (50%)	16	37	0.50	308	28	167	+4NS	Anderson et al. (2)
		wheat gluten (50%)	16	37	0.54	308				
Hypercholesterolaemic men and women (22 - 68 yr)	22[5]	animal (63%)	21	21	2.2	180	22	342	-60xx	Sirtori et al. (178)
		soybean concentrate (63%)	21	26	2.7	0-6				
Healthy women (21 - 25 yr)	10[5]	animal (58%)	15	34	0.43	168	37-41	168	-9x	Carroll et al. (21)
		soybean isolate (58%)	16	33	0.44	161				
Mildly hypercholesterolaemic young men	-	animal	13-16	30-35	0.4	100-250	42	221	+6NS	Shorey and Davis (177)
	-	soybean isolate	13-16	30-35	0.4	100-250		241		
Healthy men (av. 50 yr)	17[5]	animal	18-19	39-41	0.32-0.44	650	*	212	no consistent effects	Bodwell et al. (14)
		soybean isolate	18-19	39-41	0.32-0.44	650				
		textured soybean	18-19	39-41	0.32-0.44	650				
Healthy men and women (18-28 yr)	25	casein (65%)	13	37	0.6	387	28	152	0	van Raay et al. (161, 162)
	24	soybean isolate (65%)	13	37	0.6	365		153		
Hypercholesterolaemic men and women (28 - 60 yr)	10[5]	animal (71%)	14	34	1.36	144	21	282	0	Holmes et al. (76)
		soybean concentrate (71%)	14	35	1.34	144				
Hypercholesterolaemic men and women	127[5]	animal (80%)	20	25	1.8-2.0	-	56	351	-67xxx	Descovich et al. (32)
		soybean concentrate (80%)	19	25	1.8-2.3	-	56			

[1] Level of statistical significance: NS, not significant; x P < 0.05; xx P < 0.01; xxx P < 0.001.

[2] Casein-lactalbumine mixture: 80% casein, 20% lactalbumin.

[3] The values in parentheses denote the percentage of the total protein provided by the indicated protein.

[4] Low P/S ratio: 12% of total fat as linoleic acid; high P/S ratio: 40% of total fat as linoleic acid.

[5] In this study a cross-over design was employed.

levels of cholesterol in the serum may also have played an important role, as will be discussed later.

Beveridge *et al.* (13) found in his experiments that the addition of chole-sterol to the diet resulted in an increase in the serum cholesterol level. The difference between the diets low and high in protein was less when cholesterol was fed. However, no such effect was found by Keys and Anderson (92). Supple-mentation of the diets with 1 gram of cholesterol each day did not affect the serum cholesterol levels.

The length of the experimental periods employed in the studies summarized in Table XV varies considerably. In some studies the experiment lasted only one week. Therefore, it is possible that the data obtained in these short-term studies reflect transient changes. Leveille *et al.* (111) observed that marked elevations in serum cholesterol levels occurred initially when subjects were switched from a low protein diet to a high protein diet. However, these effects disappeared later.

Although some of these studies showed that low protein diets tend to lower the levels of serum cholesterol other studies do not confirm this or show even the reverse. Therefore, no clear cut conclusion can be drawn.

C. The Role of the Nature of Dietary Protein

Table XVI summarizes studies in man in which the effect of different diet-ary proteins on the serum cholesterol levels have been investigated. In all these experiments, comparisons have been made between proteins derived from animal and plant origin. In most of these studies no significant effects have been found. Sirtori *et al.* (178,180) and Descovich *et al.* (32), however, reported a marked decrease in the serum cholesterol levels of about 20% in hypercholesterolaemic patients fed soybean protein diets and Carroll *et al.* (21) demonstrated that the replacement of animal protein by soybean protein resulted in a slight but significant decrease in serum cholesterol level. However, the various experiments have been done under different conditions and several factors that might influenced the results should be taken into account.

The initial concentration of cholesterol in the serum might be important. The marked cholesterol-lowering effect of soybean protein as reported by Sirtori *et al.* (Table XVII) and Descovich *et al.* (32) was achieved in hyper-cholesterolaemic patients. In normocholesterolaemic subjects, no such effect could be observed (14,161,162). Therefore, it is possible that the cholesterol-lowering effect of soybean protein becomes obvious only under suitable condi-

Table XVII

EFFECT OF A DIET CONTAINING SOYBEAN PROTEIN CONCENTRATE ON SERUM LIPIDS IN HYPERLIPAEMIC PATIENTS, ACCORDING TO Sirtori *et al.* (180)[1]

	Serum concentration (mg/100 ml, mean $\pm$ S.E.M.)			
	initial	final	absolute change[2]	relative change (%)
Type IIA patients (22)[3]				
Total cholesterol	346 $\pm$ 22	289 $\pm$ 24	-57 $\pm$ 7[xxx]	-16
VLDL[4] cholesterol	28 $\pm$ 2	30 $\pm$ 3	-3 $\pm$ 2[NS]	-11
LDL cholesterol	278 $\pm$ 21	224 $\pm$ 23	-53 $\pm$ 7[xxx]	-19
Triglycerides	134 $\pm$ 7	119 $\pm$ 9	-15 $\pm$ 9[NS]	-11
Type IIB-patients (16)				
Total cholesterol	326 $\pm$ 15	262 $\pm$ 10	-66 $\pm$ 7[xxx]	-20
VLDL cholesterol	56 $\pm$ 4	47 $\pm$ 5	-11 $\pm$ 7[NS]	-20
LDL cholesterol	233 $\pm$ 14	181 $\pm$ 10	-51 $\pm$ 9[xxx]	-22
Triglycerides	246 $\pm$ 16	221 $\pm$ 24	-25 $\pm$ 24[NS]	-10
Type IIB-III patients (4)				
Total cholesterol	337 $\pm$ 20	248 $\pm$ 19	-89 $\pm$ 16[xx]	-26
VLDL cholesterol	110 $\pm$ 14	86 $\pm$ 14	-24 $\pm$ 7[x]	-22
LDL cholesterol	190 $\pm$ 35	135 $\pm$ 26	-55 $\pm$ 21[x]	-29
Triglycerides	348 $\pm$ 7	288 $\pm$ 19	-60 $\pm$ 25[x]	-17

[1] Diets were consumed for a period of 3 weeks.

[2] Level of statistical significance: NS, not significant; [x]P $\langle$ 0.05; [xx]P $\langle$ 0.01; [xxx]P $\langle$ 0.001

[3] Number of patients indicated in parentheses.

[4] VLDL, very low density lipoproteins; LDL, low density lipoproteins.

tions, *i.e.* high initial levels of serum cholesterol.

When comparing the results reported by various authors, the proportion of protein in the diet should also be taken into account. In the diets used by Sirtori *et al.* (178,180) and Descovich *et al.* (32), 19-21% of the energy intake was provided by protein whereas in the experiment of Holmes *et al.* (76) and Shorey and Davis (177) 13-16% of the energy was derived from protein. This might partly influence the results.

It should be mentioned also that the diets used in the experiments of Sirtori and coworkers (178,180) had a high P/S ratio. In a cross-over study, they observed that soybean protein diets with a low P/S ratio (0.1) were less effective in lowering the serum cholesterol levels (180). Furthermore, in the studies of Sirtori *et al.* (178,180) the intake of cholesterol was higher when diets containing animal protein were fed than when soybean protein diets were consumed. However, these authors reported that the intake of an additional 500 mg of cholesterol a day when fed the soybean protein diets did not affect the results.

In the studies of Hodges *et al.* (75) a marked decrease in the levels of serum cholesterol occurred when soybean protein diets replaced a mixed diet. However, the soybean protein diets were much lower in fat and cholesterol content compared to the control diet. Though Hodges *et al.* (75) believed that the reduction in the levels of serum cholesterol was mainly attributable to the soybean protein, Keys (93) pointed out that 90% of this decrease could be ascribed to differences in the amount of dietary fat and cholesterol.

Attention should be paid also to the nature of the soybean protein product used in the various studies. In the experiments of Sirtori *et al.* (178,180) and Descovich *et al.* (32) a textured soybean protein was used. They regard this as the only form which will reduce serum cholesterol level in man. These authors consider the non-protein components of textured soybean protein as important, but are convinced that the protein is the cholesterol-lowering factor. They argue that the structure of the protein is important, since this might affect the release and absorption of the amino acids which may be subsequently reponsible for the cholesterol-lowering effect (179).

Interesting are the findings reported by van Raaij *et al.* (162). When casein in the diet was replaced by soybean protein, no changes in total serum cholesterol concentration occurred. Nevertheless, a significant increase of the ratio of HDL to HDL cholesterol was observed when soybean protein diets were fed (Table XVIII). Since high HDL/LDL cholesterol ratios are predictive for lower risk for coronary heart disease (131) soybean protein might have a

Table XVIII

EFFECT OF DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN ISOLATE ON THE CONCENTRATION OF SERUM LIPIDS AND LIPOPROTEINS IN NORMOCHOLESTEROLAEMIC SUBJECTS, ACCORDING TO van Raay et al. (162)[1]

	Casein group[2]			Soybean protein group[2]		
	initial	final	change[3]	initial	final	change[3]
Cholesterol (mg/100 ml, mean ± S.E.M.)						
Serum	152 ± 5.4	149 ± 4.8	-3 ± 2.8	153 ± 4.7	150 ± 4.7	-3 ± 2.0
VLDL[4]	8 ± 0.6	10 ± 1.0	-1.5 ± 1.2	10 ± 0.6	10 ± 1.0	+0.8 ± 1.0
LDL	80 ± 3.4	79 ± 3.2	-0.4 ± 2.5	88 ± 3.9	81 ± 3.9	-6.6 ± 1.9[x]
HDL	59 ± 3.2	62 ± 3.0	+2.3 ± 1.7	57 ± 2.0	62 ± 2.4	+5.8 ± 1.0[x]
Serum apoprotein B (mg/l, mean ± S.E.M.)	488 ± 23	441 ± 20	-47 ± 10[x]	462 ± 22	447 ± 22	-15 ± 10
LDL cholesterol/ apoprotein B ratio (mean)	1.64	1.79	+0.15[xx]	1.90	1.81	-0.09[x]
LDL density (g/ml, mean)	1.039	1.036	-0.003[x]	1.036	1.037	+0.001

[1] The diets were consumed for a period of 4 weeks.

[2] Number of subjects was 25 and 24 for the casein and soybean protein group, respectively.

[3] Level of statistical significance: [x] P < 0.05; [xx] P < 0.01.

[4] VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

beneficial effect, even when it does not lower the level of total serum chole-
sterol.

Finally, it is worthwile to note that the age of the experimental subjects
might be an important factor in determining the results in studies on the
cholesterolaemic effects of dietary protein. Descovich et al. (32) found that
the cholesterol lowering effects of dietary soybean protein were most pronoun-
ced in young hypercholesterolaemic patients. This is in agreement with data in
rabbits, as discussed earlier.

D. The Role of Amino Acids

The possible effects of several specific amino acids on the concentration
of serum cholesterol have also been studied in man. However, in most of these
experiments no effect has been observed. The supplementation of diets with
methionine (122,134), cystine (134), histidine (53), leucine (197) or with
taurine (199) did not result in changes in serum cholesterol concentration.

Olson and coworkers (51,150,152) observed a hypocholesterolaemic effect of
glutamate. They fed subjects a formula diet containing the eight essential
amino acids at levels of three times the tentative daily requirement. Either
glutamic acid or glycine and ammonium acetate were added to provide non essen-
tial amino acids and nitrogen. When the subjects were transferred from their
home diets to the formula diets containing glutamic acid, a considerable
decrease in the serum cholesterol level occurred. No such changes were observed
when the formula diets containing glycine and ammonium acetate were fed.
However, when the same amount of glutamic acid was added to a normal diet, a
negligible effect on serum cholesterol was seen.

Furhtermore, Raja (163) observed a slight cholesterol-lowering effect in
hyperlipidaemic patients when supplements of lysine and tryptophan were fed.

V MECHANISM OF THE CHOLESTEROLAEMIC EFFECTS OF DIETARY PROTEIN

Changes in serum cholesterol concentration produced by dietary protein
might be attributable to alterations in absorption, biosynthesis, tissue distri-
bution and excretion of cholesterol which might result in either an increase or
decrease in the rate of cholesterol turnover in the whole body. The various
alternatives which could influence the rate of cholesterol turnover are reviewed
below.

A. Cholesterol Absorption

Huff and Carroll (80) showed that rabbits fed semipurified diets containing casein exhibited higher levels of serum cholesterol together with a higher absorption of cholesterol compared to animals fed soybean protein. Similar results were found by Kim *et al.* (94) in pigs which were fed either casein or soybean protein. Furthermore, it has been demonstrated in other animals that the promoting effect of casein on cholesterol absorption·is even more enhanced when higher proportions of casein are incorporated in the diet. This effect has been found to exist to a marked extent in rats (25,119) and to a less extent in chickens (90).

B. Cholesterol Synthesis

No significant difference in cholesterol synthesis was found by Kim *et al.* (94) in pigs fed either casein or soybean protein, though animals fed casein exhibited higher serum cholesterol levels than those on soybean protein diets. However in rats, Mokady and Einav (132) found a higher rate of incorporation of acetate into liver cholesterol on a gluten diet than on a casein diet. Furthermore, Reiser *et al.* (164) reported in rats that the replacement of casein by soybean protein resulted in a twofold increase in the activity of hepatic HMG-CoA reductase, a rate limiting enzyme for cholesterol synthesis. Moreover, Yeh and Leveille (209,211) observed in chickens that the cholesterol synthesis measured in liver and also in the whole body increased when higher proportions of soybean protein were incorporated in the diets. Similar results in chickens have been reported by Nishida *et al.* (146). Consistent with these findings are the data of Coccodrillo *et al.* (29) who found in calves a negative relationship between the level of dietary soybean protein and cholesterol synthesis. This might indicate that the stimulating effect of soybean protein on cholesterol synthesis can be enhanced by increasing the proportion of this protein in the diet. On the other hand, increasing the level of casein in the diet of rats, has been found to reduce the incorporation of acetate into cholesterol in the liver (19).

C. Cholesterol Excretion

In pigs, no differences in total steroid excretion were observed when diets containing casein or soybean protein were fed (94). However in rabbits fed soybean meal, Howard and Gresham (77) reported an enhanced excretion of faecal

cholesterol and coprostanol compared with animals on a casein diet. Fumagalli
et al. (48) observed that the replacement of casein by soybean protein
increased the excretion of neutral steroids but did not affect the excretion of
bile acids. An enhanced excretion of both bile acids and neutral steroids was
described by Huff and Carroll (80), when rabbits were fed soybean protein
instead of casein. Similarly in rats, a higher faecal excretion of neutral
steroids (137,171) and bile acids (171) has been measured on diets containing
soybean protein than on casein diets. Furthermore, chickens fed soybean meal
have been found to eliminate considerably more cholesterol in their faeces than
their casein-fed counterparts (90).

The excretion of cholesterol is also affected by the levels of protein in
the diet. Lutton and Chevallier (119) described in rats a lower rate of excre-
tion of total faecal steroids when the proportion of casein in the diet was
increased. However in chickens, the reverse has been reported. As discussed
above, low protein diets in chickens are generally associated with elevated
serum cholesterol levels, regardless of whether casein or soybean protein is
incorporated in the diet. This has been found to be mediated by a lower excre-
tion of cholesterol (90), bile acids (112) or of both (210). Nishida et al.
(147) studied the rate of excretion of bile acids in bile fistulated chickens
injected with ^{14}C-4-cholesterol. Animals fed a 30% casein diet had an increased
rate of excretion compared with those on a diet containing 15% casein. They
also found that the feeding of high casein diets to rats resulted in an
increased excretion of the injected ^{14}C-4-cholesterol in the bile. As in these
studies however, the excretion was measured in the bile, this does not provide
a measure of the rate of excretion of bile acids in the faeces. Under normal
physiological conditions, a great proportion of this bile acids are reabsorbed
from the intestine. This might partially explain why in rats Lutton and
Chevallier (119) observed a lower rate of excretion of total steroids in the
faeces when high casein diets were fed, whereas Nishida et al. (147) observed a
higher rate of excretion of bile acids in the bile.

D. Cholesterol Turnover

Alterations in absorption, synthesis, tissue distribution and excretion of
cholesterol might result in a change of the turnover rate of cholesterol in the
whole body. After the injection of labeled cholesterol into the blood, the rate
of decay of the specific radioactivity of cholesterol in the serum can be
measured. By using these data and making some assumptions, parameters such as

cholesterol synthesis, cholesterol excretion and size of the cholesterol pools in the body can be estimated. Increased levels of serum cholesterol due to changes in dietary protein have been found to be associated with a decrease in the cholesterol turnover rate. The studies of Huff and Carroll (80) revealed that the production rate of cholesterol was increased in rabbits fed soybean protein compared with those animals on casein diets. In chickens, Yeh and Leveille (210) observed that the feeding of high soybean protein diets resulted in a fall in serum cholesterol levels together with an increased cholesterol turnover rate.

Thus, most of the results obtained in animal experiments show that an increase in serum cholesterol levels produced by dietary protein is associated with an increase in the absorption and a decrease in the synthesis and excretion of cholesterol or the overall turnover rate of cholesterol. However, alterations in cholesterol metabolism do not necessarily implicate changes in serum cholesterol levels. In rabbits markedly elevated levels of serum cholesterol can be produced by feeding cholesterol-free diets containing casein, whereas in rats no such changes in serum cholesterol levels occur on these diets (171). Nevertheless, rats fed casein diets exhibit a reduced faecal excretion of neutral steroids and bile acids (171) and have a decreased HMG-CoA reductase activity (164) compared with those fed soybean protein diets. Similar findings have been observed in pigs by Kim et al. (95). The feeding of cholesterol-free semipurified diets containing casein resulted in a 70-80% decrease in hepatic HMG-CoA reductase activity and faecal excretion of bile acids compared to a commercial diet, in spite of no observable differences in serum cholesterol levels.

It is interesting to note the differences between species in susceptibility to hypercholesterolaemia and atherosclerosis due to dietary protein. While alterations in serum cholesterol concentration can be produced easily in rabbits by feeding cholesterol-free casein diets (22) cholesterol has to be added to the diets in rats (208), chickens (103) and pigs (94). It is likely that there is a negative correlation between the rate of cholesterol synthesis, excretion or the turnover rate of cholesterol in the whole body and the susceptibility to hypercholesterolaemia. Rats are known to be very resistant against induction of hypercholesterolaemia but have a higher rate of hepatic cholesterol synthesis than rabbits in which hypercholesterolaemia and atherosclerosis can be easily induced (19). Similarly, it has been shown that the rate of excretion of injected ^{14}C-4-cholesterol in rats was about 5 times faster than in chickens

(147). Rats obviously possess a very efficient feed back mechanism to cope
with increased cholesterol loading by diminishing hepatic cholesterol synthesis
and by enhancing bile acid formation and excretion (33,34,35).

VI CONCLUSIONS

In experimental animals, it has been known for a long time that the type of
dietary protein can profoundly influence cholesterol metabolism. In addition,
the cholesterolaemic effect of a dietary protein can be enhanced by increasing
the proportion of that protein in the diet, though not all the experiments are
consistent in this respect. Moreover, alterations in serum cholesterol level
produced by alterations of dietary protein become obvious only when a consider-
able amount of cholesterol is incorporated in the diets. Only in the rabbit,
an animal highly susceptible to the induction of hypercholesterolaemia and
atherosclerosis, is the feeding of different proteins also able to affect serum
cholesterol levels in the absence of dietary cholesterol.

Consistent with these findings are the data in man. Marked effects of the
type of dietary protein on the levels of serum cholesterol can only be observed
when the subjects are hypercholesterolaemic. On the contrary, in normochole-
sterolaemic subjects dietary protein has been demonstrated to exert a small or
no effect at all.

Alterations in serum cholesterol produced by modulations in dietary protein
have been ascribed in animals to changes in the rate of absorption, synthesis
and excretion of cholesterol. This changes are reflected in alterations of the
turnover rate of cholesterol in the whole body to maintain homeostasis.
Elevation of serum cholesterol concentration is generally found to be associated
with an increase in absorption and decrease in synthesis and excretion of chole-
sterol whereas reduction of serum cholesterol level is accompanied by the
reverse of these factors.

Finally, it should be mentioned that the effect of dietary protein can be
largely modulated by various other dietary or experimental factors. Especially
the type of dietary fat and fibre can erase or enhance the cholesterolaemic
effects of dietary protein. Furthermore, duration of the experiments and sex
and age of the probands have been reported also to influence the results.

In the light of these findings, it is questionable whether changes in the
nature of the protein in the diet will really make an important contribution
to the prevention of atherosclerosis. It may well be that only for the treat-
ment of hypercholesterolaemic patients, as now seems common practice in Italy

and Switzerland, dietary protein might provide a valuable tool. However in normocholesterolaemic subjects, dietary protein seems to play only a minor role in the regulation of cholesterol metabolism and serum cholesterol content.

Nevertheless, further studies in this field should be done in order to obtain a better understanding of the role of dietary protein in cholesterol metabolism. Studies in experimental animals and especially animals highly susceptible to changes in the diet might provide more insight in the mechanism underlying the cholesterolaemic effect of dietary protein. It should be borne in mind that some animal species do regulate cholesterol metabolism very differently from that in man. Furthermore, most animal species have serum lipoprotein patterns quite different from those in man. In contrast to man, they transport most of the serum cholesterol in the high density lipoproteins. On the other hand, exactly such differences between man and animals and between animals species might provide clues to the elucidation of the mechanisms underlying the long term effects of diet and nutrition on human performances.

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61

2. Improved Techniques for the Separation of Serum Lipoproteins by Density Gradient Ultracentrifugation: Visualization by Prestaining and Rapid Separation of Serum Lipoproteins from Small Volumes of Serum

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A method is presented for the visualization and localization of lipoprotein bands in density gradient ultracentrifugation. The lipoproteins are stained with Sudan black prior to ultracentrifugation. The technique is suitable for studying the lipoprotein patterns of normo- and hyperlipemic sera of both humans and experimental animals. The preparation and use of various types of density gradients are also described. By using smaller centrifuge tubes and high-speed rotors, a considerable saving in centrifugation time has been achieved. Formulae have been derived which facilitate the adjustment of the density of serum and the preparation of salt solutions of known density.

Methods have been reported for separating serum lipoproteins by a single ultracentrifugation in a density gradient (1,2). The samples are centrifuged in swinging-bucket rotors and the lipoprotein classes (VLDL, LDL, HDL, etc.)[4] band at their respective densities. Human lipoprotein bands can usually be observed as yellow disks, facilitating their removal from the centrifuge tube. However, this coloring is

rather variable and may not reveal the full range of lipoprotein bands. Moreover, the lipoproteins of several animal species (e.g., rabbit, rat, mouse, and calf) contain only small amounts of pigment and therefore are not visible in the gradient.

This difficulty may be overcome by prestaining the lipoproteins with Sudan black. Such a technique was described 20 years ago (3) but has not been widely used. More recently, Fat Red has been used as a stain in conjunction with differential ultracentrifugation of small amounts of serum (4). The present report describes simple and rapid procedures for prestaining lipoproteins with Sudan black prior to ultracentrifugation in readily prepared density gradients.

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[4] Abbreviations used: VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; Lp(a), sinking pre-β lipoprotein; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Sudan black B was obtained from George T. Gurr Ltd., London SW6, England. Ethylene glycol was from Boom B. V., 7940
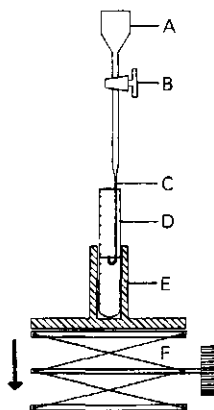
FIG. 1. Diagram of apparatus used for layering salt solutions. A, reservoir; B, tap; C, capillary; D, SW 41 centrifuge tube; E, tube holder; F, labjack.

AA Meppel, The Netherlands. The tube slicer was obtained from Nuclear Supply Company, Washington, DC. Ultracentrifugation was carried out in a Model L5-65 ultracentrifuge using either a SW 41 or a SW 50.1 rotor and cellulosenitrate tubes (Beckman Inc., Palo Alto Calif.). Densitometry was performed with a digital densitometer, Model DM 40 (Anton Paar K. G., Graz A-8054, Austria).

The Sudan black solution was prepared using a modification of the method described by Narayan (5). Sudan black (0.1 g) was added to 100 ml ethylene glycol at 65°C. The mixture was vigorously stirred for 2 min and filtered. A Sudan black solution can also be prepared by dissolving the stain in DMSO (2×10^{-4} g Sudan black/ml DMSO). However, DMSO appears to react to a small extent with the cellulosenitrate tubes, producing opaque spots. Therefore ethylene glycol is preferred, except when triglyceride is to be measured subsequently by methods involving the formation of formaldehyde (see Results).

Blood samples were taken by venipuncture from humans, rabbits, calves, and pigs, and by orbital puncture from mice. Some of

the rabbits had previously been made hyperlipemic by being fed a semipurified diet with casein as the protein source (6). The blood was allowed to clot for 1–2 h at room temperature and the serum was then separated by centrifugation at 1200g and 20°C for 15 min. Serum was stored at 4°C for a maximum of 4 days before ultracentrifugation.

KBr (0.770 g) and sucrose (0.050 g) were placed in a SW 41 cellulose–nitrate centrifuge tube on which 1-ml calibration marks had been drawn. Subsequently 2 ml of serum was pipetted into the tube and finally 0.2 ml of the Sudan black solution was added to prestain the serum. The components were carefully mixed with a spatula. The prestained serum, now with a background density of $\rho_{20} = 1.25$ g/ml (see Appendix I), was overlayered sequentially with 2 ml of a salt solution of $\rho_{20} = 1.225$ g/ml (11.42×10^{-3} g NaCl and 315.54×10^{-3} KBr/ml), 4 ml of a salt solution of $\rho_{20} = 1.10$ g/ml (11.42×10^{-3} g NaCl and 133.48×10^{-3} g KBr/ml), and 4 ml distilled water (see Appendix II). All solutions contained 10^{-4} g/ml ethylenediaminetetraacetic acid (disodium salt). The layering procedure was performed with a specially designed apparatus (Fig. 1). When starting to layer, the curved tip of the pipet is placed just under the surface of the previous layer. The tap is slowly opened and the tube is lowered by means of a labjack as the level of the liquid rises. The tubes were centrifuged for 22 h (including acceleration time and 1 h deceleration)) at 40,000 rpm (272,000 g) and 20°C. A low acceleration rate and no brake were used.

For the separation of lipoprotein classes from 1-ml serum samples, an SW 50.1 rotor was used. To the cellulosenitrate tube were added 114×10^{-3} g KBr, 25×10^{-3} g sucrose, 1 ml serum, and 0.1 ml Sudan black solution. The components were carefully mixed (final background density of $\rho_{20} = 1.10$ g/ml) and overlayered sequentially with 2.4 ml of a salt solution of $\rho_{20} = 1.06$ g/ml (11.42×10^{-3} g NaCl and 75.98×10^{-3} g KBr/ml) and 2.4 ml distilled water. After
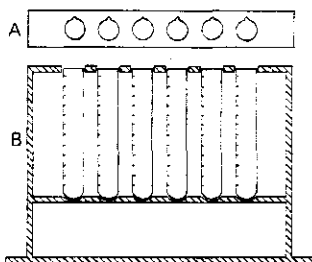
FIG. 2. Diagramatic representation of the rack used to mount the centrifuge tubes. A, from above, B, cross section. The rack was constructed from perspex of 4-mm thickness.

preparation, the gradients were spun for 7 h at 50,000 rpm (232,000g) and 20°C. The tubes were then placed in a specially designed rack (Fig. 2) and photographed. The conditions were as follows: lens, 55 mm, f/3.5 (Micro-Nikkor, Nikon, Tokyo 100, Japan); aperture, f/8; shutter speed, 1/4 s; distance, 30 cm; filter, red; illumination 12 cm behind the tubes from a diffuse light source (illuminator for X rays, Nemas B. V., 3768 BN Soest, The Netherlands); film, black-and-white negative (Agfapan 25, Agfa-Gevaert, D-5090 Leverkusen 1, West Germany).

The lipoprotein classes were removed by tube slicing and the volumes of the fractions were measured in calibrated tubes. Lipoprotein–cholesterol was estimated

enzymatically according to Röschlau et al. (7), using the kit (catalase method) supplied by Boehringer-Mannheim GmbH, West Germany. As cholesterol standards, three calibrated sera with low, medium, and high cholesterol concentrations were used; the cholesterol concentration of these sera was determined by the method of Abell et al. (8). To check for possible interference of the cholesterol determination by Sudan black, measurements were performed in whole serum and the separate lipoprotein fractions in the presence and absence of Sudan black and/or the stain solvent (ethylene glycol or DMSO). Some tests were carried out using the Abell method for cholesterol determination (8), the Lowry method for protein determination (9), and the triglyceride assay according to Soloni (10).

During centrifugation, the salt solution layers diffuse and a continuous gradient is formed. The shape of the final gradient was found by measuring with a digital densitometer the density of 1-ml fractions removed sequentially down the tube by tube slicing. Using the graphically presented form of the gradients (Figs. 3 and 4) and 1-ml fraction marks drawn on the tube before use (Fig. 5), the density range of the observed bands and the conventional boundaries of the different lipoprotein classes in the centrifuge tube could be estimated (VLDL,
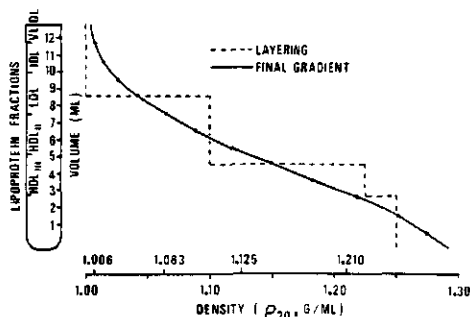

FIG. 3. The form of the density gradient in a SW41 centrifuge tube after 22-h centrifugation. Each value represents the mean of measurements in three tubes centrifuged at the same time.
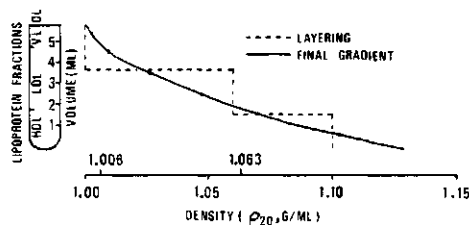
FIG. 4. The form of the density gradient in a SW 50.1 centrifuge tube after 7-h centrifugation. Each value represents the mean of measurements in three tubes centrifuged at the same time.

ρ_{20} < 1.0063 g/ml: IDL, 1.0063 < ρ_{20} < 1.019 g/ml; LDL, 1.019 < ρ_{20} < 1.063 g/ml; HDL, 1.063 < ρ_{20} < 1.21 g/ml.).

RESULTS

Stained lipoprotein bands, isolated from some human serum samples and from the sera of different species of animals, are shown in Fig. 5. In humans, four major classes are separated: VLDL at the top of the tube, LDL and HDL further down, and a heavily stained bottom fraction at the base. The lipoprotein classes can be observed as distinct bands and often two HDL subfractions are visible. The hydrated density of the LDL band is approximately 1.03 < ρ_{20} < 1.05 g/ml, whereas the two HDL bands have density ranges of approximately 1.08 < ρ_{20} < 1.10 g/ml and 1.10 < ρ_{20} < 1.13 g/ml, respectively. Figure 5B shows a serum sample with a Lp(a) or sinking pre-β band, which was verified by polyacrylamide gel electrophoresis (11). It is noteworthy that on occasions both the LDL and Lp (a) bands were subdivided into two minor components.

In most of the animals studied rather pronounced HDL bands were found, together with less stained and more diffuse LDL fractions. These visual impressions were reflected in the cholesterol concentrations in the various lipoprotein fractions (Table 1). The diffuse pattern of the LDL fraction in rabbits fed a commercial diet has also been reported by Pescador (12). However,

in rabbits made hypercholesterolemic by being fed semipurified diets containing casein the increased cholesterol was found mainly in the LDL band and to a lesser extent in the HDL fraction which often appeared as a double band (Fig. 5).

The serum lipoprotein pattern of the calf on a milk formula diet is unusual in that it shows a pronounced broad band of density intermediate between human LDL and HDL. This band was identified as an HDL by precipitation with heparin–MnCl$_2$ (13) and mobility in polyacrylamide gel electrophoresis.

Figure 6 shows the results of a 7-h centrifugation in a SW 50.1 rotor, using 1 ml serum from a human and from a hypercholesterolemic rabbit. It can be seen that a good separation of the major lipoprotein classes was achieved. Because of the nature of the
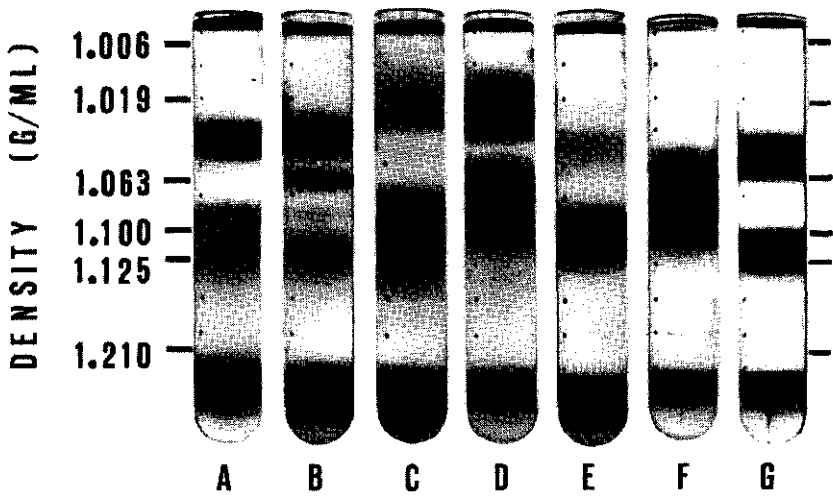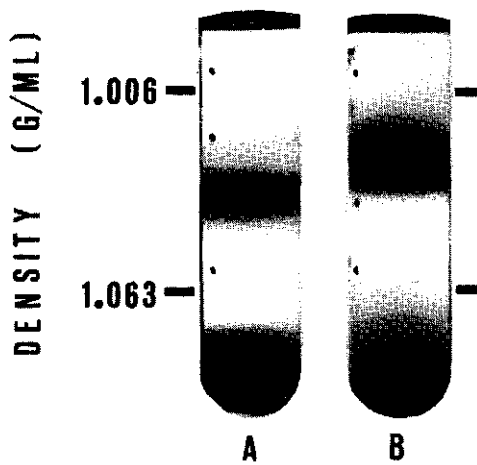
FIG. 5

FIG. 6

FIG. 5. Photograph of the lipoprotein bands isolated from prestained human serum (A), human serum with LP(a) band (B), normolipemic rabbit serum (C), hyperlipemic rabbit serum (D), mouse serum (E), calf serum (F), and Pig serum (G). Serum samples were ultracentrifuged for 22 h using a SW 41 rotor.

FIG. 6. Photograph of the lipoprotein bands isolated from prestained human serum (A) and hyperlipemic rabbit serum (B) after 7-h centrifugation using a SW 50.1 rotor.

sample is placed in the middle of the gradient. However, this can only be done satisfactorily when albumin-free lipoproteins or lipoprotein fractions are applied to the gradient as described by Anderson *et al.* (15). When whole serum is applied there is the possibility that the lipoprotein fractions in the lower part of the tube become contaminated with albumin since the sedimentation velocity of albumin is markedly reduced in a high-density medium. Therefore in these studies the sample was always placed in the bottom of the centrifuge tube.

The density of the medium down the tube was measured in 1-ml fractions in the presence and absence of serum. In the latter case, serum was replaced by a solution of NaCl (11.42×10^{-3} g/ml, ρ_{20} = 1.0063 g/ml). Identical results werre found under both conditions except that in the tubes containing serum, the bottom 1-ml fraction in the SW 50.1 tubes had a higher density. This might be due to the presence of serum proteins of high density such as albumin and HDL in a medium with a relatively low background density (ρ_{20} = 1.10 g/ml). Thus in practice the form of gradients can be measured in the presence or absence of serum. The gradients have been found to be highly reproducible.

Preparative isolation of serum lipoproteins is usually rather time consuming. However, the centrifugation time can be considerably reduced either by shortening the pathlength that the lipoproteins have to travel or by increasing the centrifugal force. For example, a 7-h spin with a SW 50.1 rotor results in a good separation of the major serum lipoprotein classes, whereas in the SW 41 rotor with a 76% longer pathlength of a 22-h centrifugation is required. However, with the short tubes of the SW 50.1 rotor it is not possible to completely separate the various lipoprotein fractions and albumin from one another. Therefore, the gradient has to be designed within a limited range to achieve the separation required. In the gradient described in this

paper VLDL, LDL, and HDL are separated from one another but the HDL fraction is included in the bottom fraction (see Fig. 6). As the tubes of the SW 50.1 rotor have a volume of about half that of the tubes of the SW 41 rotor, they also have the advantage that for a given volume of serum, the various lipoprotein fractions are not diluted to the same extent. Thus it is possible to visualize the various lipoprotein bands in a gradient using smaller volumes of serum (e.g., 1 ml). The more sensitive enzymatic methods for determining cholesterol make it possible to determine the cholesterol concentration in lipoprotein fractions isolated from smaller volumes of serum and also in very dilute fractions.

In conclusion, prestaining the serum lipoproteins with Sudan black in combination with density gradient ultracentrifugation is a helpful device in the investigation of serum lipoproteins from both humans and animals. Furthermore, the use of a suitable rotor makes it possible to separate the lipoproteins from small serum samples in a short time.

APPENDIX I: RAISING THE DENSITY OF SERUM BY ADDING KBr, SUCROSE, AND ETHYLENE GLYCOL

It can be assumed that about 6% of the serum volume consists of macromolecules (protein and lipoprotein) and that the remaining 94% serum volume is equivalent to a solution of NaCl, 11.42×10^{-3} g/ml; the density of the solution (ρ_i = the initial density) is 1.0063 g/ml (14). Calculations are based on all manipulations being carried out at 20°C; relative densities of the solutes are used.

After adding solid KBr, the final density (ρ_f) of the macromolecule-free serum is

$$\rho_f \approx \frac{M_{\text{total}}}{V_{\text{total}}} = \frac{M_{H_2O} + M_{\text{NaCl}} + M_{\text{KBr}}}{V_{H_2O} + V_{\text{NaCl}} + V_{\text{KBr}}}$$

$$\approx \frac{M_{H_2O} + M_{\text{NaCl}} + M_{\text{KBr}}}{V_{H_2O} + \dfrac{M_{\text{NaCl}}}{\rho_{\text{NaCl}}} + \dfrac{M_{\text{KBr}}}{\rho_{\text{KBr}}}},$$
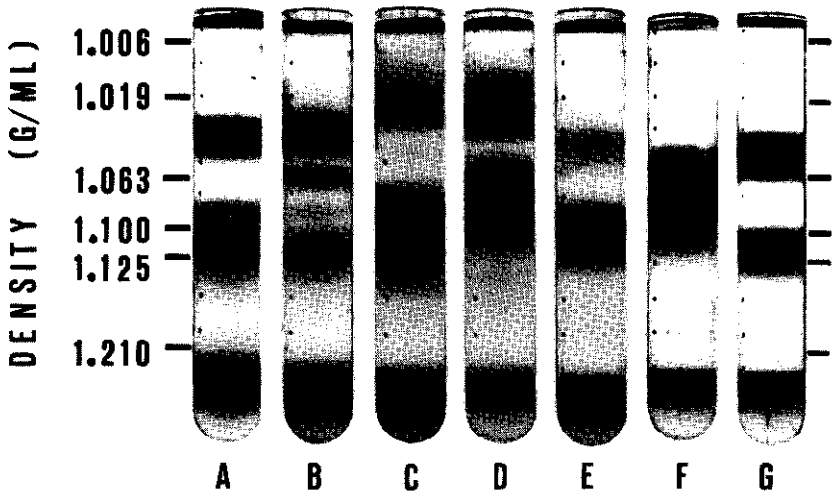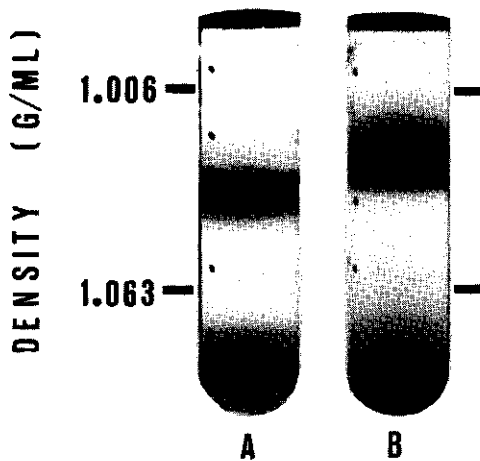
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TABLE 1

The Distribution of Cholesterol between
Lipoprotein Fractions of Serum from
Various Animals

Fraction[a]	Cholesterol concentration (mg/100 ml)				
		Rabbit			
	Mouse	1[b]	2[c]	Calf	Pig
VLDL	9	7	22	0	2
LDL	12	12	116	2	36
HDL	83	17	34	134	28
$\rho_{20>1.21}$	7	3	6	2	4
Total	111	39	178	138	70
Whole serum	113	41	183	139	75
Recovery (%)	98	95	97	99	93

[a] Fractions were collected on the basis of the appearance of the bands following ultracentrifugation.

[b] Receiving diet of commercial rabbit pellets.

[c] Receiving semipurified diet containing 20% casein for 1 month.

gradients, the HDL remains with the bottom fraction in this ultracentrifugation, while the LDL bands are approximately in the middle of the tube, and the VLDL at the top. As observed in Fig. 5, the LDL band of rabbit serum is somewhat lighter than that from human samples.

Sudan black has no effect on the estimation of cholesterol by the enzymatic method in either whole serum or the individual lipoprotein fractions (Table 2). In these tests, ethylene glycol was added to the unstained sera before ultracentrifugation in order to achieve the same density as in the stained samples. However, since the unstained sera used for cholesterol determination contained no ethylene glycol, an effect of ethylene glycol itself on the determination is unlikely.

In other studies, there was no detectable effect of Sudan black staining in the lipoprotein fractions, on either the method of Abell *et al.* for cholesterol determination (8) or on the Lowry method for protein determination

(9). However, when triglyceride is determined by the procedure of Soloni (10), the presence of ethylene glycol produced erroneously high results. As this method involves the estimation of formaldehyde liberated from the glycerol moiety of the triglyceride molecule, ethylene glycol presumably also gives rise to formaldehyde. In this case DMSO may be used as a stain solvent without any interference being observed.

DISCUSSION

Density gradient ultracentrifugation (equilibrium or isopycnic) is widely employed for the isolation and subfractionation of serum lipoproteins. One advantage of this technique compared with differential ultracentrifugation (14) is that fixed density limits need not be used to isolate the different lipoprotein classes. By staining the lipoproteins with Sudan black prior to ultracentrifugation, the lipoprotein bands can be readily detected in the density gradient and isolated using their observed positions. This is useful since, as shown in Fig. 5, the conventional density limits employed in the investigation of human serum lipoproteins are not equally applicable to other species. For example, a major lipoprotein band in rabbit serum lies partly in the conventional density range for LDL and partly in that

TABLE 2

CHOLESTEROL CONCENTRATIONS IN SERA AND LIPOPROTEIN FRACTIONS OF THREE HUMAN SAMPLES
IN THE PRESENCE OF EITHER ETHYLENE GLYCOL ALONE (UNSTAINED) OR SUDAN BLACK
DISSOLVED IN ETHYLENE GLYCOL (STAINED)

| Fraction[a] | Cholesterol concentration (mg/100 ml) | | | | | |
| | Sample 1 | | Sample 2 | | Sample 3 | |
	Unstained	Stained	Unstained	Stained	Unstained	Stained
VLDL	14	15	9	10	9	9
LDL	88	89	101	100	77	75
HDL	67	66	64	66	77	75
$\rho_{20>1.21}$	12	12	12	12	10	10
Total	181	182	186	188	173	169
Whole serum	178	179	190	189	171	175
Recovery (%)	102	102	98	99	101	97

Note. The unstained whole serum contained no ethylene glycol.

[a] Fractions were collected on the basis of the conventional density limits for human lipoproteins.

for IDL, although there is no reason to think that this lipoprotein is not homogeneous. Similarly, calf serum contains a lipoprotein band of density intermediate between human LDL and HDL classes. Another advantage of prestaining is that the minor lipoprotein classes, e.g., Lp(a), or lipoprotein subfractions, can be visualized; such bands would probably otherwise be overlooked.

In this report, the isolation of all lipoprotein classes in a single ultracentrifugation is described. However, in many instances only a single lipoprotein class, or the subfractions thereof, is required. For such applications special gradients may be constructed, for example, over the range $1.0063 < \rho_{20} < 1.10$ g/ml for the isolation of VLDL and the subfractionation of LDL, or alternatively over the range $1.05 < \rho_{20} < 1.25$ g/ml for the subfractionation of HDL. These techniques have been successfully used in this laboratory.

Two HDL bands are often seen when prestained serum from humans is separated by density gradient ultracentrifugation. The densities of these two bands range

approximately from $\rho_{20} = 1.08$ to 1.10 g/ml and from $\rho_{20} = 1.10$ to 1.13 g/ml. HDL are conventionally subdivided into HDL_{11} ($1.063 < \rho_{20} < 1.125$ g/ml) and HDL_{111} ($1.125 < \rho_{20} < 1.21$ g/ml). However, more recently Anderson *et al.* (15) provided evidence for the existence of three subfractions, HDL_{11a} ($1.063 < \rho_{20} < 1.10$ g/ml), HDL_{11b} ($1.10 < \rho_{20} < 1.125$ g/ml), and HDL_{111} ($1.125 < \rho_{20} < 1.21$ g/ml). These findings could not be confirmed by Cheung and Albers (16), employing CsCl equilibrium gradient ultracentrifugation. They observed only two absorption peaks in the HDL spectrum, one in the density range of $\rho = 1.12–1.13$ g/ml and the second at approximately $\rho_{20} = 1.08–1.09$ g/ml. These latter results are in good agreement with our findings and therefore a subfractionation of HDL at $\rho_{20} = 1.10$ g/ml may provide a better morphological and perhaps physiological basis for studies of this lipoprotein class than the conventional density limit at $\rho_{20} = 1.125$ g/ml.

Techniques for density gradient ultracentrifugation have previously been described in which the serum or lipoprotein

sample is placed in the middle of the gradient. However, this can only be done satisfactorily when albumin-free lipoproteins or lipoprotein fractions are applied to the gradient as described by Anderson *et al.* (15). When whole serum is applied there is the possibility that the lipoprotein fractions in the lower part of the tube become contaminated with albumin since the sedimentation velocity of albumin is markedly reduced in a high-density medium. Therefore in these studies the sample was always placed in the bottom of the centrifuge tube.

The density of the medium down the tube was measured in 1-ml fractions in the presence and absence of serum. In the latter case, serum was replaced by a solution of NaCl (11.42×10^{-3} g/ml, $\rho_{20} = 1.0063$ g/ml). Identical results werre found under both conditions except that in the tubes containing serum, the bottom 1-ml fraction in the SW 50.1 tubes had a higher density. This might be due to the presence of serum proteins of high density such as albumin and HDL in a medium with a relatively low background density ($\rho_{20} = 1.10$ g/ml). Thus in practice the form of gradients can be measured in the presence or absence of serum. The gradients have been found to be highly reproducible.

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After adding solid KBr, the final density (ρ_f) of the macromolecule-free serum is

$$\rho_f = \frac{M_{total}}{V_{total}} = \frac{M_{H_2O} + M_{NaCl} + M_{KBr}}{V_{H_2O} + V_{NaCl} + V_{KBr}}$$

$$= \frac{M_{H_2O} + M_{NaCl} + M_{KBr}}{V_{H_2O} + \dfrac{M_{NaCl}}{\rho_{NaCl}} + \dfrac{M_{KBr}}{\rho_{KBr}}},$$

where M, V, and ρ are the mass (g), volume (ml), and density (g/ml) of the components, respectively. Substituting $\bar{v}_{NaCl}$ and $\bar{v}_{KBr}$ (partial specific volume, ml/g) for $1/\rho_{NaCl}$ and $1/\rho_{KBr}$, respectively, and then solving for M_{KBr} gives

M_{KBr}

$= \dfrac{\rho_f(V_{H_2O} + \bar{v}_{NaCl}\cdot M_{NaCl}) - M_{H_2O} - M_{NaCl}}{1 - \bar{v}_{KBr}\cdot\rho_f}$.

The partial specific volume of a salt in solution is dependent on the density of the solution (17). Assuming that the change in $\bar{v}_{NaCl}$ after increasing the density by adding KBr is negligible, then $V_{H_2O} + \bar{v}_{NaCl}\cdot M_{NaCl}$ be-

comes equal to the initial volume of the NaCl solution, V_i; $M_{H_2O} + M_{NaCl}$ is the initial mass, which equals $V_i\rho_i$.

Thus the formula can be simplified:

$$M_{KBr} = \frac{V_i(\rho_f - \rho_i)\cdot}{1 - \bar{v}_{KBr}\cdot\rho_f}.$$

Therefore the mass of KBr (g) required to increase the density of 1 ml serum to final density ρ_f is

$$M_{KBr} = \frac{0.94(\rho_f - 1.0063)}{1 - \bar{v}_{KBr}\cdot\rho_f}.$$

The partial specific volume of KBr($\bar{v}_{KBr}$) can be calculated from published data (18) by using the following formula:

$$\bar{v}_{KBr} = \frac{(M_{H_2O}\text{ displaced by KBr per milliliter KBr solution with }\rho_f)/\rho_{H_2O}}{M_{KBr}\text{ per milliliter KBr solution with }\rho_f},$$

where $\rho_{H_2O} = 0.9982$ g/ml. Similarly, it can be shown that after adding 0.1 ml (0.111 g) ethylene glycol (etgl) and 0.025 g sucrose (suc) to 1 ml serum, the mass (g) of KBr needed to obtain a required final background density ρ_f of the mixture is

$$M_{KBr} = \frac{0.94(\rho_f - 1.0063) + \rho_f\cdot\bar{v}_{etgl}\cdot(0.111) + \rho_f\cdot\bar{v}_{suc}\cdot(0.025) - 0.111 - 0.025}{1 - \bar{v}_{KBr}\cdot\rho_f}.$$

The partial specific volumes ($\bar{v}_{etgl}$, $\bar{v}_{suc}$, and $\bar{v}_{KBr}$) can be calculated as shown before.

APPENDIX II: PREPARATION OF KBr SOLUTIONS OF REQUIRED DENSITIES CONTAINING A CONSTANT CONCENTRATION OF 11.42 × 10⁻³ g NaCl/ml

In order to increase the density of a NaCl solution containing 11.42×10^{-3} g NaCl/ml ($\rho_i = 1.0063$ g/ml), so that the final concentration of NaCl will be maintained after the addition of KBr, a given volume of H_2O must be displaced by KBr. After displacing H_2O by KBr (at constant volume) the final

density ρ_f is

$$\rho_f = \frac{M_{total}}{V_{total}} = \frac{M_{H_2O} + M_{NaCl} + M_{KBr}}{V_{H_2O} + V_{NaCl} + V_{KBr}},$$

where M, V, and ρ are the mass (g), volume (ml), and density (g/ml) of the components, respectively. In the final solution a given volume of H_2O is displaced by KBr, therefore

$$V_{f(H_2O)} = V_{i(H_2O)} - V_{KBr},$$

where $V_{f(H_2O)}$ is the final volume of H_2O and $V_{i(H_2O)}$ is the initial volume of H_2O. Substituting $M_{NaCl}\cdot\bar{v}_{NaCl}$ for V_{NaCl} and $M_{KBr}\cdot\bar{v}_{KBr}$ for V_{KBr} and solving for M_{KBr} gives

$$M_{KBr} = \frac{\rho_f(V_{i(H_2O)} + \bar{v}_{NaCl}\cdot M_{NaCl}) - M_{i(H_2O)} - M_{NaCl}}{1 - \bar{v}_{KBr}\cdot\rho_{H_2O}},$$

where $M_{i(H_2O)}$ is the initial mass of H_2O. This formula can be simplified by assuming that the change in the partial specific volume of $NaCl(\bar{v}_{NaCl})$ after increasing the density is negligible. This means that the factor $V_{i(H_2O)} + \bar{v}_{NaCl} \cdot M_{NaCl}$ becomes equal to the initial volume of the NaCl solution (V_i).
Thus

$$M_{KBr} = \frac{\rho_f \cdot V_i - M_i}{1 - \bar{v}_{KBr} \cdot \rho_{H_2O}} = \frac{V_i(\rho_f - \rho_i)}{1 - \bar{v}_{KBr} \cdot \rho_{H_2O}} \ ,$$

where M_i is the total initial mass. Therefore, the mass (g) of KBr to be dissolved per milliliter of solution to obtain a salt solution of required density, in the presence of a constant concentration of NaCl (11.42×10^{-3} g/ml), can be calculated with the following formula:

$$M_{KBr} = \frac{1(\rho_f - 1.0063)}{1 - \bar{v}_{KBr} \cdot (0.9982)} \ .$$

The partial specific volume of $KBr(\bar{v}_{KBr})$ can be calculated as shown before.

ACKNOWLEDGMENTS

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3. TIME COURSE OF THE DEVELOPMENT OF HYPERCHOLESTEROLEMIA IN RABBITS FED SEMIPURIFIED DIETS CONTAINING CASEIN OR SOYBEAN PROTEIN

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SUMMARY

The time course of the development of hypercholesterolemia was studied in rabbits fed semipurified diets containing casein or soybean protein. When the rabbits were transferred from a commercial diet to semipurified diets, a rapid increase in the concentration of serum cholesterol and phospholipids occurred. After only one day on the semipurified diets, the cholesterol and phospholipid levels increased by about 50% on the diet containing soybean protein and more than doubled on the diet containing casein. Further elevations were observed after one and two weeks on the diets. However after one month, a decrease in the group on the diet containing soybean protein was found. The increases in serum cholesterol and the differences between the soybean protein and casein group were mainly attributable to differences in the LDL fraction and to a minor extent to differences in the HDL fraction.
The feeding of semipurified diets resulted in a steep increase in the ratio of cholesterol to protein in all the lipoprotein fractions after only one day. This suggests that lipoprotein particles relatively rich in cholesterol were formed. Marked variations in the density profile of the serum lipoproteins were observed between individual rabbits fed semipurified diets.

INTRODUCTION

It has been known for a long time that feeding casein to rabbits results in atherosclerosis, whereas this does not occur when soybean protein is incorporated in the diets (1,2). Recent studies have also provided strong evidence that the protein source in the diet of rabbits plays an important role in the

regulation of cholesterol metabolism. Carroll (3) has studied the effect of a
large variety of dietary proteins on the concentration of serum cholesterol in
rabbits and found that some proteins, such as casein were hypercholesterolemic,
while other proteins, for example soybean protein were able to maintain rela-
tively low levels of serum cholesterol. Hermus (4) showed that in rabbits, the
replacement of casein in semipurified diets by a mixture of casein, gelatin
and fish protein resembling the amino acid composition of a commercial diet
resulted in a reduction of the concentration of serum cholesterol.
Kritchevsky *et al.* (5) also reported that semipurified diets containing casein
induced elevated levels of serum cholesterol compared with diets containing
soybean protein.

The serum lipoproteins of rabbits fed semipurified diets containing casein
or soybean protein have been characterized by several authors (4,6,7,8).
However, the mechanism underlying the hypercholesterolemic properties of semi-
purified diets containing casein compared with semipurified diets containing
soybean protein is still poorly understood.
The present study was undertaken to obtain an insight into the etiology of
hypercholesterolemia induced by feeding casein. Therefore, the time course of
the changes in the lipoprotein pattern was studied when rabbits were trans-
ferred from a commercial diet to semipurified diets containing either casein
or soybean protein. Changes both in the density profile and the composition of
the serum lipoproteins are reported.

MATERIALS AND METHODS

In the experiment two groups of 6 male New Zealand White rabbits, aged
13 weeks and weighing about 1800 grams, were used. The animals were housed in-
dividually in cages with wire mesh bases constructed of galvanised steel and
were kept in a room with airconditioning and controlled lighting (12 h/day).
The rabbits had been fed commercial rabbit pellets (Trouw and Co N.V.
3881 LP Putten, The Netherlands) and were subsequently transferred, without
an adaptation period, to pelleted semipurified diets containing casein and soy-
bean protein, respectively. The composition of the diets was similar to that
described previously (4,9) and contained (g/kg): maize starch 360, maize oil 10,
coconut oil 138, casein or soybean protein 200, saw dust 210, vitamin premix 12,
mineral premix 10, $KHCO_3$ 18, $Ca_2HPO_4.2H_2O$ 29, NaCl 8, MgO 2 and $MgCO_3$ 3.
Water was provided *ad libitum* but the food intake was restricted to a maximum
of 100 g/day. The animals were fed daily at 9.00 a.m.

Blood samples from non-fasted animals were collected ten days before the change-over to the semipurified diets and 1, 7, 14 and 31 days after the change-over.

Blood samples were taken from a marginal ear vein between 9.00 and 11.00 a.m. and the rabbits were weighed immediately afterwards. The serum lipo-protein classes were isolated by density gradient ultracentrifugation using a modification (10) of the method of Redgrave *et al.*(11). The lipoproteins in the gradient were visualized by prestaining the serum with Sudan Black prior to ultracengrifugation. The various lipoprotein fractions were collected by tube slicing on the basis of their appearance in the gradient. The concentration of cholesterol in the fractions was determined by the method of Abell *et al.* (12) and protein by a method based on that of Lowry *et al.* (13) in which the lipids were removed by extraction with diethyl ether immediately prior to the measure-ment of the optical density. The concentration of lipoprotein phosphorus was measured on a lipid extract (14) as described by Bartlett (15) and modified by Böttcher *et al.* (16).

The results were analysed statistically using a modified Student's one--tailed t-test (17).

RESULTS

Body weight

The body weight and weight gain of the rabbits during the experiment are presented in Table 1. As a result of the restricted feeding regime, a similar growth rate was obtained in both experimental groups.

Table 1
BODY WEIGHTS OF RABBITS FED SEMIPURIFIED DIETS CONTAINING CASEIN OR SOYBEAN PROTEIN

	Body weight (g)				Weight gain (g/day)
	Day 1[a]	Day 7	Day 14	Day 31	
Casein	1869 ± 59^{b}	1979 ± 52	2244 ± 35	2657 ± 52	25 ± 1.8
Soybean protein	1839 ± 74	1883 ± 63	2160 ± 58	2566 ± 67	23 ± 0.5

a. Animals were changed from the commercial diet to the semipurified diets on day 0 of the experiment.

b. Values expressed as mean $\pm$ S.E.M. (6 animals/group).

Serum cholesterol and phospholipids

When the rabbits were transferred from a commercial rabbit diet to the semi-purified diets, a rapid elevation in the concentration of serum cholesterol was observed (Table 2). Irrespective of the protein source in the diets, significantly higher levels of serum cholesterol were observed one day after the change-over (P < 0.01). However, this increase was more pronounced in the rabbits fed diets containing casein than in those on diets containing soybean protein (P < 0.01). After one and two weeks on the experimental diets, a further increase in the concentrations of serum cholesterol was found in both groups. Subsequently a decline occurred in the soybean protein group (P < 0.01), whereas the concentration of serum cholesterol in the casein group remained high. Similar patterns of response in the concentration of serum phospholipids also occurred (Table 2).

Density profile of the serum lipoproteins

Fig. 1 shows the density profile of the serum lipoproteins of a normolipemic rabbit compared with the density profile of the lipoproteins of a normolipemic human serum sample observed by density gradient ultracentrifugation. It can be seen that the density profile of the lipoproteins of a normolipemic rabbit is different from that observed in humans. The LDL band of rabbit serum was found to have a density range intermediate between the density limits of the IDL (1.006 < d < 1.019) and LDL (1.019 < d < 1.063) as defined for human serum lipoproteins while no large differences in density were observed between the HDL fractions of human and rabbit serum. When the rabbits were fed semipurified diets containing casein or soybean protein marked changes in the density profile occurred and variations between individual rabbits were also observed (Fig. 1). Both the LDL and HDL band showed large individual differences in their position in the gradient (Fig. 1). No such variation between rabbits was found when commercial diets were fed. It is noteworthy that in several cases the HDL fraction was observed as two distinct bands both in hyperlipemic and normolipemic rabbits. These two bands may be analogues to the HDL_{II} and HDL_{III} observed in human serum.

Composition of the serum lipoproteins

When the rabbits were fed a commercial diet, most of the serum cholesterol was found in the HDL fraction. However, the ingestion of the semipurified diets caused a steep elevation of the levels of LDL cholesterol whereas relatively minor changes occurred in the HDL fraction (Table 2). No marked
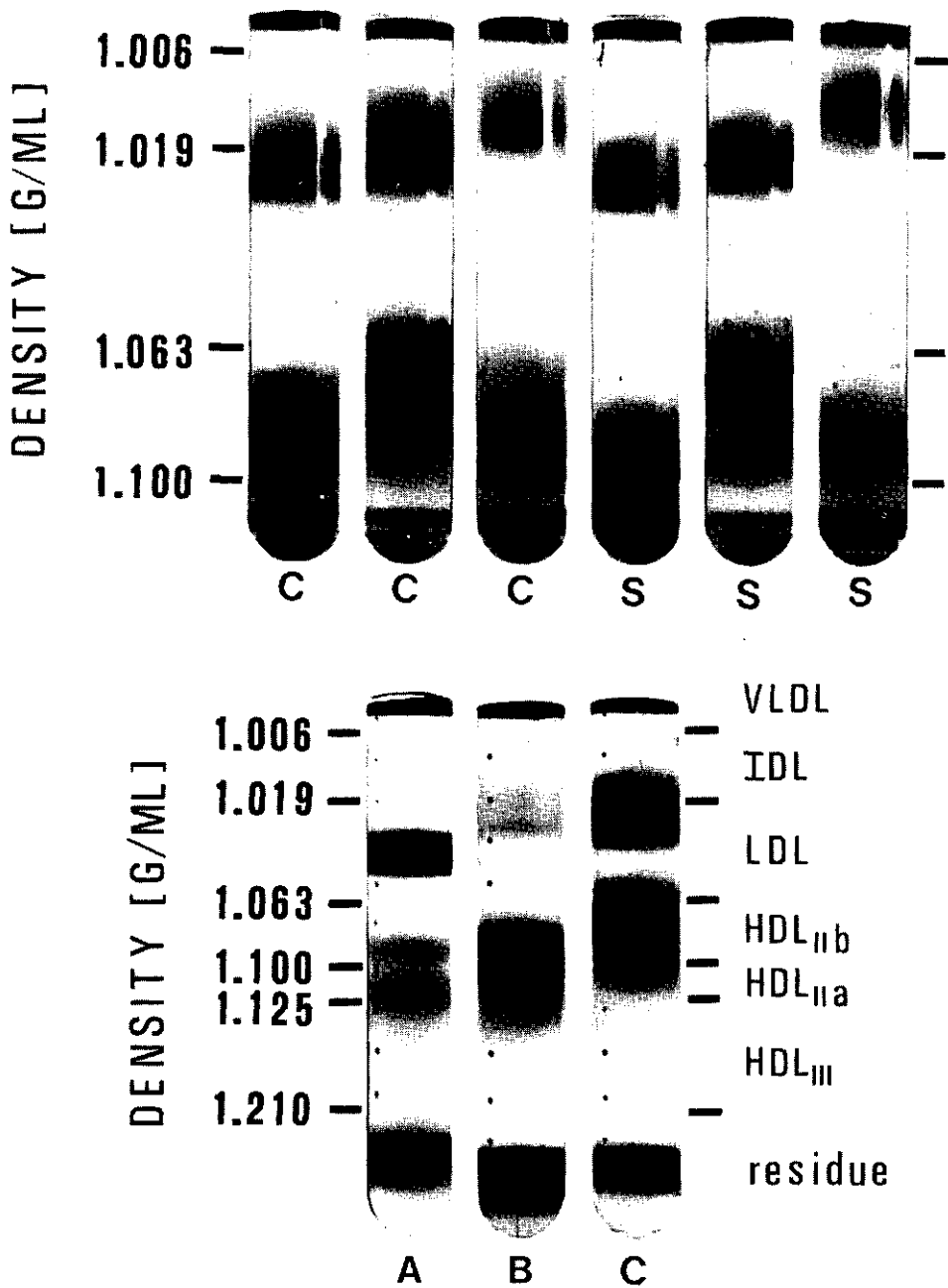
Fig. 1. Photograph of the density profile of serum lipoproteins observed after density gradient ultracentrifugation; (A) normocholesterolemic human, (B) normocholesterolemic rabbit and rabbits fed semipurified diets containing either (C) casein or (S) soybean protein.

TABLE 2

CONCENTRATION OF CHOLESTEROL AND PHOSPHOLIPIDS IN WHOLE SERUM AND CONCENTRATION OF CHOLESTEROL, PHOSPHOLIPIDS AND PROTEIN AND RATIO OF CHOLESTEROL TO PROTEIN IN THE LIPOPROTEIN FRACTION OF RABBITS FED SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN

	Day -10[a]	Day 1	Day 7	Day 14	Day 31
Cholesterol					
Casein					
VLDL	5.4 ± 1.8[b,c,d]	3.7 ± 0.7	11.2 ± 2.8	14.6 ± 5.9	12.3 ± 3.8
LDL	12.2 ± 1.2[+++]	49.1 ± 5.0[x]	71.3 ± 14.3	70.2 ± 15.4	76.6 ± 12.6[xx]
HDL	17.0 ± 2.2[++]	34.8 ± 3.2	47.7 ± 6.3	47.7 ± 6.6	49.9 ± 6.2
Whole serum	40.2 ± 4.2[+++]	91.0 ± 5.2[xx]	140.1 ± 21.0	152.8 ± 14.0[xx]	146.1 ± 14.9[xx]
Soybean protein					
VLDL	6.8 ± 2.0	3.5 ± 0.5	8.2 ± 1.6	15.1 ± 4.7	5.6 ± 1.2
LDL	14.8 ± 1.9[++]	32.0 ± 4.1	46.3 ± 5.1	39.3 ± 5.8	26.9 ± 1.9
HDL	20.3 ± 1.6	26.9 ± 4.0	41.8 ± 7.9	34.5 ± 7.1	35.2 ± 6.6
Whole serum	45.8 ± 2.8[++]	68.2 ± 4.0	100.0 ± 14.0	98.8 ± 5.2	72.2 ± 3.8
Phospholipids					
Casein					
VLDL	2.2 ± 1.3[b,c,d]	2.3 ± 0.6	5.7 ± 1.9	11.0 ± 4.6	7.2 ± 2.4
LDL	7.9 ± 1.0[++]	41.4 ± 7.2	57.3 ± 16.5	64.7 ± 14.7	45.7 ± 6.4[xx]
HDL	31.3 ± 3.2[++]	59.8 ± 6.1	93.3 ± 14.7	106.7 ± 12.1	98.1 ± 10.5[x]
Whole serum	49.3 ± 5.2[+++]	105.4 ± 5.7	149.1 ± 18.5	185.2 ± 24.1	164.2 ± 8.2[xx]
Soybean protein					
VLDL	3.9 ± 2.3	2.1 ± 0.7	8.1 ± 1.8	10.7 ± 5.3	3.4 ± 0.6
LDL	8.9 ± 1.3[+]	24.1 ± 6.4	34.6 ± 4.8	40.9 ± 9.0	18.2 ± 2.8
HDL	36.3 ± 2.1[+]	52.2 ± 6.1	93.3 ± 13.5	77.5 ± 10.6	59.7 ± 8.4
Whole serum	57.4 ± 7.4[+]	84.5 ± 9.1	131.5 ± 14.0	138.5 ± 12.4	99.6 ± 10.8

Protein					
Casein					
VLDL	7.3 ± 1.4^{b,c,d}	3.0 ± 0.2	5.2 ± 0.9	5.9 ± 1.5	6.4 ± 1.4
LDL	24.9 ± 4.6	33.3 ± 2.8	52.2 ± 9.8	54.1 ± 7.3^x	46.8 ± 5.5^{xx}
HDL	87.8 ± 9.9	93.7 ± 6.1	130.6 ± 17.8	139.1 ± 15.2	150.3 ± 9.2^x
Soybean protein					
VLDL	10.8 ± 2.9	3.5 ± 0.4	6.8 ± 1.1	10.8 ± 3.0	3.9 ± 0.8
LDL	24.3 ± 2.6	24.9 ± 2.3	36.6 ± 3.3	35.3 ± 5.3	22.5 ± 2.4
HDL	99.9 ± 6.1	92.2 ± 6.6	137.4 ± 17.7	121.8 ± 17.6	110.6 ± 9.0
Cholesterol: protein ratio					
Casein					
VLDL	0.65 ± 0.14$^{b,d,+}$	1.25 ± 0.18	2.03 ± 0.23^x	2.23 ± 0.38^x	1.79 ± 0.31
LDL	0.54 ± 0.07^{+++}	1.47 ± 0.06	1.34 ± 0.10	1.23 ± 0.14	1.39 ± 0.07
HDL	0.20 ± 0.03^{++}	0.37 ± 0.02^x	0.38 ± 0.05	0.35 ± 0.04	0.33 ± 0.03
Soybean protein					
VLDL	0.61 ± 0.09^{++}	1.01 ± 0.05	1.23 ± 0.15	1.38 ± 0.15	1.65 ± 0.30
LDL	0.63 ± 0.07^{++}	1.29 ± 0.10	1.26 ± 0.06	1.12 ± 0.03	1.30 ± 0.24
HDL	0.21 ± 0.02^{+}	0.29 ± 0.02	0.29 ± 0.02	0.28 ± 0.03	0.31 ± 0.04

a,b. See foonotes a, b respectively of Table 1.

c. Values expressed as mg/dl of whole serum.

d. Comparison by Student's one-tailed t-test:

between rabbits fed diets containing casein and soybean protein: ^{x} P < 0.05; ^{xx} P < 0.01;

between rabbits on same diet at Day 1 and Day -10: $^+$ P < 0.05; $^{++}$ P < 0.01; $^{+++}$ P<0.001.

changes were found in the VLDL class. The differences in serum cholesterol during the experiment between the rabbits fed diets containing casein and soybean protein were mostly reflected in the differences of the cholesterol content in the LDL fraction and to a smaller extent in the HDL fraction.

Throughout the experiment most of the serum phospholipids was transported in the HDL fraction (Table 2). Increases in serum phospholipids as response to the feeding of semipurified diets were attributable to elevated levels in both the HDL and LDL fractions. No marked changes were observed in the VLDL fractions.

Compared to the marked increases in the levels of the HDL and LDL cholesterol and phospholipids, minor changes in the protein component were observed (Table 2) This resulted in a significant elevation of the ratio of cholesterol to protein only one day after the change-over. Due to an initial decrease of the protein concentration in the VLDL fractions of both experimental groups (P < 0.05), an increased ratio of cholesterol to protein was found also in this lipoprotein fraction (Table 2). The most striking changes in the ratio of cholesterol to protein in the HDL and LDL fractions were observed during the change-over from the commercial diet to the semipurified diets. Later on relatively minor alterations in this ratio were found. Although the differences were not always significant, the ratios in all the lipoprotein fractions of the rabbits fed diets containing casein during the experiment were higher than in the lipoproteins of the group fed diets containing soybean protein.

DISCUSSION

The feeding of the semipurified diets to rabbits resulted in elevated levels of serum cholesterol and phospholipids and marked alterations in the composition of the lipoproteins. However, these changes were more pronounced in the casein group than in the rabbits fed diets containing soybean protein. Thus two major effects were observed. Firstly, alterations produced by the semipurified diets and secondly a differential effect of the protein component in the diets. The composition of the commercial diet differs in many aspects from the semipurified diets. As components other than protein have also been found to play an important role in the regulation of the cholesterol metabolism in rabbits (5,18), these differences in the diets may be important.

The most striking changes in the composition of the LDL and HDL fractions in both the group fed casein and soybean protein occurred during the change-over from the commercial diet to the semipurified diets. During this period a steep increase in the ratio of cholesterol to protein was found. Later on, relatively minor alterations in this ratio were observed despite considerable changes with-

in each group in the levels of cholesterol. This might indicate that initially the principal change was in the composition of the LDL and HDL fractions while subsequently, changes in the number of lipoprotein particles were probably a more relevant factor.

Remarkable is the rapidity with which the changes in the levels of serum cholesterol and phospholipids and the composition of the lipoproteins induced by diet occurred. Other experiments in our laboratory have shown that within 12 hours after the ingestion of semipurified diets, these changes were already detectable (A.H.M. Terpstra and C.J.H. Woodward, unpublished data). Rapid responses to changes in diet have also been found by other investigators. Portman and Alexander (19) reported that large alterations in the metabolism of serum lipoproteins could be detected in rabbits from 24 to 48 h after adding cholesterol to the diet. The rapidity of the changes induced by the feeding of semipurified diets would indicate that mechanisms such as alterations in the intestinal flora and transit time are not involved.

No significant changes and differences between the casein and soybean protein group were found in the concentration of the VLDL cholesterol. This might be explained by the rather low levels of total serum cholesterol observed in this experiment. Brattsand (20), using similar casein diets, observed that most of the serum cholesterol was transported in the LDL fraction when the levels of total serum cholesterol were lower than 600 mg/100 ml. At higher levels of total serum cholesterol, the VLDL became the major carrier of cholesterol. Lacombe and Nibbelink (8) found that rabbits fed diets containing casein and with a level of total serum cholesterol of about 100 mg/100 ml also had only slightly higher concentrations of VLDL cholesterol compared with rabbits fed a commercial diet, while no differences at all were found between rabbits fed diets containing either casein and soybean protein.

Despite the absence of significant changes in the level of VLDL cholesterol when the rabbits were fed semipurified diets, marked elevations were observed in the ratio of cholesterol to protein in this lipoprotein fraction. Moreover, this ratio was higher in the rabbits fed casein than soybean protein. It is noteworthy that in the LDL fraction minor differences in this ratio between the casein and soybean protein group were observed together with major differences in the level of cholesterol. These findings are consistent with those reported by Lacombe and Nibbelink (8).

In rabbits hypercholesterolemia can also be induced by feeding cholesterol (6) and by starvation (21-24). During a prolonged fast, the rabbit exhibits hypercholesterolemia which appears to be associated with an elevation in LDL

cholesterol. This could be ascribed to a decreased clearance of plasma chole-
sterol (25) and to a mobilization of tissue cholesterol (24). The feeding of
cholesterol results primarily in an elevation of the VLDL cholesterol level
which has been shown to be caused by an inhibited catabolism of remnants of
chylomicrons rich in cholesterol esters (26,27). However, the mechanism of
hypercholesterolemia due to feeding casein is not clear. The accumulation of
cholesterol in the LDL fraction, as found in our study, might suggest an im-
paired clearance of LDL cholesterol similar as found in fasting rabbits (25).
Other studies have shown that rabbits fed casein do have a lower excretion of
faecal neutral sterols than when fed soybean protein (28). Furthermore chole-
sterol turnover studies have revealed a lower excretion of cholesterol in
rabbits fed casein diets compared with rabbits on diets containing soybean
protein (29). Further studies should be carried out on this subject.

In order to study the early changes in lipoprotein composition the rabbits
were switched from the commercial diet to the semipurified diets without an
adaptation period. Despite the abrupt change-over, the experimental diets were
accepted very well from the beginning. Therefore, it can be ruled out that the
initial steep increase in the levels of serum cholesterol were attributable to
starvation (21-24) resulting from rejection of the diets.

To reduce the possible effect of differences in total protein intake on the
levels of lipids in whole serum and the various lipoprotein fractions between
the two experimental groups, a restricted feeding regime was applied. In the
light of the findings of Ammerman et al. (22) it seems unlikely that restricting
the feed intake to a maximum of 100 g/day has influenced the levels of serum
lipids. Ammerman et al. reported that, in rabbits completely deprived of feed
for several days, marked elevations in the levels of serum cholesterol were
observed. However, when rabbits were fed a limited amount of feed, essentially
no changes in concentrations of both serum cholesterol and phospholipid
occurred, even when the animals had been losing weight. An explanation for this
discrepancy between starved and restricted fed animals might be the markedly
decreased production of faeces in starved animals. Swanor and Connor (24)
reported that in rabbits, the output of faeces was diminished after only one
day and that after 5 days no further faeces at all were produced. This means
that in starving rabbits, the main pathway for the excretion of cholesterol is
blocked whereas restricted fed rabbits are still able to excrete cholesterol by
this route.

Hitherto, it has been tacitly assumed that the density limits defined for
human serum lipoproteins could be equally applied to the serum lipoproteins

of rabbits. However, the visualization of the lipoproteins in the gradient by
means of a staining technique revealed that the density profile of the serum
lipoproteins in normolipemic rabbits is different from that in humans. The
normolipemic rabbit has a faint lipoprotein band with a density range inter-
mediate between the LDL (1.019 < d < 1.063) and IDL (1.006 < d < 1.019) as defined
for human serum lipoproteins. This might explain why in normolipemic rabbits
relatively high concentrations of cholesterol have been measured in the IDL
fraction compared with the LDL fraction (7,8), while in normolipemic human
serum the concentration of LDL cholesterol is much higher in the LDL than in
the IDL fraction (11).

When the rabbits were transferred to semipurified diets containing casein
or soybean protein, a pronounced and distinct LDL band was observed. The posi-
tion of the band in the gradient varied considerably between individual
rabbits. In some rabbits, the LDL band had a density within the range of the
IDL as defined for humans while in other rabbits a density intermediate
between the LDL and IDL was found (Fig. 1). Thus individual rabbits differ in
their response to the feeding of semipurified diets. These findings throw some
light on the different results found by several authors on the relative con-
centration of cholesterol in the IDL and LDL fractions in rabbits fed casein.
Roberts *et al*. (7) reported that the increase of cholesterol in the density
range of 1.063 < d < 1.006 was mostly attributable to increased levels of IDL.
On the other hand, Ross *et al*. (6) and Lacombe and Nibbelink (8) reported
higher levels of cholesterol in the LDL compared with the IDL. However, these
contradictory results might be explained by the large variations between
individual rabbits in the density profile of the lipoproteins.

Individual differences between rabbits in the position of the HDL in the
gradient were also observed and in several cases the density range of this
band as observed in the gradient overlapped the convential density limit
between LDL and HDL (Fig. 1). Thus it might be more appropriate to collect the
lipoproteins of both normo- and hyperlipemic rabbits on the basis of their
appearance in the gradient, rather than using predetermined density limits.

It is noteworthy that in our laboratory similar individual variations in
the density profile of the serum lipoproteins have been observed in cholesterol-
-fed guinea pigs. Furthermore, in humans van Raaij *et al*. (30) also reported
that small but significant changes in the density of the LDL-band occurred as
a result of feeding diets containing casein.

Serum samples from the rabbits were taken in the non-fasting state. Hermus
(4) reported that in hypercholesterolemic rabbits, no changes in serum chole-

sterol levels were observed when blood samples were taken in the fasting and the non-fasting state. Nevertheless, we felt that data from analyses of lipoproteins in non-fasting animals were more likely to reflect the real metabolic situation. Furthermore, we were also interested in the changes in the lipoproteins produced by the ingestion of semipurified diets for only one day. These studies would have been hampered if the animals were fasted in order to take blood samples.

It is possible that the large individual variations in the density profile of serum lipoproteins in the rabbits fed semipurified diets were affected by taking blood samples in the non-fasting state. However, it seems unlikely that these variations were due to the non-fasting state as samples taken when the rabbits were on the commercial diet, also in non-fasting state, showed no such variation.

In conclusion, the results of this study showed that in rabbits a very rapid response was observed in the levels of serum cholesterol and in the composition of the serum lipoproteins to changes in the diet. These findings could be usefully pursued in further studies on the mechanism underlying the hypercholesterolemic action of casein in rabbits.

ACKNOWLEDGEMENTS

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4. THE EFFECT OF DIFFERENT PROPORTIONS OF CASEIN IN SEMIPURIFIED DIETS ON THE CONCENTRATION OF SERUM CHOLESTEROL AND THE LIPOPROTEIN COMPOSITION IN RABBITS

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SUMMARY

The effect of different proportions of casein in semi-purified diets on the concentration of serum cholesterol and the lipoprotein composition was studied in rabbits. Low casein diets (10% w/w) resulted in lower serum cholesterol levels and growth rates than high casein diets (40%). An intermediate proportion of casein (20%) produced intermediate concentrations of serum cholesterol, but only minor differences in food intake and weight gain, compared with the high casein group. In the animals with the highest values of total serum cholesterol (the 40% casein group) most of the serum cholesterol was transported in the VLDL, while with moderate hypercholesterolemia (the 20% casein group) the LDL was the main carrier of cholesterol. Elevation in lipoprotein cholesterol was associated in all the groups with an increased ratio of cholesterol to protein, suggesting the formation of particles relatively rich in cholesterol. When the rabbits on the diet containing 10% casein were subsequently transferred to the 40% casein diet, a steep increase in the level of serum cholesterol occurred. Conversely, switching the rabbits on the 40% casein diet to the 10% casein diet resulted in a decrease in the level of serum cholesterol.

INTRODUCTION

It has been well established that the protein source in the diet of rabbits plays an important role in the regulation of the concentration of serum cholesterol (1-3). The feeding of rabbits with semi-purified diets containing casein results in hypercholesterolemia, whereas diets containing other proteins such as soybean protein are able to maintain low levels of serum cholesterol. The hypercholesterolemic properties of casein in rabbits have been found to be

reproducible in other experimental animals such as swine (4), rats (5) and chickens (6). Hitherto, the focus of attention has mainly centered upon the influence of the quality of the protein source in the diets on the levels of serum cholesterol, while less research has been carried out studying the variation in the quantity of the protein source. The objective of this study was to investigate if increasing proportions of casein in the diet result in higher levels of serum cholesterol. Therefore, rabbits were fed semi-purified diets containing three different levels of casein for a period of 4 weeks. In order to accentuate possible differences, the rabbits fed the high casein diet were changed to the low casein diet, while the animals upon the low casein diet were switched to the high casein diet. The concentrations of cholesterol and protein in the different lipoprotein fractions of the rabbits fed the three levels of casein are also reported.

MATERIALS AND METHODS

Animals and Experimental Design

In the experiment, 21 male New Zealand White rabbits, aged 10 weeks and weighing about 1300 grams, were used. The animals were housed individually in cages with wire mesh bases constructed of galvanised steel and were kept in a room with air conditioning and controlled lighting. On arrival the rabbits were fed a commercial diet (Hope Farms, 3442 EH Woerden, The Netherlands) for a period of two weeks to enable them to adapt to the new environment. The rabbits were divided into three groups of six animals and one group of three animals on the basis of their weight and levels of serum cholesterol. Subsequently, the three groups of six animals were changed, without further adaptation, to pelleted semi-purified diets containing 10%, 20% and 40% (w/w) casein. The group consisting of three animals continued to receive the commercial diet. The composition of the semi-purified diets was similar to that described previously (3) and the difference in protein content was varied at the expense of maize starch. The diets contained (g/kg feed): maize starch 360, maize oil 10, coconut oil 138, casein 200, saw dust 210, vitamin premix 12, mineral premix 10, $KHCO_3$ 18, $Ca_2HPO_4-2H_2O$ 29, NaCl 8, MgO 2, $MgCO_3$ 3. The individual food consumption was measured daily and body weights were determined three times a week. Food and water were offered *ad libitum*. However, the animals fed the commercial diet continuously were offered a restricted amount of 110 g/day over the first ten days of the experiment and 130 g/day later on in order to obtain a similar growth rate as in the animals fed the semipurified diets. After feeding the

semi-purified diets for 4 weeks, the group receiving the 10% casein diet was
changed to the 40% casein diet, while the rabbits on the diet with 40% casein
were transferred to the 10% casein diet. The rabbits on the 20% casein diet
continued to receive this diet. After the cross-over the animals were kept on
the diets for another three weeks.

Sampling of Blood and Analytical Methods

Blood samples for the determination of total serum cholesterol were taken
by incision from the marginal ear vein one week before the change over to the
semi-purified diets and one day afterwards. Subsequently, blood samples were
collected twice a week. Lipoprotein analyses were carried out in the individual
samples taken one week before and four weeks after the beginning of the experi-
ment. The serum lipoproteins were isolated by density gradient ultracentrifuga-
tion, employing a modification of the method described by Redgrave *et al.* (7).
In an SW 50-1 cellulose nitrate centrifuge tube (Beckman Inc., Palo Alto CA
94304) was placed 1 ml serum. In order to prestain the lipoproteins, the serum
was mixed carefully with a Sudan Black solution (0.1 ml) prepared as described
by Narayan (8). The background density of the prestained serum was raised to
1.21 g/ml by adding 0.313 g solid KBr. Subsequently, the mixture was over-
layered with equal volumes (2.1 ml) of salt solutions of densities 1.063 g/ml
(11.42 g NaCl and 80.26 g KBr/l) and 1.0063 g/ml (11.42 g NaCl/l), respectively.
All solutions contained 0.1 g/l ethylenediaminetetraacetic acid (disodium salt).
The samples were centrifuged for 16 hr at 234,000 g(av.) at 20^0C. The clearly
visible lipoprotein bands were collected by tube slicing and analysed for chole-
sterol and protein concentration. Cholesterol in whole serum and the lipo-
protein fractions was measured according to the method of Röschlau *et al.* (9),
using the kit supplied by Boehringer Mannheim, Germany (Catalase kit, cat.no.
124.087). The protein content of VLDL and LDL was estimated by the method
described by Markwell *et al.* (10), which is a modification of the method of
Lowry *et al.* (11). Since the HDL fractions were contaminated with serum
albumin when analysed by gradient polyacrylamide gel electrophoresis (3%-12%
slabgel, a modification of the method described by Masket *et al.* (12)), no
determinations of protein were carried out in this lipoprotein class. Statisti-
cal analysis was performed using Student's one tailed t-test as adapted by
Cochran (13).

RESULTS

Food Consumption and Growth

The body weight, weight gain and food consumption throughout the experiment are presented in Table I. During the first period the growth rate of the rabbits on the 20% and 40% casein diets was higher than the growth rate observed in the rabbits fed the 10% casein diet (P ‹ 0.01). After the cross-over, the highest weight gain was seen in the 40% casein group (P ‹ 0.001), whereas similar growth rates occurred in the 20% and 10% group. This higher growth rate, associated with a higher food intake, probably can be explained as a compensatory response to the poor growth on the low casein diet during the first period. In contrast to the first period, no differences in weight gain between the 10% and 20% groups were found in the second period of the experiment. The rabbits fed the restricted amount of commercial diet exhibited, during the first period, a growth rate which was higher than the growth rate of the animals fed the 10% casein diet (P ‹ 0.01) and lower than that of the group fed the semi-purified diet containing 20% casein (P ‹ 0.01). Similarly, after the cross-over, the rabbits fed the restricted amount of commercial diet exhibited a higher growth rate than the rabbits fed the 20% casein diet (P ‹ 0.05) but lower than that of the rabbits fed the 40% casein diet (P ‹ 0.001).

Serum Cholesterol

When the rabbits were changed from a commercial rabbit diet to semi-purified diets containing casein, a rapid elevation in the concentration of serum cholesterol was observed in all three groups (Fig. 1). In the 10% casein group the maximum level of serum cholesterol was reached after only three days on the experimental diet, whereas the concentrations of serum cholesterol in the 20% casein group plateaued after 14 days. The levels in the rabbits fed 40% casein diets continued to increase. At the end of the first period significantly higher concentrations of serum cholesterol were observed in the 40% group compared to the 20% group (P ‹ 0.05) and the 20% group compared with the 10% group (P ‹ 0.01). When the rabbits on the diet containing 10% casein were transferred to the 40% casein diet, a significant increase of the concentration of serum cholesterol occurred (P ‹ 0.001). Conversely, switching the group on the 40% casein diet to the 10% casein diet resulted in a decreased level of serum cholesterol (P ‹ 0.05). The group receiving the 20% casein diet and the commercial diet remained at a constant level of serum cholesterol.

Table I

BODY WEIGHT, WEIGHT GAIN AND FOOD CONSUMPTION IN RABBITS FED DIETS CONTAINING DIFFERENT PROPORTIONS OF CASEIN

Mean ± S.E.M.[a]

	First period (28 days)			Second period (21 days)			
	Initial Body Weight (g)	Weight Gain (g/day)	Food Intake (g/day)	Initial Body Weight (g)	Weight Gain (g/day)	Food Intake (g/day)	Final Body Weight (g)
10-40% casein (6)[b,c]	1358 ± 38	10.7 ± 1.5ˣˣˣ	63.0 ± 1.3ˣˣˣ	1659 ± 71ˣˣ	40.4 ± 1.9ˣˣˣ	77.2 ± 2.8	2507 ± 98
20% casein (6)	1326 ± 36	29.3 ± 1.6	76.7 ± 1.3	2148 ± 51	10.5 ± 1.6	56.7 ± 1.7	2368 ± 45
40-10% casein (6)[d]	1325 ± 50	25.0 ± 2.3	73.3 ± 1.0	2025 ± 102	11.3 ± 4.3	66.5 ± 1.7ˣˣ	2265 ± 137
Commercial Diet (3)	1330 ± 98	20.1 ± 0.9	110/130[e]	1895 ± 93	19.3 ± 2.9	130	2297 ± 105

a. Differences between means of the 10-40% casein group and the 20% casein group and between means of the 40-10% casein group and the 20% casein group were analysed for significance by Students' two tailed t-test: xxP ⟨ 0.01; xxxP ⟨ 0.01

b. Number of rabbits.

c. The rabbits were fed 10% casein diets during the first period and 40% casein diets during the second period.

d. The rabbits were fed 40% casein diets during the first period and 10% casein diets during the second period.

e. The rabbits were fed during the first period of 10 days an amount of 110 g of pellets; subsequently 130 g was administered; these amounts were finished by all the rabbits every day.
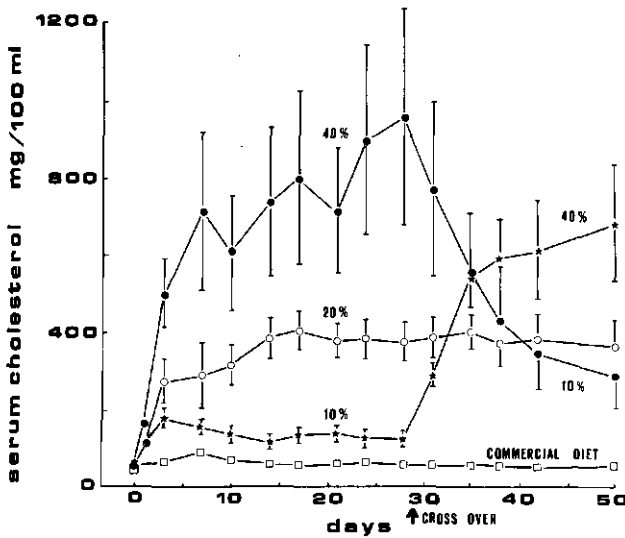
Fig. 1. Serum cholesterol concentrations in rabbits fed semipurified diets
containing different proportions of casein; ✶——✶, group receiving
diet containing 10% casein before the cross-over and 40% casein
after the cross-over; ●——● , 40% and 10% respectively;
○——○ , 20% throughout the experiment; □——□ , commercial diet.
The vertical bars represent the standard error of the mean at each
time point.

Cholesterol and Protein in the Lipoprotein Fractions

The cholesterol and protein concentrations and the ratios of cholesterol to
protein in the different lipoprotein fractions immediately before the cross-over
are given in Table II. The increase in serum cholesterol was mainly reflected
in the LDL and VLDL fractions, while in the HDL fractions there were only
relatively minor changes. An increase in the protein concentration of the VLDL
and LDL fractions also was found but this was less pronounced than the increase
of the cholesterol. Therefore, an elevation of the ratio of cholesterol to
protein could be observed in the VLDL and LDL fractions of the rabbits fed the
semipurified diets. This suggests that lipoprotein particles relatively rich in
cholesterol were formed.

Table II

CHOLESTEROL AND PROTEIN CONCENTRATION AND CHOLESTEROL : PROTEIN RATIO IN SERUM
LIPOPROTEINS IN RABBITS FED DIETS CONTAINING DIFFERENT PROPORTIONS OF CASEIN

	MEAN ± S.E.M.			
	Initial (18)[a,b]	Casein 10% (6)[c]	Casein 20% (6)[c]	Casein 40% (6)[c]
Cholesterol[d]				
VLDL	17.8 ± 3.4	40.7 ± 5.4[x]	119.1 ± 33.7	542.2 ± 197.1[x]
LDL	14.0 ± 2.0	30.9 ± 13.7[xx]	146.8 ± 15.3	210.0 ± 57.4
HDL	18.4 ± 1.2	15.1 ± 2.8[xxx]	60.2 ± 5.5	44.7 ± 11.5
Protein[d]				
VLDL	16.0 ± 2.9	26.8 ± 5.2	46.1 ± 14.9	137.2 ± 41.9[x]
LDL	18.9 ± 2.3	22.0 ± 4.1[xx]	82.9 ± 8.6	116.2 ± 27.9
Cholesterol: Protein Ratio[e]				
VLDL	1.10 ± 0.05	1.77 ± 0.33[+]	2.73 ± 0.29[+++]	3.61 ± 0.47[+++]
LDL	0.75 ± 0.05	1.38 ± 0.41	1.79 ± 0.10[+++]	1.76 ± 0.08[+++]

a. Number of animals in parentheses.

b. These figures represent the initial values of all the animals before the
change-over to the semi-purified diets.

c. Values after feeding the diets for 28 days.

d. Concentrations expressed in mg/100 ml. Comparison by Student's t-test of the
increases in concentration from the initial values of the 10% and 40% group
with the 20% casein group: [x]P < 0.05; [xx]P < 0.01; [xxx]P < 0.001.

e. Comparison by Student's paired t-test of the differences from the initial
values within each group: [+]P < 0.05; [+++]P < 0.001.

DISCUSSION

The growth of rabbits on semipurified diets containing casein is usually
less than on commercial rabbit pellets (3,14). Hove and Herndon (15) reported
that in rabbits aged 4 weeks higher growth rates could be obtained by increasing
the proportion of casein in the diet in the range from 10% to 50%. In our study,
during the first period a higher weight gain was also found upon the 20% casein
diet than on the 10% casein diet, but a further increase of the proportion of
dietary casein to 40% slightly, but not significantly depressed the growth.
Huff et al. (14), on the other hand, actually observed a loss of weight when
feeding diets containing 54% casein compared to 27%. The reason for these contra-

dictory results is not clear. The low growth rate on the 10% diet probably can be explained by a deficiency of amino acids, as the proportion of most of the essential amino acids present in the 10% casein diet is lower than that recommended for rabbits (16).

The rabbits fed the commercial diet consumed a much greater amount of food than the animals fed the semi-purified diets, although the growth rate in some cases was lower. However, the composition of the commercial diet differs in many aspects from that of the semi-purified diets. The semi-purified diets had a higher caloric density (3400 kcal/kg feed) than the commercial diet (2800 kcal/kg feed) and the difference can be partly attributed to the high fat content of the semi-purified diets. Differences in the availability of the energy also may be important.

It has been well established in rabbits that the feeding of semi-purified diets containing casein results in hypercholesterolemia, whereas this effect is not observed with diets containing soybean protein (1,2). The results of our study additionally show that increasing the proportions of casein in the diet results in higher levels of serum cholesterol. Huff *et al.* (14) reported that doubling the proportion of soybean protein from 27% to 54%, at the expense of the carbohydrate source (dextrose) had no significant effect on the concentration of serum cholesterol. Furthermore, Hamilton and Carroll (17) observed that the replacement of dextrose in a semi-purified diet by maize starch, as used in our diets, did not result in changes in the levels of serum cholesterol. Therefore, it is suggested that the differences in the levels of serum cholesterol observed in our rabbits fed diets containing different proportions of casein can be attributed to the changes in the proportion of casein rather than to changes in the proportions of either total protein or of maize starch.

The results obtained in our study are in agreement with those reported by Huff *et al.* (14). However, in their study, doubling the amount of casein in the diets resulted in higher concentrations of serum cholesterol, which was associated with loss of weight. It is known that, in rabbits, hypercholesterolemia occurs during periods of starvation (18,19). In our study no loss of weight was observed. Furthermore, by feeding a group of rabbits a restricted amount of commercial diet it was demonstrated that no consistent relationship existed between the growth rates and the levels of serum cholesterol. During the first period of the experiment, the rabbits fed the commercial diet exhibited a lower level of serum cholesterol and a lower growth rate than the group fed the 20% casein diet. However after the cross-over, the group fed the commercial diet also had lower concentrations of serum cholesterol but this was

associated with a higher growth rate. Therefore, it is unlikely that the differences in serum cholesterol levels can be attributed to differences in growth rate rather than to differences in the diets *per se*.

It was observed over fifty years ago that the atherogenic properties of a particular protein could be enhanced by the incorporation of a higher proportion of the protein in the diet of rabbits. Newburgh and Clarkson (19) found that rabbits consuming a diet containing 36% protein derived from lean beef muscle developed atherosclerosis sooner than did rabbits receiving 27% of the protein in their diet. Similar results have been obtained in rats. Nath *et al.* (20) showed that, in rats fed casein diets, the levels of serum cholesterol were higher when the diet contained 70% casein compared with 40%. They found the highest levels of serum cholesterol in rats on 6% casein diets. However, oi this low protein diet weight loss was also observed, which might have contributed to the elevation of the levels of serum cholesterol. Hevia *et al.* (22) also reported that in rats the feeding of diets containing casein in increasinç proportions (7.5%, 15% and 30%) resulted in higher levels of serum cholesterol.

Other authors (5,14) have provided strong evidence that the amino acid composition of the protein under investigation plays an important role in the etiology of hypercholesterolemia. Hermus (3) indicated that the amino acids present in a 20% casein diet meet the tentative requirements of the rabbit. Therefore, an amino acid imbalance might be more relevant than an absolute deficiency of some particular amino acids. The data obtained from our study underline this hypothesis, since increasing the proportion of casein in the diets resulted in further elevation of the serum cholesterol levels and not the reverse.

Higher proportions of casein in the diets resulted in higher levels of serum cholesterol, which was mainly attributable to increased cholesterol in the VLDL and LDL fractions. A relatively minor elevation of the HDL cholesterol occurred whereas in the rabbits fed 10% casein diets no significant changes at all were detectable in this lipoprotein fraction. At moderate levels of serum cholesterol the LDL was the major carrier of cholesterol (20% casein group), but at higher concentrations of serum cholesterol (40% casein group) most of the cholesterol was transported by the VLDL. These findings agree with those reported by other authors (23,24).

Hypercholesterolemia in rabbits, due to feeding semi-purified diets containing casein resulted in the formation of lipoprotein particles with a high ratio of cholesterol to protein suggesting the appearance of cholesterol-rich lipoprotein particles. Similar observations have been reported in rabbits made

hypercholesterolemic by feeding cholesterol (25) or by starvation (19).

In conclusion, the results of this study show that in rabbits, the hyper-cholesterolemic properties of semi-purified diets containing casein can be enhanced by increasing the proportion of dietary casein. This finding might be very useful in studies on the mechanism underlying the hypercholesterolemic action of casein. By the use of diets with higher proportions of casein more pronounced and more rapid elevations in serum cholesterol levels can be produced, which might facilitate the elucidation of the mechanism of the casein-induced hypercholesterolemia.

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5. A CROSS-OVER STUDY OF SERUM CHOLESTEROL AND LIPOPROTEINS IN RABBITS FED SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOYBEAN PROTEIN

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SUMMARY

1. Two groups, each of six rabbits, were fed semipurified diets containing
   either 40% (w/w) casein or 40% soy-bean protein for 20 days and then the
   diets of the two groups were crossed over.

2. Just before the cross-over, the serum cholesterol concentration was
   307 ± 59 mg/100 ml (mean ± SE) and 80 ± 14 mg/100 ml for the groups fed casein
   and soy-bean protein, respectively.

3. Changes in the serum cholesterol concentration were observed 1 day after
   crossing over the diets. By 10 days, the cholesterol levels in the two
   groups had also crossed over.

4. The changes in serum cholesterol level after the cross-over were reflected
   in the very-low-density lipoproteins and low-density lipoproteins.

5. Lipoprotein protein concentrations in the low-density lipoproteins changed
   in the same way as cholesterol. In the very-low-density lipoproteins,
   however, the protein concentration decreased in both groups after the change
   in diet.

6. The cholesterol: protein ratios for the low-density and very-low-density
   lipoproteins markedly increased in the rabbits changed from the soy-bean
   protein diet to the casein diet, reaching a maximum 2 days after the cross-
   over. In the animals switched from casein to soy-bean protein, the ratios
   progressively declined.

7. The source of dietary protein exerts a rapid effect on the composition of
   both the very-low-density and low-density lipoproteins which is proposed to
   be attributed to changes in the number and size of lipoprotein particles.

INTRODUCTION

Over 70 years ago, it was observed that rabbits fed a diet rich in animal products developed atherosclerotic lesions, and it was suggested that the protein might be the component responsible (Ignatowski, 1909). However, this idea was largely abandoned when Anitschkow & Chalatow (1913) found that the inclusion of cholesterol in the diet resulted in similar lesions. Nevertheless, several investigators still adhered to the idea that dietary protein might play an important role in the aetiology of hypercholesterolaemia and atherosclerosis. Newburgh & Squier (1920) observed that feeding high casein diets to rabbits produced atherosclerosis, whereas the feeding of soy-beans did not. Similar results were later reported by Meeker & Kesten (1941). More recently it has been clearly shown that cholesterol metabolism in rabbits can be influenced by dietary protein (Carroll, 1975, Kritchevsky, 1979, Hermus, 1975). Rabbits fed semipurified diets containing casein exhibit hypercholesterolaemia and atherosclerosis whereas substituting soy-bean protein for casein in such a diet results in these effects being much reduced.

The excess of serum cholesterol in rabbits fed a diet containing casein has been found to be carried mainly in the low-density lipoproteins (LDL) and the very-low-density lipoproteins (VLDL) (Brattsand, 1976, Ross *et al.* 1978, Roberts *et al.* 1979, Lacombe & Nibbelink, 1980, Terpstra & Sanchez-Muniz, 1981, Terpstra, Harkes *et al.* 1981). With moderate hypercholesterolaemia, the LDL fraction is the main carrier of serum cholesterol, whereas at more elevated levels of serum cholesterol most of the cholesterol is transported in the VLDL particles (Terpstra, Harkes *et al.* 1981).

Lipoprotein protein and phospholipid concentrations also change when rabbits are switched from commercial diets to semipurified diets containing either casein or soy-bean protein (Roberts *et al.* 1979, Terpstra & Sanchez-Muniz, 1981). However, the different lipoprotein components do not change proportionately, so that the composition of lipoproteins from rabbits fed casein differs from that of animals fed soy-bean protein; the cholesterol: protein ratio is greater in animals fed casein diets.

In a previous study (Terpstra & Sanchez-Muniz, 1981) the time course of alterations in serum lipids and lipoprotein composition was examined when rabbits were switched from a commercial diet to semipurified diets containing either casein or soy-bean protein. It was found that significant changes occurred after only 1 d of feeding the semipurified diets. In the present experiment, the time course of changes in serum lipoproteins has been studied after

switching rabbits from a semipurified diet containing casein to a semipurified diet containing soy-bean protein and vice versa. High protein diets have been used to enhance the differences between the groups; by raising the proportion of dietary protein, hypercholesterolaemia due to casein is enhanced, whereas changes in the proportion of soy-bean protein in the diet do not affect serum cholesterol levels (Huff et al. 1977, Terpstra, Harkes et al. 1981).

METHODS

Animals and experimental design

In the experiment, two groups each of six male New Zealand White rabbits, aged 13 weeks, were used. The animals were housed individually in cages with wire mesh bases constructed of galvanised steel and were kept in a room with air conditioning and a 12 h light/dark cycle. The rabbits had been fed commercial rabbit pellets (Trouw en Co N.V., 3881 LB Putten, The Netherlands) and were subsequently transferred, without an adaptation period, to pelleted semipurified diets containing either 40% (w/w) casein or 40% soy-bean protein. The composition of the diets was (g/kg): maize starch 180, dextrose 122, coconut oil 40, soy-bean oil 10, sawdust 12, casein or soy-bean protein 400, vitamin premix 12, mineral premix 10, $KHCO_3$ 18, $Ca_2HPO_4-2H_2O$ 29, NaCl 4, MgO 2, $MgCO_3$ 3 and molasses 50. The composition of the vitamin and mineral premix has been described earlier (Hermus, 1975). After feeding the semipurified diets for 20 d, the group receiving the casein diet was changed to the diet containing soy-bean protein, whereas the animals fed the soy-bean protein diet were switched to the casein diet. After the cross-over the rabbits were kept on the diets for a further 35 d (Fig. 1).
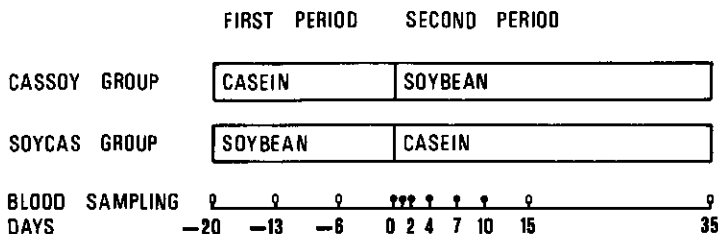
FIRST PERIOD   SECOND PERIOD

CASSOY GROUP	CASEIN	SOYBEAN

SOYCAS GROUP	SOYBEAN	CASEIN

BLOOD SAMPLING   ♀   ♀   ♀   ♀♀♀ ♀ ♀ ♀   ♀   ♀

DAYS   **—20**   **—13**   **—6**   **0 2 4 7 10   15**   **35**

Fig. 1. Design of the experiment. Blood samples were taken as indicated for either serum cholesterol (o) or for serum cholesterol and lipoprotein fractionation (•). Six rabbits per group.

Food and water were provided *ad libitum*. The individual food consumption was measured daily and body weight weekly. Blood samples from non-fasted animals were taken by incision from the marginal ear vein between 08.00 and 10.00 hours. The days on which blood samples were collected for the estimation of total serum cholesterol and the separation of serum lipoproteins are indicated in Fig. 1.

Analytical methods

The serum lipoproteins were separated by density gradient ultracentrifugation, employing a slight modification of the method described previously (Terpstra, Woodward *et al.* 1981). In a polyallomer centrifuge tube (Beckman Inc., Palo Alto CA 94304) designed to fit the Beckman SW 50.1 roter was placed 1 ml serum. The serum lipoproteins were prestained by mixing the serum carefully with 0.1 ml of a Sudan Black solution prepared as described by Narayan (1975). The background density of the prestained serum was raised to 1.10 g/ml by adding 114 mg KBr and 25 mg sucrose. Subsequently, the mixture was overlayered with equal volumes (2.1 ml) of a salt solution of density 1.05 g/ml (11.42 g NaCl and 61.65 g KBr/l) and distilled water.The samples were spun for 16 h in an SW 50.1 rotor at 20^{0}C and 50,000 rpm (234,000 g_{av}), using a Beckman L5-65 ultracentrifuge. After centrifugation, three stained bands were visible in the density gradient; on top of the gradient the VLDL fraction, further down in the tube the LDL particles and on the bottom the high-density lipoproteins (HDL) together with the serum proteins. The three lipoprotein fractions were collected by tube slicing on the basis of their visible positions in the gradient and analysed for cholesterol and protein. Cholesterol in whole serum and the lipoprotein fractions was measured by the method of Röschlau *et al.* (1974), using the kit supplied by Boehringer Mannheim, Germany (Catalase kit, cat.no. 124.087). The protein in the VLDL and LDL was estimated by the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978).

Statistical analysis was preformed using a modified Student's one-tailed t test (Snedecor & Cochran, 1967).

RESULTS

Food consumption and growth

The body weight, weight gain and food consumption of the two groups of rabbits are presented in Table 1. Both diets were well accepted throughout the experiment and supported adequate growth rates. However, the food intake

tended to be higher, when the rabbits were fed the semipurified diet containing soy-bean protein than that containing casein. These differences in food intake paralleled the growth rates. Nevertheless, throughout the whole experiment both groups had achieved a similar weight gain.

TABLE 1.
BODY WEIGHT, WEIGHT GAIN AND FOOD INTAKE OF RABBITS FED SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOY-BEAN PROTEIN[a].
(Mean values with their standard errors for 6 rabbits/dietary group).

	Cassoy group[b]		Soycas group[c]	
Initial body weight (on day -20) (g)	1667	± 96	1859	± 69
Body weight on day 0 (g)	2163	± 84	2556	± 89
Final body weight (on day 35) (g)	3073	± 131	3268	± 118
Weight gain during first period (g/d)	23.7	± 2.3	31.8	± 5.2
Weight gain during second period (g/d)	26.0	± 4.1	21.2	± 2.4
Weight gain during whole experiment (g/d)	25.1	± 2.6	25.2	± 2.6
Food intake during first period (g/d)	82.7	± 5.9	112.7	± 8.7
Food intake during second period (g/d)	116.6	± 9.3	106.0	± 5.2

a Individual food intake was measured daily and body weight weekly.

b The cassoy group was fed the casein diet during the first period of 20 d and then after the cross-over the soy-bean protein diet during the second period of 35 d.

c The soycas group was fed the soy-bean protein diet during the first period of 20 d and then after the cross-over the casein diet during the second period of 35 d.

Serum cholesterol

The semipurified casein diet resulted in markedly elevated levels of serum cholesterol whereas the soy-bean protein diet maintained the serum cholesterol concentration low (Fig. 2). At the end of the first period this difference was highly significant (P < 0.01). When the rabbits on the casein diet were trans-ferred to the diet containing soy-bean protein (the cassoy group), a rapid decrease in serum cholesterol occurred. After only 2 d a decrease in serum cholesterol of 54 mg/100 ml was observed (P < 0.01). Conversely, changing the rabbits from the diet containing soy-bean protein to the casein diet (the soycas group) resulted in a significant increase (P < 0.01) in serum cholesterol of 17 mg/100 ml after only one day. These changes continued; the serum cholesterol level in the cassoy group progressively decreasing and that

in the soycas group increasing. By 10 d after the change-over, the cholesterol levels in the two groups had crossed over. By the end of the whole experiment, the concentration in the cassoy group had returned essentially to its initial value. At the same time, the cholesterol concentration in the soycas group had increased to 210 mg/100 ml which is rather less than the maximum for the cassoy group.
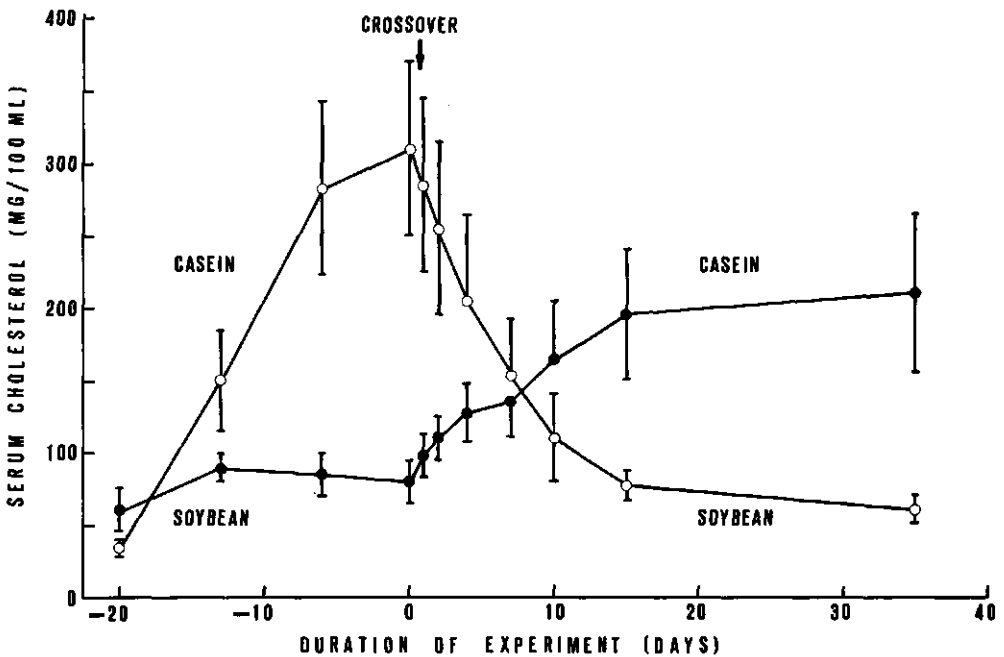

Fig. 2. Serum cholesterol concentration in rabbits fed semipurified diets containing either casein or soy-bean protein.
o——o, group receiving diet containing casein before the cross-over and soy-bean protein after the cross-over (cassoy group);
●——●, soy-bean protein and casein, respectively (soycas group).
Each point denotes the mean from 6 rabbits; the vertical bars correspond to one S.E.

Lipoprotein composition

The cholesterol and protein levels in the different lipoprotein fractions are shown in Table 2. Immediately before the dietary cross-over, the cassoy group exhibited significantly higher concentrations of cholesterol in the VLDL and LDL fractions when compared to the soycas group. After the cross-over, the cassoy group showed a progressive decrease in both VLDL and LDL cholesterol.

TABLE 2

CONCENTRATION OF CHOLESTEROL AND PROTEIN (mg/100 ml) IN SERUM LIPOPROTEIN FRACTIONS OF RABBITS FED SEMIPURIFIED DIETS CONTAINING EITHER CASEIN OR SOY-BEAN PROTEIN[a].

(Mean values with their standard errors for 6 rabbits/dietary group)[b].

	day 0	day 1	day 2	day 4	day 7	day 10
Cholesterol						
Cassoy group						
VLDL	94.1 ± 24.2[xx]	77.9 ± 32.1[x]	45.0 ± 16.0[x]	34.9 ± 22.0	22.7 ± 4.9	18.1 ± 4.9
LDL	166.9 ± 44.9[x]	166.9 ± 44.1[x]	154.0 ± 41.4[x]	125.8 ± 40.8	92.7 ± 40.6	62.3 ± 24.7
HDL	25.8 ± 4.2	34.9 ± 3.9	38.6 ± 2.4	48.0 ± 6.1	39.4 ± 5.1	28.5 ± 5.0
Soycas group						
VLDL	10.7 ± 2.1	11.1 ± 1.0	12.0 ± 2.1	18.3 ± 2.7	18.5 ± 4.5	21.2 ± 5.0
LDL	27.1 ± 8.9	45.6 ± 9.8	51.8 ± 10.1	65.5 ± 15.1	70.2 ± 22.8	86.5 ± 30.1
HDL	36.5 ± 7.3	39.6 ± 7.8	45.4 ± 7.3	45.9 ± 6.1	51.2 ± 9.0	52.1 ± 10.2
Protein						
Cassoy group						
VLDL	28.2 ± 7.4	22.6 ± 7.6	13.3 ± 4.2	12.3 ± 6.8	14.4 ± 3.8	11.2 ± 1.4
LDL	85.9 ± 17.2[x]	84.3 ± 16.3[x]	82.5 ± 19.0[x]	65.0 ± 17.4	61.1 ± 13.5	44.4 ± 11.3
Soycas group						
VLDL	22.7 ± 16.0	28.1 ± 22.3	4.6 ± 1.0	10.1 ± 2.4	8.1 ± 1.6	9.3 ± 1.6
LDL	29.6 ± 3.0	32.9 ± 5.5	30.9 ± 5.4	42.9 ± 7.8	41.4 ± 9.4	48.4 ± 12.9

a The cassoy and soycas group were fed semipurified diets containing casein and soy-bean protein (see footnotes b and c of Table 1) and were changed on day 0 to a soy-bean and casein diet, respectively.

b Statistical comparison between the cassoy and soycas group: ^x P < 0.05, ^xx P < 0.01.

However, initially only a decrease in VLDL cholesterol was observed, followed by a subsequent decrease of the cholesterol concentration in the LDL fraction. On the other hand, in the soycas group the progressive elevation in serum cholesterol was mainly reflected by increased LDL cholesterol, whereas the contribution of the VLDL was relatively small. HDL cholesterol showed in both groups an increasing tendency, but declined in the cassoy group on day 10 after the cross-over.

The LDL protein concentration in the cassoy group showed a progressive decline, whereas that in the soycas group increased. However, VLDL protein decreased in both groups. The first changes in protein concentration of both groups occurred in VLDL and were clearly established 2 d after the cross-over. Changes in LDL protein did not become obvious for 4 d.

In Fig. 3 the values for the ratios of cholesterol to protein in VLDL and LDL are presented. These provide an indication of the composition of the lipo-protein particles, and they reflect the concurrent alterations in cholesterol and protein levels which have been described above. Immediately before the dietary change-over, these ratios were significantly higher in the cassoy group than in the soycas group (P ⟨ 0.05). After the cross-over, the ratio in the VLDL of the cassoy group showed a progressive decrease, which first appeared on day 4 after the cross-over. In the soycas group this ratio increased significantly (P ⟨ 0.01) on the second day and thereafter remained at a higher level. In the LDL the pattern is similar: the ratio in the cassoy group began to decrease on the first day and this trend continued up to day 10. In the soycas group the initial increase on the first day continued up to the second day (P ⟨ 0.05), at which level the ratio stayed.

DISCUSSION

In a previous study (Terpstra & Sanchez-Muniz, 1981) the time course of the changes in lipoprotein composition were studied in rabbits switched from a commercial diet to semipurifed diets containing either casein or soy-bean protein. The aim of the present study was to examine the development and regression of hypercholesterolaemia when rabbits were changed from a semipuri-fied diet containing soy-bean protein to a semipurified diet containing casein and vice versa. In order to enhance the effects, high protein diets were used.

When the soycas group was switched to the casein diet, a significant increase in serum cholesterol of 17 mg/100 ml (P ⟨ 0.01) occurred after only 1 d. After 2 d significant elevations in the ratio of cholesterol to protein
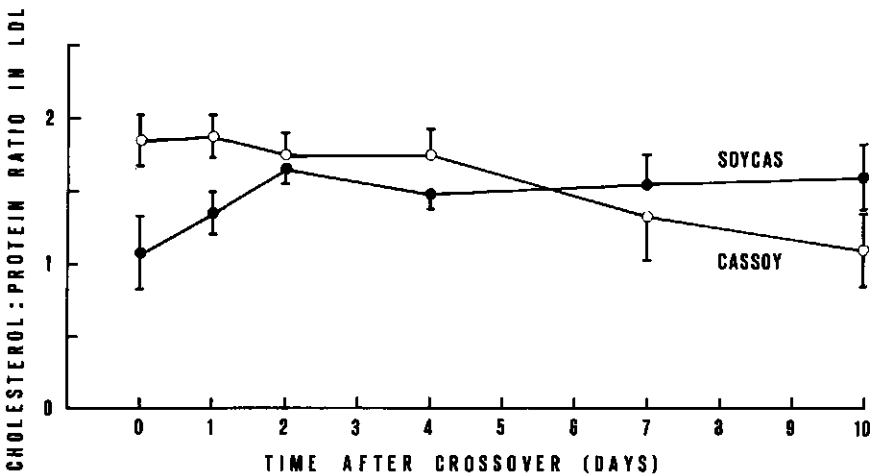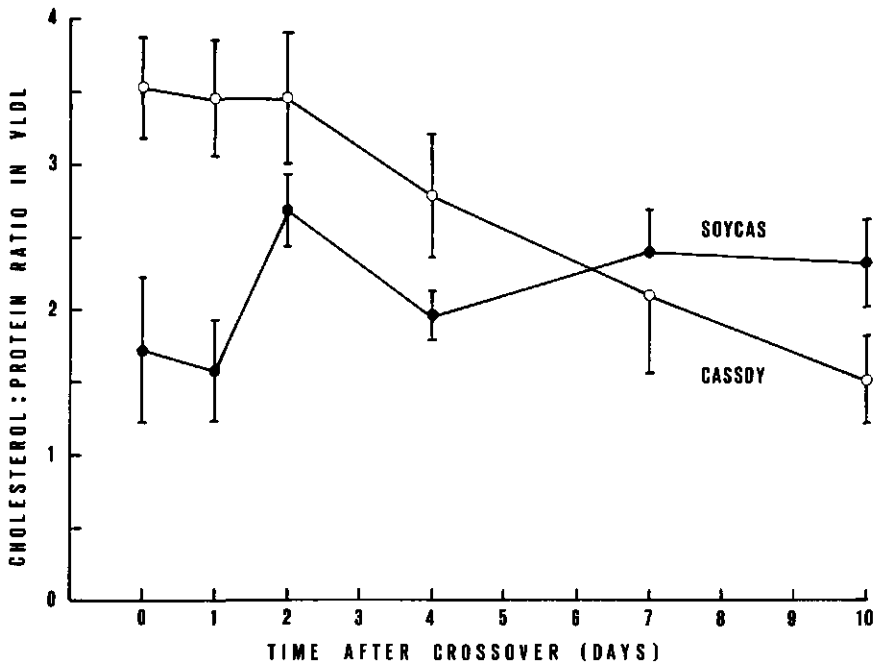
Fig. 3. Cholesterol: protein ratio in VLDL and LDL after the cross-over in
rabbits fed semipurified diets containing either casein or soy-bean
protein.
o————o, group changed from the casein diet to the soy-bean protein
diet (cassoy group);
●————●, group changed from the soy-bean protein diet to the casein
diet (soycas group).
Each point denotes the mean from 6 rabbits; the vertical bars
correspond to one S.E.

of the VLDL (P ‹ 0.01) and LDL (P ‹ 0.05) were observed. However, in the previous study (Terpstra & Sanchez-Muniz, 1981) when rabbits were switched from a commercial diet to a semipurified diet these changes were even much more pronounced and rapid, despite a lower proportion of casein in the diet (20% w/w). After 1 day the serum cholesterol levels rose by 51 mg/100 ml and the ratios of cholesterol to protein in VLDL and LDL more than doubled over the same time. There may be several explanations for these differences. Firstly, in the present study, only the protein source in the diet was changed, whereas in the previous experiment the diet as a whole was different. It has been shown that in rabbits dietary components other than protein also play an important role in determining serum cholesterol levels (Kritchevsky et al., 1977, Hamilton and Carroll, 1976). Secondly, there might be an age effect. Ignatowski (1909) already noticed that young rabbits were more susceptible to atherosclerosis produced by dietary means than their older counterparts. Furthermore, experiments in our laboratory have clearly shown, that the effect of dietary protein on serum cholesterol is markedly reduced in adult rabbits (West & Terpstra, unpublished data). When the animals in the present study were transferred to another semipurified diet, their age was 16 weeks, whereas the rabbits in the previous experiment were 13 weeks during the change-over. This age affect might also explain why the soycas group on the casein diet did not reach such a high serum cholesterol level as the cassoy group when fed casein, although the casein diet was fed for a much longer period. Finally, it should be noticed that the serum cholesterol levels of the soycas group was higher before the cross-over than in the animals in the previous experiment when changed from a commercial diet to a semipurified casein diet (40 vs 80 mg/100 ml). This might also have affected the results.

The cassoy group exhibited immediately before the dietary change-over significantly higher levels of VLDL and LDL cholesterol compared to the soycas group. Ten d after the cross-over, most of the increase in serum cholesterol of the soycas group was transported in the LDL fraction whereas the VLDL cholesterol had only slightly increase. This different pattern might be explained by the differences in total serum cholesterol in the cassoy group just before the cross-over (309 ± 59 mg/100 ml) and the soycas group 10 d afterwards (165 ± 41 mg/100 ml). It has been observed previously (Terpstra, Harkes et al., 1981) that at moderate elevated levels of serum cholesterol most of the cholesterol is transported by the LDL while at markedly increased serum cholesterol concentrations the VLDL fraction becomes a major carrier of cholesterol.

The protein concentration in the LDL fraction of both the cassoy and soycas group changed in the same way as cholesterol in these particles. On the other hand, VLDL protein declined in both groups. However, it is possible that this decrease of VLDL protein in the soycas group is only transient. As pointed out above, 10 d after the cross-over the total serum cholesterol in the soycas group had only moderately increased and only minor elevations in VLDL cholesterol had occurred. Nevertheless 2 d after the cross-over, the ratio of cholesterol to protein in the VLDL fraction had significantly increased (P < 0.01) due to a decrease in VLDL protein concentration. A similar pattern of response was observed when rabbits were changed from a commercial diet to a semipurified casein diet (Terpstra & Sanchez-Muniz, 1981). It seems likely that the concentration of VLDL protein will increase later, when there is an accumulation of cholesterol in the VLDL.

Ten d after the cross-over a similar concentration of VLDL cholesterol was measured in the soycas and cassoy group. Nevertheless, the ratio of cholesterol to protein was higher in the soycas group. This might indicate that in rabbits fed casein, VLDL particles are synthesized with a different composition than in rabbits fed soy-bean protein. Since VLDL particles are assumed to be metabolised into LDL particles (Eisenberg, 1979), this might also result in LDL particles with a high cholesterol: protein ratio. It is noteworthy, that in the cassoy group, the decrease in cholesterol: protein ratio of the LDL was preceded by a decline of this ratio in the VLDL. This might also be explained by a subsequent conversion of VLDL into LDL particles. However, it should be taken into account that blood samples were taken in non-fasted state and that the VLDL fraction also contains chylomicrons. Therefore, changes reported in the VLDL composition possibly reflect, to some extent, changes in the composition of chylomicrons.

On switching rabbits from a commercial diet to semipurified diets, the cholesterol: protein ratio in the lipoprotein fractions increased significantly on the first day and later relatively minor alterations in this ratio were observed (Terpstra & Sanchez-Muniz, 1981). This was interpreted as showing that semipurified diets produce a rapid change in lipoprotein composition, which is possibly followed by an increase of the number of lipoprotein particles. In the present experiment, a similar pattern was apparent for rabbits changed from a diet containing soy-bean protein to one containing casein, and a similar explanation may therefore be invoked. During the regression of hypercholesterol-aemia in the cassoy group, the pattern is however, somewhat different. The cholesterol and protein levels fall more in parallel, suggesting simultaneous

changes in composition and number.

The results of this study show clearly that soy-bean protein is able to reduce hypercholesterolaemia. Similar findings have been reported by Sirtori *et al.*, 1979 in hypercholesterolaemic patients, when soy-bean protein diets were consumed. These authors reported that in type IIB-III patients, who have elevated LDL and VLDL cholesterol, a reduction of cholesterol in both the LDL and VLDL fraction occurred. On the other hand in type IIA and IIB patients, characterized by elevated LDL levels, the reduction in serum cholesterol was mainly reflected in the LDL. Similar findings were observed in the present study with rabbits. The cassoy group exhibited just before the cross-over elevated levels of both LDL and VLDL cholesterol. Upon feeding soy-bean protein initially a decrease in VLDL cholesterol occurred followed by a subsequent decrease in LDL. When the cholesterol in the VLDL had reached a rather low level (7 d after the cross-over), a further decrease in serum cholesterol was mainly reflected in the LDL

In conclusion, the present study has clearly shown a differential effect of dietary casein and soybean protein on the serum cholesterol levels and lipoprotein composition. Further, the time course of regression and progression of hypercholesterolaemia induced by semipurified diets containing soy-bean protein and casein, respectively, has been studied. These data might provide a basis for further studies on the mechanism underlying the cholesterolaemic effects of various dietary proteins.

ACKNOWLEDGEMENTS

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SUMMARY

This thesis deals with the effect of dietary casein and soybean protein on the concentration of serum cholesterol and the lipoprotein composition in rabbits. Special attention has been paid to the time course of the changes produced by the protein in the diet.

After a short introduction, a review of the studies on the effect of dietary protein on serum cholesterol is presented. From these studies, it was concluded that the effect of dietary protein on the concentration of serum cholesterol is only manifested in hypercholesterolaemic subjects and in experimental animals fed a diet high in cholesterol. An exception to this generalisation is the rabbit, an animal highly susceptible to the induction of hypercholesterolaemia and atherosclerosis. In the rabbit, dietary protein is also able to influence serum cholesterol levels when cholesterol-free diets are used.

In the next chapter a method is presented for the separation of serum lipoproteins by density gradient ultracentrifugation. By staining the serum lipoproteins prior to ultracentrifugation the various lipoprotein classes can be easily localized in the gradient after the separation. By means of this technique it was observed that the density profile of the serum lipoproteins of rabbits and other experimental animals differs to that in man.

Subsequently the time course of the changes in serum cholesterol concentration and lipoprotein composition were studied, when rabbits were transferred from a commercial diet to a semipurified diet containing either casein or soybean protein. It was observed that after only one day of feeding a semipurified diet containing casein the serum cholesterol levels had more than doubled. In the rabbits fed soybean protein the serum cholesterol level increased only slightly. The ingestion of semipurified diets resulted in a steep increase in the ratio of cholesterol to protein in all the serum lipoprotein fractions. This suggests that lipoprotein particles relatively rich in cholesterol were formed. Furthermore, marked variations in the density profile of the serum lipoproteins were observed between individual rabbits fed semipurified diets.

In chapter 4 the effect of higher proportions of casein in the diet on the enhancement of the hypercholesterolaemia produced by this protein was examined. A low casein diet (10%) resulted in lower serum cholesterol levels than did a high casein diet (40%), whereas a diet containing 20% casein produced inter-mediate concentrations of cholesterol in the serum. In the animals with the highest levels of total serum cholesterol (the 40% casein group) most of the cholesterol was transported in the very low density lipoproteins. With moderate hypercholesterolaemia (the 20% casein group), the low density lipoproteins were the main carrier of cholesterol. Elevations in lipoprotein cholesterol were associated with an increased ratio of cholesterol to protein in all of the groups.

In the final experiment, the time course of the regression and progression of hypercholesterolaemia was studied when rabbits were transferred from a semi-purified diet containing casein to a semipurified diet with soybean protein and *vice versa*. In this study high protein diets (40%) were used. When casein in the diet was replaced by soybean protein, a rapid decrease in serum cholesterol occurred. This decrease in serum cholesterol was initially reflected in a decrease in the amount of cholesterol in the very low density lipoproteins followed by a subsequent drop in the cholesterol in the low density lipoproteins. Conversely, the replacement of soybean protein by casein resulted in an steep elevation in the serum cholesterol levels, which was mainly caused by an in-crease in the cholesterol in the low density lipoproteins.

These studies show that in rabbits very rapid and pronounced changes in serum cholesterol concentrations and lipoprotein composition can be produced by changing the type and amount of dietary protein. These findings underline the suitability of the rabbit as a model for studies of hypercholesterolaemia.

SAMENVATTING

Dit proefschrift handelt over de invloed van caseine en soja eiwit in het rantsoen op het serumcholesterolgehalte en de lipoproteinen samenstelling bij konijnen. Met name is aandacht besteed aan het tijdsverloop van de veranderingen die optreden o.i.v. de eiwitbron in het rantsoen.

Na een korte inleiding wordt een literatuuroverzicht gegeven van experimenten waarin de invloed van de eiwitbron in de voeding op het serumcholesterolgehalte is bestudeerd. Uit deze studie kon worden gekonkludeerd dat het effect van de eiwitbron in de voeding zich slechts manifesteert in hypercholesterolemische personen en in proefdieren op een cholesterolrijk rantsoen. Een uitzondering hierop is het konijn, een proefdier dat bijzonder gevoelig is voor de induktie van hypercholesterolemie en atherosclerosis. Bij het konijn kunnen eveneens veranderingen in het serumcholesterolgehalte d.m.v. de eiwitbron in de voeding worden geïnduceerd door gebruik te maken van cholesterolvrije rantsoenen.

In het volgende hoofdstuk wordt een methode beschreven voor de scheiding van serumlipoproteinen m.b.v. dichtheids gradient ultracentrifugatie. Door de lipoproteinen van te voren te kleuren kunnen na de scheiding de verschillende lipoproteinenfrakties gemakkelijk in de gradient worden gelokaliseerd. M.b.v. deze techniek kon worden waargenomen dat het dichtheidsprofiel van de serumlipoproteinen van het konijn en andere proefdieren verschilt van dat van de mens.

Vervolgens werd het tijdsverloop van de veranderingen in het serumcholesterolgehalte en de lipoproteinen samenstelling bestudeerd, wanneer konijnen worden overgezet van handelsvoer op een semisynthetisch rantsoen met caseine of soja eiwit. Waargenomen werd dat reeds één dag na de overgang op een caseine rantsoen het serumcholesterolgehalte zich had verdubbeld. Bij de konijnen op het rantsoen met soja eiwit was het serumcholesterolgehalte slechts in geringe mate gestegen. Het voederen van semisynthetische rantsoenen resulteerde in een sterke stijging van de cholesterol : eiwit verhouding in al de serumlipoproteinenfrakties. Dit suggereert dat lipoproteinendeeltjes werden gevormd

die relatief rijk zijn aan cholesterol. Bovendien werd een sterke variatie in het dichtheidsprofiel van de serumlipoproteinen waargenomen tussen individuele konijnen wanneer semisynthetische rantsoenen werden gevoederd.

In hoofdstuk 4 werd bestudeerd of het hypercholesterolemische effekt van caseine in het rantsoen kon worden versterkt door het gehalte aan caseine in het rantsoen te verhogen. Een laag caseine rantsoen (10%) resulteerde in een lager serumcholesterolgehalte dan een hoog caseine rantsoen (40%). Een rantsoen met 20% caseine gaf waarden die hier tussenin lagen. In de konijnen met de hoogste serumcholesterolgehalten (de groep op een rantsoen met 40% caseine) werd het grootste gedeelte van het serumcholesterol vervoerd in de lipoproteinen met een zeer lage dichtheid. Wanneer het serumcholesterolgehalte in geringe mate was gestegen (de groep op het rantsoen met 20% caseine) bevond zich het meeste cholesterol in de lipoproteinen met een lage dichtheid. Een stijging van het cholesterolgehalte in de lipoproteinen ging in al de drie proefgroepen gepaard met een verhoogde cholesterol : eiwit verhouding in deze deeltjes.

In het laatste experiment werd het tijdsverloop bestudeerd van het ontstaan en de teruggang van hypercholesterolemie wanneer konijnen worden overgezet van een rantsoen met soja eiwit naar een rantsoen met caseine en vice versa. In deze studie werden rantsoenen gebruikt met een hoog gehalte aan eiwit (40%). Wanneer caseine in het rantsoen werd vervangen door soja eiwit werd een snelle daling van het serumcholesterolgehalte waargenomen. Deze daling weerspiegelde zich aanvankelijk in een afname van het cholesterolgehalte van de lipoproteinen met een zeer lage dichtheid. Vervolgens trad een daling op van het cholesterol-gehalte van de lipoproteinen met een lage dichtheid. De vervanging van caseine door soja eiwit daarentegen resulteerde in een scherpe stijging van het serum-cholesterolgehalte. Dit werd vooral veroorzaakt door een toename in het cholesterol van de lipoproteinen met een lage dichtheid.

Deze studies tonen aan dat bij konijnen zeer snelle en duidelijke verande-ringen in het serumcholesterolgehalte en de lipoproteinensamenstelling kunnen worden geïnduceerd door het soort en de hoeveelheid eiwit in het rantsoen te veranderen. Deze resultaten geven aan dat het konijn een zeer geschikt dier-model is voor de bestudering van hypercholesterolemie.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 13 oktober 1947 te Broek
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