INFLUENCE OF HUMAN DIETS CONTAINING CASEIN, SOY PROTEIN ISOLATE, AND SOY PROTEIN CONCENTRATE ON SERUM CHOLESTEROL AND LIPOPROTEINS IN HUMANS, RABBITS AND RATS

Promotor: dr. J.G.A.J. Hautvast, hoogleraar in de voeding en voedselbereiding

Co-referent: dr. M.B. Katan, wetenschappelijk hoofdmedewerker

J.M.A. VAN RAAIJ

# INFLUENCE OF HUMAN DIETS CONTAINING CASEIN, SOY PROTEIN ISOLATE, AND SOY PROTEIN CONCENTRATE ON SERUM CHOLESTEROL AND LIPOPROTEINS IN HUMANS, RABBITS AND RATS

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. C.C. OOSTERLEE,
HOOGLERAAR IN DE VEETEELTWETENSCHAP,
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 15 OKTOBER 1982
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN

ISW = 19469 2-03

Het in dit proefschrift beschreven onderzoek werd mede mogelijk gemaakt door steun van het Nederlands Instituut voor Zuivelonderzoek.

Het verschijnen van dit proefschrift werd mede mogelijk gemaakt door steun van de Nederlandse Hartstichting.

STELLINGEN

1. Met betrekking tot beïnvloeding van de serumlipoproteïnen in de normocholesterolemische mens, is de herkomst van het eiwit in de voeding (dierlijk versus plantaardig) van slechts beperkte betekenis.
   *Dit proefschrift.*

2. Op basis van de resultaten van een enkel voedingsexperiment met proefpersonen kunnen nooit voedingsadviezen worden uitgebracht.

3. Met betrekking tot de relatie voeding en cholesterolmetabolisme mogen resultaten uit dier-experimenteel onderzoek nooit direct geëxtrapoleerd worden naar de mens.
   *Dit proefschrift.*

4. In het onderzoek naar beïnvloeding van bloedlipiden door de samenstelling van de voeding dat in het kader van preventie van hart- en vaatziekten wordt uitgevoerd, vormen voedingsexperimenten met proefpersonen onmisbare schakels.

5. De effecten op het serumcholesterolniveau die door het aanbrengen van veranderingen in de samenstelling van de voeding bereikt kunnen worden, mogen niet onderschat worden.
   *Brussaard JH, Katan MB, Knuiman JT. Voeding en coronaire hartziekten, een verzekering met slechts gedeeltelijke dekking? Ned T Geneesk 1982; 126: 291-296.*

6. In de planning van voedingsexperimenten waarbij effecten van diverse proefvoedingen met elkaar worden vergeleken, wordt bij de keuze van het proefschema en van het aantal proefpersonen onvoldoende rekening gehouden met een te verwachten spreiding in waarnemingen ten gevolge van tijdseffecten en verschillen tussen personen.

7. De bewering dat veranderingen in lichaamsgewicht voorspeld kunnen worden op basis van veranderingen in energieopneming, is op fysiologische gronden onjuist.

*Konishi F, Harrison SL. Body weight gain equivalents of selected foods. J Am Diet Ass 1977; 70: 365-368.*

*Garrow JS. Treat obesity seriously. A clinical manual. pp. 21-23. Edinburgh: Churchill Livingstone, 1981.*

8. In de behandeling van overgewicht door middel van diëten dienen gewichtsverliezen van 0,5 tot 1,0 kg per week als optimaal beschouwd te worden. Verhalen dat men met succes in één week 5 kg kan afvallen, zijn fabeltjes.

*Garrow JS. Treat obesity seriously. A clinical manual. pp. 11-13. Edinburgh: Churchill Livingstone, 1981.*

*Het sneldieet: vijf kilo mooier in één week! Libelle 1982; 34; 53-55.*

9. Het erkennen van burgerlijke ongehoorzaamheid door de overheid (het niet-strafbaar stellen van burgerlijke ongehoorzaamheid) is een stap voorwaarts naar een rechtvaardiger samenleving.

*Zie ook: Pastorale Paper no. 3 (1981), Studentenpastoraat Wageningen.*

10. Het feit dat oud-missionarissen ingeschakeld worden in Nederlandse parochies mag niet begrepen worden als een gewijzigde visie op missie.

*Proefschrift J.M.A. van Raaij*
*Influence of human diets containing casein, soy protein isolate, and soy protein concentrate on serum cholesterol and lipoproteins in humans, rabbits and rats.*
*Wageningen, 15 october 1982.*

Aan mijn Ouders

# CONTENTS

# VOORWOORD

In dit proefschrift worden twee voedingsproeven met vrijwilligers en vier voedingsproeven met proefdieren beschreven. De proeven zijn uitgevoerd tijdens mijn promotie-assistentschap bij de Vakgroep Humane Voeding van de Landbouwhogeschool (1978-1981). Velen hebben mij in die jaren met raad en daad ter zijde gestaan.

Als eersten wil ik noemen mijn promotor Prof.Dr. J.G.A.J. Hautvast en mijn co-referent Dr. M.B. Katan. Jo Hautvast ben ik dankbaar voor het in mij gestelde vertrouwen en voor zijn voortdurend stimulerend enthousiasme. Martijn Katan ben ik veel dank verschuldigd voor zijn vele kritische opmerkingen, zowel bij het opzetten en uitvoeren van de proeven, als ook bij het interpreteren van de resultaten en het schrijven van de publicaties. Ik heb erg veel van hem geleerd.

Aan de discussies voorafgaande aan de proeven met de vrijwilligers hebben ook Ruud Hermus, Marianne Stasse-Wolthuis, Clive West en vooral ook Tineke Brussaard een belangrijke bijdrage geleverd. Het opzetten en uitvoeren van de dierproeven was vooral het werk van Clive West en Ton Terpstra. Voor hun kritische opmerkingen in de beginfase van het onderzoek ben ik ook dank verschuldigd aan Renske van Beresteijn van het Nederlands Instituut voor Zuivelonderzoek NIZO te Ede en Piet van Stratum van Unilever Research te Vlaardingen, als ook aan de statistici van Unilever Research en van de Vakgroep Wiskunde van de Landbouwhogeschool.

De twee voedingsproeven met vrijwilligers zijn uitgevoerd op de Vakgroep Humane Voeding. In deze proeven is o.a. gebruik gemaakt van voedingsmiddelen die niet in de handel te koop waren en speciaal voor deze proeven ontwikkeld en bereid moesten worden. In dit opzicht ben ik dank verschuldigd aan: A. Visser, L. Schutte en W.T.M. de Groot van Unimills B.V. te Zwijndrecht; F.M.W. Visser en wijlen J. Schut van De Melkindustrie Veghel DMV te Veghel; H. Vonkeman, C. Andrea, H. Herstel, G.G. Nijholt en wijlen P. Westdorp van Unilever Research te Vlaardingen, en destijds ook Duiven; P. Sluimer van het Instituut voor Graan, Meel en Brood IGMB-TNO te Wageningen; J.W. van der Kamp en A.P. Ringers van Van Den Bergh & Jurgens B.V. te Rotterdam; H.J. van der Stege, J.H. Jansen en G. Kleter van de Sectie Zuivel van de Vakgroep Levensmiddelentechnologie van de LH; en aan H. Mesdom van Purina Protein Europe te Brussel.

De bereiding van de proefvoedingen, als ook de vele voorbereidende werkzaamheden met betrekking tot de voedselverstrekking lag in de vakkundige handen van de diëtisten Marita Engelen, Jeanne de Vries en Joke van Jeveren, daarbij geassisteerd door de diëtisten Netty van Kaathoven, Geesje Sanderink-Hulsink, Annelies Geenen, Leny Lamers en Marcella Mager. Bij de uitvoering van de proeven met de vrijwilligers heb ik ook veel steun gehad van de doctoraal-studenten Gisèle Snelder, Marja Markusse, Anke Ekkes, Mary Vlassak en Jan de Vries.

Het slagen van proeven met vrijwilligers ligt grotendeels in handen van de deelnemers. Ik ben hen allen zeer dankbaar voor hun nauwgezette en prettige medewerking. De ontspannen sfeer die hierdoor mogelijk werd, maakte het uitvoeren van de proeven voor ons tot een dankbare aangelegenheid.

De drie proeven met konijnen werden uitgevoerd op het Instituut voor Landbouwkundig Onderzoek van Biochemische Producten ILOB-TNO te Wageningen, onder toeziend oog van J.B. Schutte en K. Deuring. De proef met de ratten werd uitgevoerd op het Centrum voor Kleine Proefdieren van de LH, en was in handen van J.W.M. Haas en G. van Tintelen..

De verwerking van en de analyses in de vele bloed- en voedselmonsters werden voornamelijk gedaan door medewerkers van onze eigen laboratoria: Joke Barendse - van Leeuwen, Jannie Bos, Cock Germing - Nouwen, Ans Soffers, Elly Koot - Gronsveld, Frans Schouten, Peter van de Bovenkamp en Henny Verwey. Van buiten onze Vakgroep kregen wij hulp bij de analyses van: P.H.E. Groot en G. Dallinga - Thie van de Erasmus Universiteit te Rotterdam; P.N.M. Demacker van de Universiteit van Nijmegen; S. Visser van het NIZO te Ede en H. Koopman van de Centrale Dienst Biotechnion van de LH.

De co-auteurs van de artikelen die in dit proefschrift opgenomen zijn, zeg ik dank voor de waardevolle discussies die aan de tot standkoming van de artikelen vooraf gingen.

De leden van de Ethische Commissie bedank ik voor de tijd die zij gestoken hebben in de beoordeling van het onderzoeksprotocol van de proeven met vrijwilligers.

Het typewerk van de manuscripten was in handen van Gerfriede Hoekendijk - de Wal, Riekie van der Molen - Janssen en Riet Hoogkamer - Weijman. Helen West ben ik dankbaar voor het corrigeren van de Engelse tekst van een groot deel van dit proefschrift. Medewerkers van de tekenkamer en reproductie afdeling van de Centrale Dienst Biotechnion verzorgden de figuren. Hugo Albers maakte de foto's.

Ook alle anderen die hier niet genoemd zijn en die mij tijdens het onderzoek hebben geholpen, wil ik dankzeggen.

Tenslotte, maar zeker niet het minst, dank ik mijn vrouw Annelies voor de vele uren die zij mij gelaten heeft voor het voltooien van het onderzoek en het schrijven van dit proefschrift.

# SAMENVATTING

Het is bekend dat wanneer dieren als konijnen overgezet worden van handelsvoer op een semisynthetisch rantsoen met dierlijke eiwitten zoals caseïne, dit resulteert in hypercholesterolemia en atherosclerosis. Semisynthetische rantsoenen met plantaardige eiwitten zoals soja-eiwit zijn daarentegen in staat het cholesterolgehalte in serum laag te houden. Bij de mens is weinig bekend over dergelijke effecten van het type eiwit in de voeding.

Dit proefschrift gaat voornamelijk over de effecten van de voedingseiwitten caseïne en soja-eiwit op de gehaltes aan cholesterol en lipoproteïnen in serum, zoals deze gevonden werden in twee voedingsproeven met vrijwilligers en in vier proeven met proefdieren. In deze proeven werden als voornaamste eiwitbronnen gebruikt: caseïnaat; soja isolaat, wat het meest zuivere soja-eiwit preparaat is dat in de handel verkrijgbaar is; en soja concentraat, wat een minder zuiver soja-eiwit preparaat is.

Aan de eerste proef met de vrijwilligers (Chapter 2) namen 69 studenten deel in de leeftijd van 18 tot 28 jaar. Zij gebruikten gedurende 38 dagen een voeding waarin 13% van de energie door eiwit geleverd werd. Van het eiwit was 65% afkomstig uit óf caseïnaat, óf soja isolaat, óf een 2 op 1 mengsel van caseïnaat en soja isolaat. Gedurende de eerste 10 dagen, de controle periode, kregen alle proefpersonen de gemengde caseïne-soja voeding, waarna zij ingedeeld werden in 3 groepen. Gedurende de hierop volgende testperiode van 28 dagen, gebruikte iedere groep één van de 3 test voedingen. Aan de tweede proef met vrijwilligers (Chapter 3) namen 57 personen deel in de leeftijd van 29 tot 60 jaar. Zij gebruikten gedurende 45 dagen een voeding waarin 16% van de energie door eiwit geleverd werd. Van het eiwit was 60% afkomstig uit óf caseïnaat, óf soja isolaat, óf soja concentraat. Alle proefpersonen gebruikten gedurende de controle periode van 17 dagen de caseïne voeding, waarna ook zij ingedeeld werden in 3 groepen. Gedurende de test periode van 28 dagen gebruikte iedere groep één van de 3 test voedingen. Voor beide proeven geldt dat de test voedingen volledig identiek waren met uitzondering van het gebruikte eiwit preparaat. Afgezien van 0.5 MJ (120 kcal) per dag in de eerste proef, en 1.0 MJ (240 kcal) per dag in de tweede, werden aan de deelnemers volledige dagvoedingen verstrekt, overeenkomstig ieders energiebehoefte. Onder andere aan het eind van de controle en test perioden werd bloed afgenomen ten behoeve van analyses.

Gedurende de proeven met vrijwilligers werden dubbele porties van de test voedingen verzameld. Met deze voedingen werden na afloop 2 konijnenproeven

en één rattenproef uitgevoerd. Tenslotte werd nog een derde konijnenproef uitgevoerd waarbij gebruik gemaakt werd van semisynthetische rantsoenen. Van het eiwit in deze rantsoenen was 100% afkomstig uit óf caseïnaat, óf soja isolaat, óf soja concentraat. De proefschema's van de dierproeven waren gelijk aan die van de vrijwilligers (Chapter 4).

In beide proeven met vrijwilligers werd tussen de caseïne en soja voedingen geen duidelijk verschillend effect gevonden op het totaal serumcholesterolniveau. Vergeleken met de caseïne voeding werd op de soja isolaat voeding een kleine daling waargenomen in het cholesterolgehalte in de lage-dichtheids-lipoproteïnen (LDL) en een kleine stijging in het cholesterolgehalte in de hoge-dichtheids-lipoproteïnen (HDL). De apolipoproteïne-B en -$A_1$ resultaten wijzen erop dat deze veranderingen waarschijnlijk veroorzaakt worden door veranderingen in de aantallen lipoproteïne deeltjes en niet door veranderingen in de samenstelling van deze deeltjes. De genoemde effecten werden echter alleen gevonden met soja isolaat en niet met soja concentraat. Met betrekking tot het risico voor hart- en vaatziekten mogen deze effecten met soja isolaat als gunstig bestempeld worden, maar ze zijn klein vergeleken met de effecten van de vetsamenstelling of het cholesterolgehalte van de voeding.

De resultaten met de proeven met konijnen en ratten op menselijke voedingen bevestigen wat al eerder gevonden was met semisynthetische rantsoenen: in konijnen doet caseïne het cholesterolgehalte in serum zeer sterk toenemen vergeleken met soja eiwit, terwijl in ratten deze effecten veel minder zijn. De resultaten wijzen er op dat men met betrekking tot de relatie voedingseiwit en serum cholesterol voorzichtig moet zijn in het extrapoleren van resultaten uit dierexperimenteel onderzoek naar de mens.

In de literatuur zijn enkele studies beschreven waarin bij hypercholesterolemische patiënten op soja-eiwit voedingen zeer grote dalingen in serumcholesterolniveau waargenomen werden. Met het oog op preventie van hart- en vaatziekten is daarom wel eens gesuggereerd dat het goed zou zijn als dierlijke eiwitten in onze voeding vervangen werden door plantaardige. De resultaten van onze proeven met gezonde vrijwilligers wijzen er echter op dat de normocholesterolemische mens waarschijnlijk vrij ongevoelig is voor het type eiwit in de voeding. Wel moet gezegd worden dat onze proeven van relatief korte duur waren en dat de proefpersonen volwassenen waren. Lange termijn effecten als ook de effecten bij groeiende kinderen dienen nog nader onderzocht te worden.

Hoewel de effecten van het type eiwit in de voeding bij de normocholesterolemische mens waarschijnlijk klein zullen zijn, zal een vervanging van eiwitrijke dierlijke producten door eiwitrijke plantaardige producten in de praktijk toch gunstige effecten kunnen hebben omdat dierlijke producten veelal ook rijk zijn aan verzadigd vet en cholesterol.

Als men met het oog op beïnvloeding van bepaalde fysiologische parameters bepaalde veranderingen in de voeding aanbrengt, dient men zich te realiseren dat ook andere parameters beïnvloed kunnen worden. Een voorbeeld hiervan is gegeven in Chapter 5. Caseïnaten zijn vrijwel vrij van purines. De gebruikte soja preparaten bevatten echter, net zoals vlees, aanzienlijke hoeveelheden purines. Daar voedingspurines in het lichaam omgezet worden in urinezuur, werden bij de proefpersonen op de soja voedingen significant hogere serum uraat spiegels gevonden dan bij de proefpersonen op de caseïnevoeding. Zoals bekend is het ontstaan van jicht direct afhankelijk van het serum uraat niveau.

# SUMMARY

It is well known that feeding animals such as rabbits with semipurified diets containing animal proteins, as for example casein, results in hypercholesterolemia and atherosclerosis. On the other hand, diets containing vegetable proteins such as soybean protein maintain low levels of serum cholesterol. Little is known about the effects of the type of protein in the diet in humans.

This thesis deals with the effects of casein and soy protein on serum cholesterol and lipoproteins, as observed in two experiments with human subjects and four experiments using animals. Three principle sources of protein were compared: caseinate; soy protein isolate, which is the purest soy protein preparation available commercially; and soy protein concentrate, a less refined soy protein preparation.

In the first human experiment (Chapter 2), 69 young healthy students aged between 18 and 28 years were given for a period of 38 days diets containing 13% of energy as protein of which 65% consisted of protein from caseinate, or soy protein isolate, or a 2:1 mixture of caseinate and soy protein isolate. After a control period of 10 days during which all the subjects received the casein-soy diet, the subjects were divided into three groups, each group receiving one of the test diets for a test period of 28 days. In the second human experiment (Chapter 3), 57 healthy subjects aged between 29 and 60 years were given for a period of 45 days diets containing 16% of energy as protein of which 60% consisted of protein from caseinate, or soy protein isolate, or soy protein concentrate. After a control period of 17 days during which they received the casein diet, the subjects were divided into three groups, each group receiving one of the test diets for a test period of 28 days. Subjects in both studies were under strict dietary control. Apart from 0.5 MJ (120 kcal) per day in the first study and 1.0 MJ (240 kcal) per day in the second, all of the food eaten was supplied daily to the subjects in amounts appropriate to individual energy requirements. Food records and chemical analysis of the diets indicated that within each study, there were essentially no differences between the experimental diets except for the type of protein and/or the amount of non-protein material derived from the protein preparations. Blood samples were taken at the end of the control and test periods.

During the experiments with human volunteers, two extra portions of each diet were collected. These were later homogenised and fed to animals in

two experiments with rabbits and one with rats. A further experiment with rabbits was carried out using semipurified diets, the total protein content of which was test protein. The designs of the animal experiments were similar to those of the human experiments (Chapter 4).

In the two human studies, there was no clear difference in response with respect to total serum cholesterol between the casein and the soy diets. A decrease in low-density-lipoprotein (LDL)-cholesterol and an increase in high-density-lipoprotein (HDL)-cholesterol were observed in those on the soy isolate diet when compared with those on the casein diet. The results on apolipoprotein-B and -$A_I$ suggest that the changes in cholesterol concentrations in the lipoprotein fractions resulted mainly from changes in the number of lipoprotein particles. However, these effects were only observed in those on the diets containing soy isolate and not in those on diets containing soy concentrate. With regard to the risk of coronary heart disease, the soy isolate diet can be considered to be beneficial, but the effects are small when compared with the effects of the fat composition and cholesterol content of the diet.

The results of the animal studies using human diets confirm the results reported for semipurified diets. In rabbits, casein was found to be highly hypercholesterolemic when compared with the soy preparations, while in rats the effects were much smaller. The results stress the danger of extrapolating too readily from data from animal experiments on the effect of protein on serum cholesterol directly to man.

Large decreases in level of serum cholesterol have been reported in hypercholesterolemic patients on diets containing soybean protein. Therefore, with regard to prevention of coronary heart disease, it has been suggested that animal protein in the diet should be replaced by vegetable protein. However, the results of the present studies with healthy subjects suggest that the normocholesterolemic human is probably relatively insensitive to the type of protein in his diet. It should be noted, however, that these experiments were of short duration and that the subjects were adults. Effects of a long-term intake of various proteins commencing at an early age have still to be determined.

Although the effects of the type of protein are probably small in normocholesterolemic subjects, the replacement of foods containing animal protein with foods containing vegetable protein in the normal diet may still lead to changes in the concentration of serum lipids, because the main sources of

animal protein are often also rich in saturated fat and cholesterol.

In modifying the diet in order to influence certain physiological parameters, it should be realised that other parameters may also be affected (see Chapter 5). Caseinates are practically free of purines, while soy protein preparations like other protein sources such as meat, contain considerable amounts of purines. Uric acid is the end product of human purine metabolism and the subjects on the soy diets showed significantly higher levels of serum urate than the subjects on the casein diets. It is well known that the prevalence of gout is directly related to serum urate concentration.

# 1. INTRODUCTION

*Diet, serum cholesterol, lipoproteins and coronary heart disease (CHD)*

Atherosclerosis and complications, that is coronary heart disease (CHD), stroke, and peripheral vascular disease, are main causes of death in many Western countries. Present evidence strongly supports the concept that the composition and quantity of dietary constituents can influence atherosclerosis and the risk of developing CHD. Cholesterol in serum probably plays a crucial role in the relationship between diet and atherosclerosis.

Cholesterol is transported through the blood stream in lipoproteins. Lipoproteins which contain lipids and apolipoproteins are discrete particles with finite dimensions. The main classes of lipoproteins which can be distinguished are: chylomicrons, the lightest lipoproteins; very-low-density lipoproteins (VLDL); low-density-lipoproteins (LDL); and high-density-lipoproteins (HDL). Lipoproteins differ in composition and have distinct physiological roles. The main carriers of cholesterol are LDL and HDL, LDL transporting about 70% of the serum cholesterol, and HDL about 15-30%.

A high concentration of LDL-cholesterol is atherogenic, and is used as a risk indicator for CHD. Since most of the cholesterol in serum is in the LDL, the total cholesterol concentration in serum is also often used as a risk indicator. A high concentration of HDL-cholesterol is a negative risk indicator for CHD (1, 2). High concentrations of LDL are known to promote deposition of cholesterol in the arterial wall and other tissues, while HDL may do the reverse.

In the prevention of CHD, current dietary modifications are based on the concept that modification of risk indicators should decrease the risk of CHD. It is well established that dietary modifications may influence levels of serum cholesterol and lipoproteins, and that high total cholesterol and LDL-cholesterol levels in serum contribute directly to atherosclerosis and CHD. However, it has not been proven convincingly in humans that lowering of level of cholesterol in serum can prevent or delay cardiovascular disease. The "ideal experiment" which would reveal such information is practically impossible to carry out (2, 3). However, the evidence from many animal experiments, epidemiological surveys and clinical trials, indicates that such a link is almost inescapable (1, 2).

On the other hand, the protection against CHD which can be obtained by dietary means, must not be overstated. Dietary modifications may lead to a decrease in level of serum cholesterol and to a decrease in mortality

from CHD when the population as a whole is considered. However, for the individual only the effects on the risk of CHD can be estimated. Even dramatic changes in food habits will not give an individual 100% protection against CHD (3). Furthermore, it should be clearly recognised that apart from serum cholesterol, there are many other factors involved in cause of atherosclerosis and its complications.

*Dietary protein, serum cholesterol and lipoproteins*

The influence of dietary factors such as type and amount of dietary fat and amount of cholesterol on serum cholesterol and lipoproteins are well established. Recently more attention has been given to the role of dietary fiber and the type of protein in the diet.

In animal studies, dietary proteins derived from animal sources are generally found to be hypercholesterolemic when compared with proteins from plant sources. In man, epidemiological studies and nutritional studies in vegetarians have also suggested that there is a relationship between intake of animal protein and serum cholesterol. Such epidemiological observations should be interpreted with caution as there may also be differences in other nutritional factors. Only a few controlled trials on the relationship between dietary protein and serum cholesterol have been carried out in humans, mainly with hypercholesterolemic subjects (for a review, see Ref. 4). On the basis of the results of animal studies and several studies on hypercholesterolemic patients, it has been suggested that replacing animal protein in the diet by vegetable protein will be beneficial in the prevention of atherosclerotic diseases (5). However, more information on the possible effects of the type of protein on normocholesterolemic subjects is required.

*Experiments described in this thesis*

In the experiments described in this thesis, the effects of three sources of protein on serum cholesterol and lipoproteins were compared: caseinate; soy protein isolate, the purest soy protein preparation available commercially; and soy protein concentrate, a less refined soy protein preparation. These protein preparations were chosen because the proteins they contain produce largely different effects in certain animal species and because these preparations are available commercially in a highly purified form suitable for human consumption.

In the first experiment with human volunteers (Chapter 2), three diets

were compared: a casein diet, a soy isolate diet, and a diet containing a 2:1 mixture of casein and soy isolate. The last diet was included because in animal experiments it has been observed that the addition of a relatively small amount of soy protein to casein eliminates the effect casein has of raising cholesterol levels. As the possible effect of soy protein on cholesterol may be influenced by the type of protein preparation used, a second experiment with volunteers (Chapter 3) was carried out, in which in addition to the casein and soy isolate diets, a soy concentrate diet was included. This experiment was carried out with middle-aged volunteers because the possible effect of soy protein may be dependent on the lipid status of the subjects; it is known that in Western countries cholesterol levels increase with age. In order to compare the results of experiments carried out with humans and those with animals, diets collected during the human experiments were given to rabbits and rats. In addition an experiment with rabbits was carried out in which the protein preparations were incorporated into semipurified diets (Chapter 4). Most of the results presented in this thesis have been published or presented earlier (6-11).

The casein and soy protein preparations differed not only in the type of protein, but also in a number of other substances, the nucleic acid content for example. The purine bases of dietary nucleic acids are degraded in man to uric acid. Soy protein preparations unlike caseinates contain considerable amounts of nucleic acids. Therefore the effects of soy protein preparations on levels of serum urate were also measured (Chapter 5).

It has been suggested that an increased consumption of animal protein is accompanied by increased blood pressure (12). In the first study, the effects of the various test diets on blood pressure were systematically investigated, but no differences were observed between those on the casein diet and those on the soy test diet. These results have been published elsewhere (13).

The results of many nutritional experiments would have been more meaningful if more appropriate designs had been used. The advantages and disadvantages of experimental designs often used in dietary trials are discussed in Appendix I. The planning, preparation and carrying out of strictly controlled nutritional experiments with humans are discussed in Appendix II.

REFERENCES

1. Grundy SM, Bilheimer D, Blackburn H, et al. Rationale of the Diet-Heart Statement of the American Heart Association. Circulation 1982; 65: 839A-854A.
2. Levy RI. Cholesterol, Lipoproteins, Apoproteins, and Heart Disease: Present Status and Future Prospects. Clin Chem 1981; 27: 653-662.
3. Brussaard JH, Katan MB, Knuiman JT. Voeding en coronaire hartziekten, een verzekering met slechts gedeeltelijke dekking? Ned T Geneesk 1982; 126: 291-296.
4. Terpstra AHM, Hermus RJJ, West CE. The role of dietary protein in cholesterol metabolism. Wld Rev Nutr Diet (in press).
5. Lewis B. Dietary prevention of ischaemic heart disease - a policy for the '80s. Br Med J 1980; 281: 177-180.
6. Raaij JMA van, Engelen MR, Vries JHM de. Effect van caseine versus soja-eiwit op het serum cholesterolniveau van gezonde proefpersonen. Voeding 1980; 41: 260-264.
7. Raaij JMA van, Katan MB, Hautvast JGAJ. Casein, soya protein, serum--cholesterol. Lancet 1979; ii: 958.
8. Raaij JMA van, Katan MB, Hautvast JGAJ, Hermus RJJ. Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. VII Int Symp on Drugs Affecting Lipid Metabolism, Milan, May 28-31, 1980.
9. Raaij JMA van, Katan MB, West CE, Hautvast JGAJ. Influence of diets containing casein, soy isolate and soy concentrate on serum cholesterol and lipoproteins in middle-aged volunteers. XII Int Congress of Nutrition San Diego, August 16-21, 1981.
10. Raaij JMA van, Katan MB, Hautvast JGAJ, Hermus RJJ. Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. Am J Clin Nutr 1981; 34: 1261-1271.
11. Raaij JMA van, Katan MB, West CE, Hautvast JGAJ. Influence of diets containing casein, soy isolate and soy concentrate on serum cholesterol and lipoproteins in middle-aged volunteers. Am J Clin Nutr 1982; 35: 925-934.
12. Sacks FM, Rosner B, Kass EH. Blood pressure in vegetarians. Am J Epidemiol 1974; 100: 390-398.
13. Brussaard JH, Raaij JMA van, Stasse-Wolthuis M, Katan MB, Hautvast JGAJ. Blood pressure and diet in normotensive volunteers: absence of an effect of dietary fiber, protein, or fat. Am J Clin Nutr 1981; 34: 2023-2029.

# 2. Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers[1, 2]

*Joop M. A. van Raaij, M.Sc., Martijn B. Katan,[3] Ph.D., Joseph G. A. J. Hautvast, M.D.D.N. (Cantab), and Ruud J. J. Hermus,[4] Ph.D.*

ABSTRACT The effects of casein and soy protein on serum cholesterol levels and lipoprotein composition were studied in 69 healthy volunteers (18 to 28 yr of age) under strict dietary control. Subjects were fed for 6 wk on diets containing 13% of energy as protein, 38% as fat (P/S ratio = 0.6) and about 380 mg cholesterol per day. Of the protein in the diets 65% consisted of casein or soy protein or a 2:1 mixture of casein and soy protein. After a control period of 10 days during which all the subjects received the casein-soy diet, 20 subjects continued on this diet for the next 4 wk as a base-line control, 25 subjects switched to the casein diet, and the remaining 24 subjects switched to the soy diet. Both food records and chemical analysis of double portions revealed that the diets were completely identical except for the type of protein. Average serum cholesterol levels at the end of the control period were 152 ± 27 mg/dl (3.93 ± 0.69 mmol/l) and 153 ± 23 mg/dl (3.95 ± 0.60 mmol/l) (mean ± SD) for the casein and soy group, respectively. At the end of the test period the levels were 149 ± 24 and 150 ± 23 mg/dl, respectively; thus there was no significant change on either diet. On the casein diet there was no change in the low-density lipoprotein cholesterol concentration, and only a slight, nonsignificant increase in the high-density lipoprotein cholesterol concentration. On the soy diet, however, there was a significant decline in low-density lipoprotein-cholesterol (−6.6 mg/dl; −0.17 mmol/l) and a significant increase in high-density lipoprotein-cholesterol (+5.8 mg/dl; +0.15 mmol/l). The decline in low-density lipoprotein cholesterol in the soy group was significantly different from the small change in the casein group, but the difference in increase in high-density lipoprotein cholesterol in the soy and the casein group was only weakly significant. This suggests that soy protein could have a slight beneficial effect on the distribution of cholesterol over the various lipoprotein fractions, even at constant total cholesterol concentration. *Am. J. Clin. Nutr.* 34: 1261–1271, 1981.

KEY WORDS Cholesterol, serum lipids, casein, soy protein

## Introduction

Diet is thought to play an important role in provoking hypercholesterolemia and atherosclerosis. The influence of nutritional factors such as type and amount of dietary fat, carbohydrate, and dietary fiber on plasma cholesterol concentrations has been widely emphasized. Recently more attention has been devoted to the role of the type of dietary protein.

In rabbits proteins derived from animal sources as part of semipurified diets are generally found to produce hypercholesterolemia, whereas usually little or no elevation of

[1] From the Department of Human Nutrition, Agricultural University, 6703 BC Wageningen, The Netherlands.

[2] Supported in part by the Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands.

[3] Author to whom request for reprints should be addressed.

[4] Present address: Central Institute for Nutrition and Food Research (CIVO-TNO), P.O. Box 360, 3700 AJ Zeist, The Netherlands.

serum cholesterol is observed with proteins from plant sources (1–5). Similar effects have been observed in rats (6) and pigs (7); the data for chickens are conflicting (8, 9).

In man, epidemiological data show a strong positive correlation between animal protein consumption and mortality from coronary heart disease (10). Furthermore, in several countries the trend toward increasing mortality from coronary heart disease during this century coincides with a doubling in the ratio of animal to vegetable protein in the diet (11–13). In nutritional studies of vegetarians, Hardinge and Stare (14) found lower serum cholesterol levels among those who were pure vegetarians than among lacto-ovo-vegetarians. Both groups had significantly lower levels than non-vegetarians. Sacks et al. (15) found similar effects in vegetarians.

In humans, only a few controlled trials relating dietary protein to serum cholesterol have been carried out. While differences in the concentrations of serum cholesterol have been consistently produced in rabbits (2–5, 16), the results of studies on humans are conflicting (17–19). We have, therefore, investigated the effect of casein and soy protein on the concentrations of serum cholesterol and lipoproteins in healthy young volunteers using highly purified proteins and with strict control of nutrient intake.

## Subjects and methods

*Subjects*

Forty-six male and 30 female university students, aged 18 to 28 yr, volunteered and were selected for the study. All subjects were healthy and had normal values for serum cholesterol, serum triglycerides, blood pressure, and percentage of body fat as described for previous experiments (20). In addition the subjects were found to have normal values for blood hemoglobin and erythrocyte sedimentation rate and to have no detectable glucose or protein in the urine; none had received any medication known to affect serum lipids for at least 2 months before the study.

The experimental protocol was fully explained to the participants and informed consent was obtained. The subjects were not offered payment and were free to stop participating at any moment. They were seen daily by one of the investigators (J.M.A.v.R.) and by two research dietitians. During the experiment two subjects became ill, two decided to withdraw, and three did not fully adhere to the dietary protocol; their data were eliminated.

*Diets and control of food intake*

The amount (and type) of fat, cholesterol, protein, and carbohydrates in the diets were planned to simulate an average Western diet (11). The diets were completely identical except for the type of dietary protein. Of the protein 65% was replaced by either casein (casein diet), soy protein (soy diet), or a 2:1 mixture of casein and soy protein (cassoy diet). To keep the diets acceptable 35% of the protein came from other sources (mainly from wheat, rice, potatoes, and other vegetables).

The 65% replacable protein was incorporated as caseinate or soy isolate (**Table 1**) into specially developed products. Milk like beverages were prepared from butter concentrate, lactose, milk minerals and test protein (caseinate or soy isolate, 4.4% by weight); these beverages were in part fermented to yoghurts; both were developed by Unilever Research Laboratory, 3130 AC Vlaardingen, and by DMV-Veghel, and manufactured by the Department of Food Technology of the Agricultural University. The Institute for Cereals, Flour and Bread (IGMB-TNO), 6701 AN Wageningen, prepared brown breads from flour, bran, and test protein (75, 10, and 15 parts respectively) and small amounts of minerals, yeast, etc.; gluten-free breads from gluten-free flour and test protein (85 and 15 parts, respectively) and small amounts of minerals, yeast, etc; cookies from gluten-free flour, test protein, sugar, and fat (51, 10, 22, and 17 parts, respectively). Sandwich spreads were manufactured by DMV-Veghel and by Unilever Research, and consisted of test protein (14% by weight), fat (16% by weight) and either vegetables or fruits plus sugar. Cheese was used in the casein and the cassoy diets because it contains casein as practically the only protein. For the soy diet, a gelated product was prepared by Unilever Research from soy isolate (20% by weight) flavoring and spices (2.5% by weight) and water; butterfat was also added to the soy diet to balance the fat in cheese. Fresh egg yolk was used in all diets to adjust cholesterol intakes and each subject consumed one multivitamin tablet per day. Each diet met the nutritional requirements for adults (21). Special margarines were prepared by VanDenBerg & Jurgens B.V., 3000 AD Rotterdam. All supplies were purchased in bulk except for fresh products which were purchased from the same distributors throughout the experiment.

TABLE 1
Composition of casein and soy protein isolate used in the experimental diets

| | Casein* | Soy isolate† |
|---|---|---|
| | *g/100 g* | |
| Protein (N × 6.25)‡ | 90.5 | 92.8 |
| Water | 3.0 | 3.1 |
| Lactose | <0.2§ | |
| Fat | <0.8§ | <0.1§ |
| Ash | 4.2 | 3.5 |

* Calcium and sodium caseinate (spray dried, bland), DMV Milk Industries, 5460 BA Veghel. Data expressed as mean value for calcium and sodium caseinate.

† UNISOL NH 70, UNIMILLS B.V., 3330 AA Zwijndrecht.

‡ The true Kjeldahl nitrogen-to-protein conversion factors for casein and soy protein are 6.38 and 5.70, respectively (52). Using the figure of 5.70 the protein content of soy isolate is 84.6 g/100 g, which by difference gives an unavailable carbohydrate content of about 8.8 g/100 g.

§ Data provided by manufacturer.

The three diets consisted of the same regular foodstuffs and of highly similar special products. Breakfast and evening meal consisted of breads, margarine, spreads, sweetenings, fruit and fruit juices. The hot meal at noon consisted of soup, potatoes (or rice), other vegetables, sauces, egg yolk, cheese (or gelated soy product), and dessert. The soups, sauces, and desserts were prepared from the analogs of milk and yoghurt. None of the following products was used: dairy products (except for cheese), meats, egg white, fish, legumes, onions, and garlic.

All foodstuffs were weighed out for each person in quantities appropriate to his or her energy needs, except for 500 kJ (120 kcal) per day, which the subjects were free to choose from a list of foodstuffs not containing protein. Individuals were allowed unlimited tea, coffee and selected low-calorie beverages. Sugar was provided and up to 6 g/day of coffee whitener was allowed. Hot meals were served on weekdays at noon in the department. All other foods were provided as packages daily; food for the weekend including ingredients for midday hot meals, was provided each Friday afternoon. Food rejections or deviations of any kind were recorded in diaries. Most of the special products were well accepted and adherence to the diets was excellent.

During the control and test period the actual intake of nutrients was calculated for each individual on 2 and 4 separate days, respectively, by multiplying the weights of foods on the questionaire by values on a computerized food composition table (22). In addition, double portions of each diet were analyzed as described earlier (20, 27).

*Experimental design*

All subjects consumed the cassoy diet for a control period of 10 days, after which they were divided into three groups; the groups were matched for initial serum cholesterol, energy intake, and sex. During the test period of 28 days, the casein group (n = 25) received the casein diet, the soy group (n = 24) received the soy diet, and the cassoy group (n = 20) continued on the cassoy diet, as shown in **Figure 1**.

Body weight was recorded weekly. For each individual, energy intake was adjusted to avoid changes in body weight of more than 2 kg/2 wk. During the control period body weights decreased by 1.1, 0.8, and 1.1 kg in the casein, cassoy, and soy group, respectively. These slight weight losses continued during the test period, with mean weight reductions of 1.3, 1.0, and 1.6 kg, respectively. The largest declines were found in three subjects on the soy diet (3.1, 4.0, and 4.1 kg). There were no differences in weight loss between the groups during the test period.

The subjects were asked to note in diaries illness, drug use and departures from the diets. Hemoglobin and erythrocyte sedimentation rate were measured fortnightly and found to remain normal.

*Blood sampling and analysis*

Specimens of blood were obtained from an arm vein after an overnight fast. Blood was collected weekly and serum was obtained by low-speed centrifugation. All the samples were assayed for total cholesterol and high-density lipoprotein (HDL)-cholesterol. At the end of the control and test period two samples were taken at 1-day intervals (Fig. 1), and the results for total cholesterol and HDL-cholesterol were averaged. Serum cholesterol was measured with the reagent of Huang et al. (23) using serum calibrators as described earlier (24). Reproducibility for blind control sera provided by the Center for Disease Control, Atlanta, GA, was ± 1.4% (CV) and accuracy was within 1.8% of the "true" (target) values. HDL-cholesterol was determined after Mn-heparin precipitation of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (24, 25) at an Mn-concentration of 92 mmol/l. At the end of both periods, lipoproteins in sera of the subjects from the casein and soy group were separated by density gradient ultracentrifugation (26–28). Before preparing the gradient prior to the ultracentrifugation, Sudan Black was mixed with the serum in order to stain the lipoproteins (28). After centrifugation the lipoprotein bands were photographed and then separated by use of a tube slicer into VLDL ($d < 1.015$ g/ml), LDL ($1.015 < d < 1.060$), and HDL ($d > 1.060$ g/ml). For each individual the mean LDL density (center of the colored LDL band) was calculated from the photograph with the aid of the known density gradient in the centrifuge tubes (A. H. M. Terpstra, C. J. H. Woodward, and F. J. Sanchez-Muniz, unpublished data). Lipoprotein fractions were assayed for cholesterol by an enzymatic method (29) using a kit (No. 124087, Boehringer-Mannheim GmbH, West Germany) and using serum calibrators as described earlier (24).

The mean recovery was 97.8% (CV = 4.6%). Day-to-day reproducibility was ±1.0% (CV) and bias was less than 1.0% compared with values obtained by the Abell method (30). Apolipoprotein-B was measured in whole sera by rocket immunoelectrophoresis as described previously (27). For one person the samples obtained at the

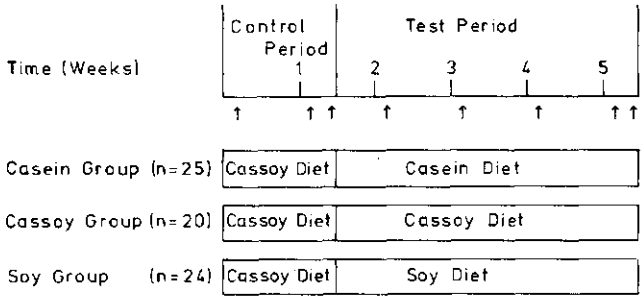



FIG. 1. Experimental design. Blood sampling is indicated by *arrows*.

start and at the end of the test period were always analyzed on one plate in order to minimise the effect of interassay variability. Analytical error was monitored by analysing a sample from a control pool on each plate. The combined within-day and between-day coefficient of variation for this control serum was 7%.

*Statistical evaluation*

The major hypothesis to be tested was that soy protein would lower serum cholesterol concentration, if compared with casein. A minor hypothesis was that a 2:1 mixture of casein and soy protein would also lower serum cholesterol, when compared with casein. The effect of diet was examined by testing the difference between the mean responses of the groups by unpaired one tailed *t* tests (31). The experiment was designed to protect against an error of the second type, i.e., missing a significant difference through chance fluctuations: it was calculated that the casein and the soy group would each need to contain at least 24 subjects in order to detect differences between groups in total cholesterol response of at least 0.25 mmol/l (10 mg/dl) at a significance level $\alpha$ of 5% and with a power $\beta$ of 80%. The effect of sex was examined by analysis of variance (31).

## Results

*Nutrient intake*

The results of nutrient intake, as measured by food records, are shown in **Table 2**. Food records and the chemical analysis of double portions indicated that there were essentially no differences between the experimental diets with respect to the amount of protein, fat, carbohydrate, alcohol, cholesterol, and dietary fiber and to the composition of the fatty acids, plant sterols and carbohydrates. The calcium content of the casein diet was slightly higher than that of the soy diet, but it is unlikely that this influenced serum lipids. The analysis did reveal a slightly higher total protein content and a lower sugar content than the calculations based on the records for all diets. The amino acid composition of casein was, as expected considerably different from that of soy protein (**Table 3**). The amino acid composition of the whole diet was diluted by those proteins that were already present and similar for all diets. However, the differences between the diets were still pronounced (Table 3) and established beyond doubt that the planned differences in protein amino acid composition had been achieved. Both from the food records and from the amino acid analysis it could be calculated that about 65% of the total dietary protein consisted of soy protein or casein.

*Serum total cholesterol*

The time course of the mean serum total cholesterol concentration is presented for each group in **Figure 2**. The values of the

TABLE 2
Mean daily intake of nutrients before and during the experiment according to individual food records*

| | Habitual intake before experiment† | Control period | Test period | | |
|---|---|---|---|---|---|
| | | Cassoy diet‡ | Casein diet§ | Cassoy diet§ | Soy diet§ |
| Energy¶ (kcal) | 2710 | 2595 | 2515 | 2670 | 2565 |
| (MJ) | 11.3 | 10.8 | 10.5 | 11.2 | 10.7 |
| Total fat (energy %) | 33.1 | 38.5 | 38.2 | 38.6 | 38.4 |
| Saturated | 15.7 | 13.8 | 13.2 | 13.8 | 12.9 |
| Mono-unsaturated | 12.0 | 17.0 | 17.0 | 17.0 | 17.4 |
| Poly-unsaturated | 5.4 | 7.7 | 7.9 | 7.8 | 8.1 |
| Carbohydrates (energy %) | 49.3 | 47.4 | 47.2 | 47.5 | 47.4 |
| Sugars | 22.6 | 23.3 | 23.2 | 23.4 | 22.4 |
| Polysaccharides | 26.7 | 24.1 | 24.0 | 24.1 | 25.0 |
| Protein (energy %) | 13.7 | 12.6 | 12.9 | 12.3 | 12.6 |
| Casein | | 5.6 | 8.8 | 5.4 | |
| Soy protein | | 2.6 | | 2.5 | 8.4 |
| Alcohol (energy %) | 3.9 | 1.6 | 1.7 | 1.6 | 1.7 |
| Cholesterol (mg/day) | 334 | 394 | 387 | 398 | 365 |
| Dietary fiber (g/day) | 38.2 | 28.8 | 28.7 | 29.4 | 28.2 |

* The food records were elaborated using Netherlands food composition tables supplemented with analyses of special products.
† Three-day records.
‡ Two-day records.
§ Four-day records.
¶ 1000 kcal = 4.2 MJ.

TABLE 3
Amino acid composition of casein, soy protein isolate, and the duplicate portions of the diets*, †

| Amino acid | Pure protein | | Complete diets | | |
|---|---|---|---|---|---|
| | Casein§ | Soy Isolate | Casein | Cassoy | Soy |
| | | | *g/100 g amino acid* | | |
| L-alanine | 2.8 | 4.1 | 3.2 | 3.5 | 4.0 |
| L-arginine | 3.3 | 7.6 | 3.8 | 4.8 | 6.8 |
| L-aspartic acid‖ | 6.5 | 11.3 | 7.5 | 8.5 | 10.8 |
| L-glutamic acid‖ | 21.4 | 19.6 | 23.2 | 22.9 | 22.1 |
| Glycine | 1.7 | 4.0 | 2.3 | 2.9 | 3.9 |
| L-histidine | 2.8 | 2.5 | 2.7 | 2.7 | 2.5 |
| L-isoleucine | 5.1 | 5.0 | 4.7 | 4.6 | 4.7 |
| L-leucine | 8.8 | 7.8 | 8.3 | 8.0 | 7.6 |
| L-lysine | 7.6 | 6.2 | 6.4 | 6.1 | 5.5 |
| L-methionine | 2.5 | 1.2 | 2.0 | 1.6 | 1.2 |
| L-phenylalanine | 4.7 | 5.2 | 4.6 | 4.7 | 4.9 |
| L-proline | 9.5 | 4.9 | 9.0 | 8.1 | 5.8 |
| L-serine | 5.5 | 5.3 | 5.5 | 5.6 | 5.1 |
| L-threonine | 3.9 | 3.7 | 3.7 | 3.7 | 3.6 |
| L-tyrosine | 5.2 | 3.6 | 4.1 | 3.7 | 3.3 |
| L-valine | 6.4 | 5.0 | 6.0 | 5.5 | 5.0 |
| L-cystine‡ plus L-tryptophan‡ | 3 | 3 | 3 | 3 | 3 |
| | 100 | 100 | 100 | 100 | 100 |

* Amino acid analysis of a hydrochloric acid digest of the lyophilized material using a Jeol JLC-5AH amino acid analyzer.
† Expressed as g amino acid per 100 g amino acid.
‡ Cystine and tryptophan were not analyzed; their total contribution was estimated (52) as 3 g/100 g.
§ Average value for sodium and calcium caseinate.
‖ Including amide.

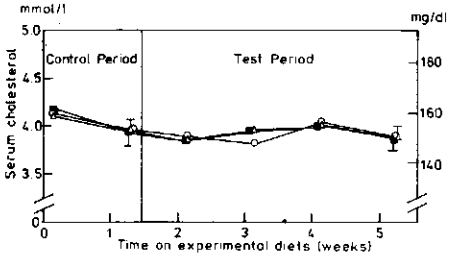



FIG. 2. Effects of casein and soy protein diets on mean total serum cholesterol concentrations in humans throughout the experiment. *Vertical bars* indicate 1 SEM. ■——■, casein group (25 subjects); O——O, cassoy group (20 subjects); Δ——Δ, soy group (24 subjects).

mean concentrations at the end of both periods are shown in **Table 4**. During the cassoy control period of 10 days serum total cholesterol concentration decreased slightly in all groups compared with the uncontrolled preexperimental situation. The control group that continued on the cassoy diet did not show any change in serum total cholesterol during the test period, proving that levels had stabilized already at the end of the base-line

TABLE 4
Effects of casein and soy protein diets on serum total cholesterol concentrations (Mean ± SD)*

| | Casein group (n = 25) | Cassoy group (n = 20) | Soy group (n = 24) |
|---|---|---|---|
| | | *mg/dl*† | |
| Control period | 152 ± 27 | 153 ± 24 | 153 ± 23 |
| Test period | 149 ± 24 | 150 ± 25 | 150 ± 23 |
| Change | −3 ± 14 | −3 ± 13 | −3 ± 10 |

* Each value represents the average of two separate determinations at the end of the respective period.
† 100 mg/dl = 2.59 mmol/l.

period of 10 days. The soy and the casein diets also induced hardly any change in average cholesterol concentrations, except for minor oscillations of about 4 mg/dl (0.1 mmol/l). No sex effects were found.

### *Distribution of cholesterol over the lipoprotein fractions*

The mean cholesterol concentrations in the lipoprotein fractions are given in **Table 5**. Comparison of HDL-levels determined by ultracentrifugation and by the Mn-heparine

TABLE 5
Effects of casein and soy protein diets on cholesterol concentrations in lipoprotein fractions and on serum apolipoprotein-B levels (mean ± SD)

| | Casein group (n = 23) | Soy group (n = 23) |
|---|---|---|
| | *mg/dl** | |
| Cholesterol | | |
| Ultracentrifugation: | | |
| VLDL control period | 8 ± 3 | 10 ± 3 |
| test period | 10 ± 5 | 10 ± 5 |
| change | +1.5 ± 5.8 | +0.8 ± 5.0 |
| LDL control period | 80 ± 17 | 88 ± 19 |
| test period | 79 ± 16 | 81 ± 19 |
| change | −0.4 ± 12.4 | −6.6 ± 9.3† ‡ |
| HDL control period | 59 ± 16 | 57 ± 10 |
| test period | 62 ± 15 | 62 ± 12 |
| change | +2.3 ± 8.5 | +5.8 ± 5.0† |
| Mn-heparin precipitation: | | |
| HDL control period | 58 ± 12 | 55 ± 9 |
| test period | 60 ± 12 | 58 ± 8 |
| change | +1.9 ± 5.4 | +3.1 ± 3.5† |
| Serum apoprotein-B | *mg/l* | |
| control period | 488 ± 119 | 462 ± 107 |
| test period | 441 ± 99 | 447 ± 107 |
| change | −47 ± 52†‡ | −15 ± 51‡ |

* 100 mg/dl = 2.59 mmol/l.
† Significantly different from 0 (paired $t$ test; $p < 0.05$).
‡ Significantly different from casein group (unpaired $t$ test; $p < 0.05$).

procedure yielded a correlation coefficient of $r = 0.91$, which is similar to published values (32, 33). Values obtained by the ultracentrifugational method were in general a few percent higher than those obtained by the precipitation procedure (cf. Table 5). This is a common finding (32, 33) and is probably explained by the presence of small amounts of sinking pre-$\beta$ lipoprotein (lipoprotein (a)) in the ultracentrifugal HDL fraction when isolated within the classical density limits of 1.063 and 1.210 g/ml. In the casein group there were no significant changes in concentrations in any of the lipoprotein fractions. In the soy group, however, there was a significant decline in LDL-cholesterol and a significant increase in HDL-cholesterol.

There were no changes in VLDL-cholesterol concentration. The decline in LDL-cholesterol in the soy group is significantly different from the small change of LDL-cholesterol in the casein group, but the difference in increase in HDL-cholesterol in the soy and the casein group is only weakly significant according to the ultracentrifugal data and not significant according to the precipitation method. Analysis of variance failed to show significant sex effects on the changes during the test period. The individual changes in serum total cholesterol, HDL- and LDL-cholesterol were not correlated with the initial values (at the end of the control period).

*Serum apolipoprotein-B concentration*

In both the casein and the soy group the apoprotein-B concentration declined during the test period (Table 5), but the decline in the casein group was significantly greater than that in the soy group. In the casein group the LDL-cholesterol/apoprotein-B ratio increased significantly from 1.64 to 1.79 g/g ($p < 0.01$), and in the soy group this ratio declined from 1.90 to 1.81 g/g ($p < 0.05$).

It should be noted that at the end of the control period the soy group happened to have a lower mean concentration of apo-B

and a higher concentration of LDL-cholesterol than the casein group. This resulted in a significantly higher initial LDL-cholesterol/apo-B ratio in the soy group than in the casein group. Such a difference is not surprising, because we did not explicitly match the groups for this ratio.

During the test period the mean densities of the LDL particles of the subjects in the casein group declined significantly from 1.039 ± 0.005 to 1.036 ± 0.003 g/ml (mean ± SD; paired $t$ test; $p < 0.05$). In the soy group, the slight increase in mean densities from 1.036 ± 0.002 to 1.037 ± 0.002 g/ml was not significant.

## Discussion

Recent experiments, both in various animal species (2, 3, 6, 7) and in humans (17, 18) have shown profound effects on serum cholesterol levels of soy versus meat and/or milk proteins. This has led to the expectation that replacement of animal by vegetable protein in the human diet might aid in lowering serum total cholesterol levels, thereby providing an extra tool in the prevention of atherosclerotic vessel disease. Our results do not provide support for these expectations: in a carefully controlled short-term trial using nearly 70 healthy young subjects we found no effect on serum total cholesterol of either casein or soy protein compared with a 2:1 mixture of these proteins. However, when duplicate portions of the same diets were fed to 12 New Zealand White rabbits the casein diet caused much higher serum cholesterol concentrations than the soy diet; the difference was already significant after 2½ wk (**Fig. 3**; Reference 34).

There are several ways to explain the differences in response between humans and animals. It is known that animal species differ greatly in resistance to diet-induced hypercholesterolemia. Apparently, healthy humans are less sensitive to changes of dietary protein than rabbits, rats, or pigs. Furthermore, most animal experiments have used young animals which have spent a considerable part of their lifespan on the test diets. Our results do not rule out the possibility that a long-term intake of animal protein, starting at an early age, will reveal similar cholesterol-raising effects in normocholesterolemic humans. All the same, our results do stress the risk of extrapolating too readily animal data concerning the effect of protein on serum cholesterol directly to man. However, these species differences may provide clues to the mechanism of cholesterol homeostasis.

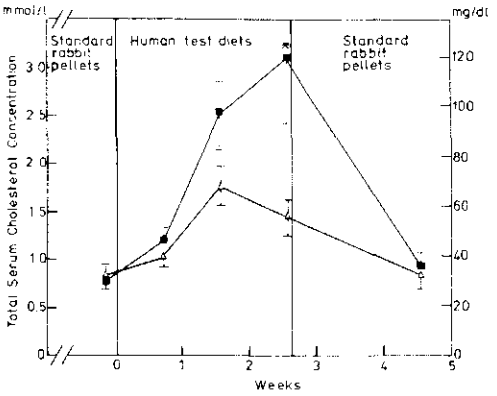



FIG. 3. Effects of casein and soy protein diets on mean total serum cholesterol concentrations of rabbits. *Vertical bars* indicate 1 SEM. Throughout the experiment on humans, duplicate portions of the casein and soy diets were collected and stored at −40°C. These were homogenized, freeze-dried, and pelletted afterward, and fed to 12 New Zealand White rabbits (3 months of age). Before and after the test period of 2½ wk the rabbits were fed a commercial rabbit pellet diet. The increase in serum total cholesterol on the casein diet was significantly greater than that on the soy diet ($p < 0.05$). As the groups had unequal variances $t$ was tested at 5 instead of 10 degrees of freedom, as suggested by Cochran (Reference 31, p. 115). After switching back to the commercial diet serum cholesterol concentrations returned to baseline values. ■——■, casein group (n = 6); △——△, soy group (n = 6).

Epidemiological studies have suggested, when comparing nations or whole populations, a relation between intake of animal protein, serum cholesterol level, and cardiovascular disease (10).

Epidemiological data, however, should be interpreted with caution as differences in other nutritional factors may be present. As an example, the main sources of animal protein (meat and dairy products) also contain much saturated fat and cholesterol.

Very few strictly controlled experiments in man relating the source of dietary protein to the concentration of cholesterol in serum have been carried out, and the sparse results are contradictory (**Table 6**). No clear effects of the type of dietary protein were found by Walker et al. (35), Campbell et al. (36), An-

**TABLE 6**
**Summary of studies with humans on the effect of dietary protein on the serum (plasma) cholesterol concentration**

| Subjects | n | Protein source | Composition of test diets | | | | Duration of test periods | Effect of plant diet | Authors |
|---|---|---|---|---|---|---|---|---|---|
| | | | Protein* | Fat* | P/S | cholesterol | | | |
| | | | | | | *mg/day* | *days* | *mg/dl*† | |
| Healthy women (17–22 yr) | 6 | Mixed animal | 8 | 36 | | | 42 | | Walker et al. (35) |
| | 6 | Mixed plant | 8 | 36 | | | 42 | −13 NS | |
| Healthy men (53–70 yr) | | Casein/Lactalb.‡ (75%)§ | 7 | 40 | low‖ | | 25 | | Campbell et al. (36) |
| | | Gluten (75%) | 7 | 40 | low‖ | | 25 | −18 NS | |
| | 6¶ | | | | | | | | |
| | | Casein/lactalb. (75%) | 7 | 40 | high‖ | | 25 | | |
| | | Gluten (75%) | 7 | 40 | high‖ | | 25 | +9 NS | |
| Healthy men (21–26 yr) | 11¶ | Egg white (50%) | 16 | 37 | 0.5 | 308 | 28 | | Anderson et al. (37) |
| | | Gluten (50%) | 16 | 37 | 0.5 | 308 | 28 | +4 NS | |
| Hypercholesterolemic patients (22–68) | 20¶ | Mixed animal (63%) | 21 | 21 | 2.2 | 140–200 | 21 | | Sirtori et al. (17)** |
| | | Soy (63%) | 21 | 26 | 2.7 | 0 | 21 | −50†† | |
| Healthy women (19–25 yr) | 10¶ | Mixed animal (58%) | 15 | 34 | 0.4 | 168 | 37–41 | | Carroll et al. (18) |
| | | Soy (58%) | 16 | 33 | 0.4 | 161 | 37–41 | −9‡‡ | |
| Mildly hypercholesterolemic young men | ? | Mixed animal | 13–16 | 30–35 | 0.4 | 100–250 | 42 | | Shorey and Davis (19) |
| | ? | Soy | 13–16 | 30–35 | 0.4 | 100–250 | 42 | +6 NS | |
| Healthy men and women (18–28 yr) | 25 | Casein (65%) | 13 | 37 | 0.6 | 387 | 28 | | present results |
| | 24 | Soy (65%) | 13 | 37 | 0.6 | 365 | 28 | 0 | |

* Percentage of total energy intake.
† 100 mg/dl = 2.59 mmol/l.
‡ Casein/lactalbumin mixture: 80% casein, 20% lactalbumin.
§ The values within parentheses indicate the percentage of the total protein provided by the indicated protein.
‖ Low P/S ratio: 12% of total fat as linoleic acid; high P/S ratio: 40% of total fat as linoleic acid.
¶ In this study a cross-over design was used.
** Addition of 500 mg cholesterol per day to the soy diet did not modify the hypocholesterolemic response (17). The soy diet was less effective at a P/S ratio of 0.1 (38).
†† $p < 0.01$.
‡‡ $p < 0.05$.

derson et al. (37), and Shorey and Davis (19). On the other hand, small but significant effects have been observed by Carroll et al. (18), while striking cholesterol-lowering properties of soybean protein have been reported by Sirtori et al. (17, 38). How can one explain these different results?

Many soy products contain appreciable amounts of nonprotein material and it has been suggested that this is partly responsible for the observed effects in some experiments (39–45). Even the soy protein isolate used in our experiment, which is representative of the purest commercially available material, still contains about 10% of nonprotein material, mainly carbohydrates. The customary Kjeldahl nitrogen-to-protein conversion factor of N × 6.25 obscures this fact (Table 1). It has also been suggested that the form in which the soy protein is incorporated in the diets is important (46). The test diets in some studies differed not only in the source of protein but also in cholesterol content and the amount and composition of fat because of the strong coupling of protein with other nutrients in ordinary foodstuffs (17). The lipid status of the subjects could be an important factor: our subjects had low to very low serum cholesterol levels, while Sirtori et al. (17, 38) studied hypercholesterolemic patients. Normocholesterolemic subjects are possibly less sensitive to changes in dietary protein than hypercholesterolemic individuals (38), which remains to be established. All these factors may have played a role in producing conflicting results in the different studies with humans.

Another important factor is the use in the studies of widely different "animal" and "vegetable" proteins. It has been shown that animal or vegetable proteins cannot be grouped in that way regarding their cholesterolemic action in rabbits (1, 3, 41, 43). Animal experiments suggest that effects of dietary protein on serum cholesterol can be explained, at least partly, by the amino acid composition of the proteins (2, 3, 34, 43, 45, 47–49). The role of the individual amino acids or of specific combinations of amino acids, however, still remains unclear. Attention has been particularly focused upon the lysine/arginine ratio (3, 49, 50), glycine (43, 49), glutamic acid (51), and methionine (39) but the results are not conclusive (49). The amino acid composition of soy protein is intermediate in many regards between casein and meat protein (52). As a result, our soy protein diet contained more alanine, arginine and glycine and less glutamic acid, tyrosine, and valine than the casein diet, but the reverse was true for the plant protein diet (containing soy protein) in Carrol's experiment (18); there was less alanine, arginine, and glycine and more glutamic acid, tyrosine, and valine in their plant protein diet than in their meat/milk protein diet. Therefore, it is conceivable that had we chosen other plant and animal proteins, we might have been able to demonstrate an effect on the concentration of cholesterol in serum.

Our data suggest a small decline in LDL-cholesterol and a small increase in HDL-cholesterol concentration on soy protein but not on casein. In view of the inverse relation between HDL-cholesterol concentration and obesity, the lipoprotein data were reanalyzed after exclusion of possible interference by weight loss. Deletion of the results of the subjects who lost more than 2 kg of body weight during the test period (six subjects from the casein and nine from the soy group) left the conclusions essentially unchanged.

The effect on HDL- and total cholesterol remained the same after exclusion of those people who lost more than 2 kg. Only the difference in the effect on LDL-cholesterol became less pronounced (3 instead of 6 mg/dl). Sirtori et al. (38) found that soybean protein exerted its hypocholesterolemic effect mainly by lowering LDL-cholesterol, while only a minor decline of HDL-cholesterol was observed. Our results for the LDL-cholesterol/apoprotein-B ratio suggest that ingesting the casein diet caused a shift to LDL-particles richer in cholesterol, while consuming the soy diet caused a shift to LDL-particles containing less cholesterol. The interpretation of these changes is complicated, however, because persons randomized into the soy group happened to have higher LDL-cholesterol/apoprotein-B ratios than the subjects in the casein group; thus the starting levels were not comparable, although the direction of the individual diet effects was unmistakeable. These findings are confirmed by the changes in the density of the LDL. The data on the LDL composition and density are

preliminary only and this finding should be investigated further. However, it seems clear that the absence of an effect on total cholesterol levels by dietary casein and soy protein does not rule out the possibility that changes in composition and/or concentration of individual cholesterol-transporting lipoproteins do occur. Although it has not definitely been "proven" that an increase of the HDL/LDL cholesterol ratio by dietary manipulation will decrease the incidence of coronary artery diseases, it is a fact that high HDL/LDL cholesterol ratios are predictive for lower risk for these diseases (53, 54). If our results can be confirmed, this would suggest that soy protein could have qualitatively a beneficial effect, even at constant total cholesterol concentrations.

The authors thank the volunteers for their excellent cooperation and keen interest. We wish to acknowledge for developing and preparing the special products: DMV-Milk Industries (F. Visser and J. Schut), Unilever Research (P. van Stratum, H. Vonkeman, C. Andreae, H. Herstel, G. G. Nijholt, and P. Westdorp before his untimely death), IGMB-TNO (P. Sluimer), VanDen-Bergh & Jurgens (J. W. Van der Kamp), The Department of Food Technology of the Agricultural University (H. J. van der Stege and G. Kleter), and UNIMILLS (L. Schutte and A. Visser). We thank S. Visser (NIZO) for the amino acid analyses; P. H. E. Groot (Erasmus University, Rotterdam) for providing us with the methodology and materials for apolipoprotein assays; A. H. M. Terpstra for supervising the rabbit eyperiment; C. E. West for help in preparing the manuscript; the dietitians Marita Engelen, Jeanne de Vries, Annelies Geenen and Lenie Lamers for preliminary dietary planning and for dietary supervision; the analysts Mrs. J. Barendse, Mrs. J. Bos, Mrs. C. Germing, F. Schouten, P. van de Bovenkamp, H. Verwey; Gisèle Snelder, Marja Markusse and Anke Ekkes for their excellent assistance; and all the other workers of our Department. Workers from NIZO (Mrs. E. C. H. van Beresteijn), CIVO-TNO, Unilever Research and the Department of Statistics of the Agricultural University generously gave helpful advice and discussion.

## References

1. Carroll KK, Hamilton RMG. Effects of dietary protein and carbohydrate on plasma cholesterol levels in relation to atherosclerosis. J Food Sci 1975;40:18–23.
2. Hamilton RMG, Carroll KK. Plasma cholesterol levels in rabbits fed low fat, low cholesterol diets. Effects of dietary proteins, carbohydrates and fibre from different sources. Atherosclerosis 1976;24:47–62.
3. Hermus RJJ. Experimental atherosclerosis in rabbits on diets with milk fat and different proteins. Agric Res Report 838. Wageningen, Netherlands: Centre for Agricultural Publishing and Documentation, 1975.
4. Kritchevsky D, Tepper SA, Williams DE, Story JA. Experimental atherosclerosis in rabbits fed cholesterol-free diets. Part 7. Interaction of animal or vegetable protein with fiber. Atherosclerosis 1977;26: 397–403.
5. Kritchevsky D, Tepper SA, Story JA. Influence of soy protein and casein on atherosclerosis in rabbits (abstr). Fed Proc 1978;37:747.
6. Yadav NR, Liener IE. Reduction of serum cholesterol in rats fed vegetable protein or an equivalent amino acid mixture. Nutr Rept Int 1977;16:385–9.
7. Kim DN, Lee KT, Thomas WA. Effect of soy protein on serum cholesterol in swine fed fat and cholesterol. Exp Mol Pathol 1978;29:385–99.
8. Kritchevsky D, Kolman RR, Guttmacher RM, Forbes M. Influence of dietary carbohydrate and protein on serum and liver cholesterol in germ-free chickens. Arch Biochem Biophys 1959;85:444–51.
9. Hevia P, Visek WJ. Dietary protein and plasma cholesterol in chickens. J Nutr 1979;109:32–8.
10. Stamler J. Population studies. In: Levy RI, Rifkind BM, Dennis BH, Ernst RD, eds. Nutrition, lipids and coronary heart disease. New York: Raven Press, 1979;25–88.
11. Bosman W. De voeding in Nederland in 1976. Voeding 1979;40:351–360.
12. Mulder T. De voeding in Nederland gedurende de jaren 1960–1969. Voeding 1973;34:54–97.
13. Kritchevsky D. Diet and cholesteremia. Lipids 1977;12:49–52.
14. Hardinge MG, Stare FJ. Nutritional studies of vegetarians. 2. Dietary and serum levels of cholesterol. Am J Clin Nutr 1954;2:83–8.
15. Sacks FM, Castelli WP, Donner A, Kass EH. Plasma lipids and liqoproteins in vegetarians and controls. N Engl J Med 1975;292:1148–51.
16. Belton EA, Truswell AS. Effect of casein compared with soya-bean protein isolate on plasma cholesterol in the rabbit. Proc Nutr Soc 1978;37:12A.
17. Sirtori CR, Agradi E, Conti F, Mantero O, Gatti E. Soy bean-protein diet in the treatment of Type-II hyperlipoproteinaemia. Lancet 1977;1:275–7.
18. Carroll KK, Giovannetti PM, Huff MW, Moase O, Roberts DCK, Wolfe BM. Hypocholesterolemic effect of substituting soy-bean protein for animal protein in the diet of healthy young women. Am J Clin Nutr 1978;31:1212–21.
19. Shorey RL, Davis JL. Effects of substituting soy for animal protein in the diets of young mildly hypercholesterolemic males (abstr). Fed Proc 1979;38:551.
20. Stasse-Wolthuis M, Albers HFF, Jeveren JGC van, et al. Influence of dietary fiber from vegetables and fruits, bran or citrus pectin on serum lipids, fecal lipids and colonic function. Am J Clin Nutr 1980;33: 1745–56.
21. Nederlandse Voedingsmiddelen Tabel. Aanbevolen hoeveelheden energie en voedingsstoffen. 1979, 32e druk.
22. Hautvast JGAJ. Ontwikkeling van een systeem om gegevens van voedingsenquetes met behulp van de computer te verwerken. Voeding 1975;36:356–61.
23. Huang TC, Chen CP, Wefler V, Raftery A. A stable reagent for the Liebermann-Burchard reaction. Ap-

plication to rapid serum-cholesterol determination. Anal Chem 1961;33:1405–7.
24. Haar F van der, Gent CM van, Schouten FM, Voort HA vd. Methods for the estimation of high density cholesterol, comparison between two laboratories. Clin Chim Acta 1978;88:469–81.
25. Burstein M, Samaille J. Sur un dosage rapide du cholesterol lié au $\alpha$- et $\beta$- lipoproteins du serum. Clin Chim Acta 1960;5:609.
26. Redgrave TG, Roberts DCK, West CE. Separation of plasma lipoproteins by density gradient ultracentrifugation. Anal Biochem 1975;65:42–9.
27. Brussaard JH, Dallinga-Thie G, Groot PHE, Katan MB. Effects of amount and type of dietary fat on serum lipids, lipoproteins and apolipoproteins in man: a controlled 8-week trial. Atherosclerosis 1980;36:515–27.
28. Deleted in proof.
29. Röschlau P von, Bernt E, Gruber W. Enzymatische Bestimmung des Gesamt-Cholesterins im Serum. Z Klin Chem Klin Biochem 1974;12:403–7.
30. Abell LL, Levy BB, Brody BB, Kendall FE. A simplified method for the estimation of total cholesterol in serum and a demonstration of its specificity. J Biol Chem 1952;195:357–66.
31. Snedecor GW, Cochran WG. Statistical methods. 6th ed. Ames: Iowa State University Press, 1967.
32. Warnick GR, Cheung MC, Albers J. Comparison of current methods for high-density lipoprotein cholesterol quantitation. Clin Chem 1979;25:596–604.
33. Demacker PNM, Vos-Jansen HE, Jansen AP, Laar A van't. Evaluation of the dual-precipitation method by comparison with the ultracentrifugation method for measurement of lipoproteins in serum. Clin Chem 1977;23:1238–44.
34. Raaij JMA van, Katan MB, Hautvast JGAJ. Casein, soya protein, serum-cholesterol. Lancet 1979;11:958.
35. Walker GR, Morse EH, Overley VA. The effect of animal protein and vegetable protein diets having the same fat content on the serum lipid levels of young women. J Nutr 1960;72:317–21.
36. Campbell AM, Swendseid ME, Griffith WH, Tuttle SG. Serum lipids of men fed diets differing in protein quality and linoleic acid content. Am J Clin Nutr 1965;17:83–7.
37. Anderson JT, Grande F, Keys A. Effect on man's serum lipids of two proteins with different amino acid composition. Am J Clin Nutr 1971;24:524–30.
38. Sirtori CR, Gatti E, Mantero O, et al. Clinical experience with the soybean protein diet in the treatment of hypercholesterolemia. Am J Clin Nutr 1979;32:1645–58.
39. Helms P, Gatti E, Sirtori CR. Soybean-protein diet and plasma-cholesterol. Lancet 1977;1:805–6.
40. Sautier C, Doucet C, Flament C, Lemonnier D. Effects of soy protein and saponins on serum, tissue and feces steroids in rat. Atherosclerosis 1979;34: 233–41.
41. Hermus RJJ, Stasse-Wolthuis M, Hautvast JGAJ. Soybean-protein diet and plasma-cholesterol. Lancet 1977;1:905.
42. Oakenfull DG, Fenwick DE, Hood RL, Topping DL, Illman RL, Storer GB. Effects of saponins on bile acids and plasma lipids in the rat. Br J Nutr 1979;42:209–16.
43. Hermus RJJ, Dallinga-Thie GM. Soya, saponins and plasma-cholesterol. Lancet 1979;1:48.
44. Potter JD, Topping DL, Oakenfull D. Soya, saponins and plasma-cholesterol. Lancet 1979;1:223.
45. Huff MW, Hamilton RMG, Carroll KK. Effects of dietary proteins and amino acids on the plasma cholesterol concentrations of rabbits fed cholesterol-free diets. In: Manning GW, Haust MD, eds. Adv Exp Biol Med. Vol. 82, Atherosclerosis: metabolic, morphologic and clinical aspects. New York: Plenum Press 1977:275–277.
46. Sirtori CR, Descovich G, Noseda G. Texturated soy protein and serum-cholesterol. Lancet 1980;1:149.
47. Huff MW, Hamilton RMG, Carroll KK. Plasma cholesterol levels in rabbits fed low fat, cholesterol-free, semipurified diets: effects of dietary proteins, protein hydrolysates and amino acid mixtures. Atherosclerosis 1977;28:187–95.
48. Hermus RJJ, Stasse-Wolthuis M. Lipids and lipoproteins in rabbits fed semisynthetic diets containing different proteins. In: Peeters H, eds. Protides of the biological fluids. Vol. 25. Oxford: Pergamon Press, 1978:457–60.
49. Hermus RJJ. Dietary proteins, amino acids and serum cholesterol. XIth International Congress of Nutrition, 1978. Rio de Janeiro-Brasil, abstr. 124.
50. Kritchevsky D. Vegetable protein and atherosclerosis. J Am Oil Chem Soc 1979;56:135–40.
51. Bazzano G, Olson RE. Effect of glutamic acid on sterol metabolism in man. Am J Clin Nutr 1969;22: 667–8.
52. Amino acid content of foods and biological data on proteins. Rome: Food Policy and Food Science Service, Nutrition Division, Food and Agriculture Organization of the United Nations, 1970.
53. Blackburn H, Chapman J, Dawber TR, et al. Revised data for 1970 ICHD report. Am Heart J 1977;94:539–40.
54. Miller GJ, Miller NE. Plasma-high-density lipoprotein concentration and development of ischaemic heart-disease. Lancet 1975;1:16–9.

# 3. Influence of diets containing casein, soy isolate, and soy concentrate on serum cholesterol and lipoproteins in middle-aged volunteers[1, 2]

*Joop M. A. van Raaij,[3] M.Sc., Martijn B. Katan, Ph.D., Clive E. West, Ph.D., and Joseph G. A. J. Hautvast, M.D., Dip.Nut.(Cantab)*

ABSTRACT Fifty-seven healthy volunteers (mean age 46 yr) were fed for 45 days on diets containing 16% of energy as protein, 35% as fat (polyunsaturated/saturated fat ratio = 0.5) and about 375 mg cholesterol/day. Of the protein in the diets 60% was provided as caseinate, as soy protein isolate, or as soy protein concentrate. After a control period of 17 days during which all the subjects received the casein diet, 17 subjects continued on this diet for the next 28 days (test period), 20 subjects switched to the soy isolate diet, and the remaining 20 subjects switched to the soy concentrate diet. Serum cholesterol levels at the end of the control period were 207 ± 36, 205 ± 40, and 199 ± 35 mg/dl (mean ± SD) for the casein, isolate, and concentrate groups, respectively. Mean changes over the test period were −2 ± 10, −8 ± 12, and +1 ± 10 mg/dl, respectively. Compared with the casein diet, the isolate diet caused a small, nonsignificant decline in both serum total cholesterol and low-density lipoprotein cholesterol (−6.5 mg/dl) and an increase in high-density lipoprotein cholesterol (+5.8 mg/dl) ($p < 0.05$). These effects may have been more obvious if there had been no differences between groups in weight loss. No correlation was found between the response and the initial cholesterol level. No differences in lipoprotein composition were found between the casein and soy concentrate groups. Our data suggest that soy protein preparations do not have dramatic effects on the serum total cholesterol concentration in healthy subjects. However, pure soy protein might have some beneficial effect on the distribution of cholesterol over the lipoproteins. The lack of effect of the less refined soy protein concentrate suggests that the dietary fiber and other nonprotein components of soy concentrate do not have, at least in the short-term, a favorable effect on serum cholesterol and lipoproteins in healthy adults. *Am J Clin Nutr* 1982;35:925–934.

KEY WORDS Casein, soy protein, serum cholesterol, serum lipoproteins, high-density lipoproteins, dietary fiber

## Introduction

Several clinical investigations in hypercholesterolemic patients have shown that replacement of animal proteins in the diet by textured soy protein can lower the concentration of serum low-density lipoprotein (LDL) cholesterol to a marked degree (1–3). This had led to the expectation that the replacement of animal protein by vegetable protein in the human diet might aid in lowering serum total cholesterol levels, and hence, be of benefit in the prevention of atherosclerosis (4–6). However, we were unable to confirm the hypocholesterolemic effect of soy protein compared with casein in a strictly controlled nutritional trial with 69 volunteers aged 18 to 28 yr. We did observe a small but significant increase in the ratio of high-density lipoprotein (HDL) over low-density lipoprotein cholesterol on the soy protein diet (7).

Several factors, such as the lipid status of the subjects and the type of soy protein used, may explain the difference in results between the studies of other workers and ourselves. This is supported by the results of Sirtori et

[1]From the Department of Human Nutrition, Agricultural University, De Dreijen 12, 6703 BC Wageningen, The Netherlands.

[2]Supported in part by grants from the Netherlands Institute for Dairy Research (NIZO) and the Netherlands Heart Foundation (79.045, 26.003).

[3] Author to whom requests for reprints should be addressed.

Received July 9, 1981.
Accepted for publication October 27, 1981.

al. (8, 9) who found that the therapeutic response was related to the initial serum cholesterol level of their patients. In our previously reported study, the volunteers were students with low serum cholesterol levels. Thus it seemed appropriate to examine whether the results of our earlier experiment could be confirmed in subjects with a higher mean serum cholesterol level. As serum cholesterol increases with age in Western countries we decided to repeat out previous experiment with middle-aged volunteers. We also decided to examine if different types of soy preparation vary in effectiveness in lowering serum cholesterol. In addition to the highly purified soy protein preparation that we used previously (soy isolate), a diet containing soy preparations with substantial amounts of nonprotein material (soy concentrate) was now also tested. It has been suggested that the nonprotein material from legumes such as dietary fiber and saponins may lower serum cholesterol and might be responsible for the observed effects in some studies (10–14).

## Subjects and methods

*Subjects*

Thirty-two male and 29 female volunteers were recruited from the town of Wageningen and surrounding areas through advertisements in local newspapers. The subjects ranged in age from 29 to 60 yr (mean 46 ± 9 yr). They were apparently healthy as determined by a medical questionnaire and were found to have normal values for blood Hb and no detectable glucose or protein in their urine. Erythrocyte sedimentation rate was slightly but persistently elevated (20 to 30 mm/h) in five subjects, while another six subjects showed a slightly elevated blood pressure (diastolic between 95 and 105 mm Hg or systolic between 150 and 160 mm Hg). Four men and eight women were overweight (weight/height$^2$ more than 27 and 26 kg/m$^2$, respectively). Serum total cholesterol in casual blood samples ranged from 135 to 305 mg/dl (mean 215 mg/dl) and HDL-cholesterol from 34 to 86 mg/dl (mean 55 mg/dl). None had received any medication known to affect serum lipids for at least 2 months before the study.

Before approval for the study was obtained from the Ethical Committee of the Department of Human Nutrition of the Agricultural University. The experimental protocol was fully explained to the participants and informed consent was obtained. The subjects were not offered payment and were free to end their participation at any moment wanted. They were seen by one of the investigators (JMA van R) and by four research dietitians each day during the week. During the experiment, two subjects decided to withdraw. Participants were asked to note in diaries any illness, drugs use, and departures from the diets. On the basis of this information, it was decided to eliminate the data of another two subjects from the analysis, so that data on 57 subjects were used.

*Diets and control of food intake*

The amount (and type) of fat, cholesterol, protein, and carbohydrates in the test diets were planned to simulate an average Western diet, but with slightly higher intakes of protein and polyunsaturated fatty acids. Of the protein 60% was replaced by protein from caseinate (casein diet), from soy protein isolate (isolate diet), or from soy protein concentrate (concentrate diet). Details of these proteins are given in **Table 1**. To keep the diets palatable, it was necessary to provide 40% of the protein from other sources (mainly from wheat, rice, potatoes, and other vegetables). The caseinates, isolates, and concentrates were incorporated into highly similar specially developed products. Brown bread and gluten-free bread and analogues of milk and yogurt were prepared as described previously (7). Half of the protein in brown breads consisted of test protein; the protein in the gluten-free breads and in the milk-like beverages consisted of test protein alone. The analogues of milk and yogurt were incorporated into soups, sauces, puddings, and sandwich spreads. Cheese was used in the casein diet because it contains casein as practically the only protein. A gelated product was prepared from soy isolate as a counterpart for cheese in the isolate diet (7), and a commercial textured product was used in the concentrate diet. Butter fat was added to both soy diets to balance the fat in cheese. The gelated and textured soy products were added to sauces and sandwich spreads. Fresh egg yolk was used in all diets to adjust cholesterol intakes, and each subject consumed one multivitamin tablet (Davitamon 10, N.V. Organon, 5349 AB Oss) per day.

TABLE 1
Composition of the protein preparations used

| | Caseinate* | Soy isolate† | Soy concentrate‡ |
|---|---|---|---|
| | *g/100 g* | *g/100 g* | *g/100 g* |
| Protein | 92.4§ | 79.7§ | 57.2§ |
| Moisture | 3.0 | 5.5 | 5.9 |
| Fat | 0.3 | 0.5 | 1.6 |
| Ash | 4.2 | 4.0 | 6.4 |
| Carbohydrates | 0.2‖ | 10.3¶ | 28.9¶ |

* Calcium and sodium caseinate (spray dried, bland), DMV Milk Industries, 5460 BA Veghel. Data expressed as mean of the values for calcium and sodium caseinate.

† Soy protein isolate PP500E and PP610, Purina Protein Europe, B-1050 Brussels, Belgium. Data expressed as mean of the values for PP500E and PP610.

‡ Soy protein concentrates Unico (powder) and Dubit (textured, prepared from Unico), Unimills BV, 3330 AA Zwijndrecht. Data expressed as mean of the values for Unico and Dubit.

§ Kjeldahl nitrogen-to-protein factors of 6.38 for casein and 5.70 for soy protein were used. The convential factor of 6.25 would have given values of 90.5 for casein, 87.4 for soy isolate and 62.7 for soy concentrate.

‖ Lactose.

¶ By difference.

Only small amounts of meat (providing about 7% of total protein intake) and no egg white, fish, legumes, or dairy products apart from cheese were used in the preparation of the diets. Each diet met the recommended nutritional allowances for adults (15). The daily menu is given in **Table 2**. As the purity of the protein preparations differed (see Table 1), it was necessary to incorporate different amounts of the protein preparations in the diets in order to keep the proportion of protein constant. The diets were completely identical except for the type of protein and the amount of nonprotein material derived from the preparations used, because the diets consisted of the same regular foodstuffs and similar special products.

Total diets were provided for the participants except for 240 kcal/day, which the subjects were free to choose from a list of foodstuffs not containing protein. All foodstuffs were weighed out for each person in quantities appropriate to his or her energy needs. Hot meals were served on weekdays in the evening at the Department.

TABLE 2
Example of a daily menu for a subject consuming 2240 kcal/day*

| | Item | Amount | |
|---|---|---|---|
| | | Total | Test protein |
| | | g | g |
| Breakfast and lunch | Brown bread† | 245 | 18 |
| | Gluten-free bread† | 80 | 6 |
| | Margarine | 30 | |
| | Sandwich spread† | 75 | 6 |
| | Meats | 30 | |
| | Sugar‡ | 25 | |
| Dinner | Soup† | 200 | 6 |
| | Potatoes (or rice) | 100 | |
| | Other vegetables | 175 | |
| | Soy sauce† | 150 | 13 |
| | (or Casein sauce† | 125 | 3 |
| | plus cheese | 40 | 10) |
| | Egg yolk | 20 | |
| | Salad | 30 | |
| | Pudding† | 175 | 6 |
| Snacks | Fruit§ | | |
| | Juice‡ | | |
| Total (kcal) | | 2000 | 55 |

* Food provided for subjects. In addition to these items subjects were allowed to choose 240 kcal/day of foodstuffs from a list of low-protein products. Subjects were allowed unlimited tea, coffee, selected low-calorie beverages, and up to 6 g of coffee-whitener/day.

† Special product containing test protein.

‡ Sugar was provided as sugar, apple syrup, honey, apple juice, or grapefruit juice, as determined by individual preference.

§ The amount of fruit varied with the type of fruit provided.

All other foods were provided as packages daily; food for the weekend including ingredients for the hot meals was provided each Friday. Nonadherence to the diets was recorded in diaries. The special products were well accepted and adherence to the diets was excellent. Each subject weighed and recorded his or her food intake on 2 random days during the control period and on 4 random days during the test period. The actual nutrient intake was calculated for each individual from these food records using a computerized food composition table supplemented with analyses of special products (16). In addition, double portions of each diet were collected and analyzed as described earlier (17, 18).

*Experimental design*

All subjects consumed the casein diet for a control period of 17 days after which they were divided into three groups; the groups were matched for initial serum total cholesterol, HDL-cholesterol, energy intake, and sex. During the test period of 28 days, the soy isolate group ($n = 20$) received the soy isolate diet, the soy concentrate group ($n = 20$) received the soy concentrate diet, and the casein group ($n = 17$) continued on the casein diet, as shown in **Figure 1**.

Body weight was recorded weekly. For each individual, energy intake was adjusted to avoid changes in body weight of more than 2 kg/2 wk. During the control period, body weights decreased by 0.8, 0.7, and 0.4 kg in the casein, isolate, and concentrate groups, respectively. These weight losses continued during the test period, with mean weight losses of 1.9, 1.4, and 1.2 kg, respectively. The mean weight reduction in the casein group was significantly higher than in the concentrate group ($p < 0.01$). Sixteen subjects lost more than 3 kg of weight over the entire experimental period; two of them, both from the casein group, lost more than 5 kg (5.4 and 5.6 kg, respectively).

*Blood sampling and analysis*

Blood samples were obtained fortnightly from an arm vein after an overnight fast. At the end of the control period, and also at the end of the test period, two samples were taken at 1-day intervals (Fig. 1). Serum was obtained by low-speed centrifugation and all serum samples were assayed for total cholesterol and HDL-cholesterol. At the end of both periods, lipoproteins in sera were separated by density gradient ultracentrifugation, and recovered by aspiration into very low-density lipoprotein ($d < 1.006$ g/ml), LDL + sinking pre-$\beta$ lipoproteins ($1.006 < d < 1.075$ g/ml), HDL-2 ($1.075 < d < 1.125$ g/ml) and HDL-3 ($d > 1.125$ g/ml). The mean recovery of cholesterol was 97.2 ± 5.0%. All methods used were those described for our previous experiment (7). Comparison of HDL-levels determined by ultracentrifugation and by the Mn-heparin procedure yielded a correlation coefficient of $r = 0.91$. Values obtained by the ultracentrifugation method were about 3% higher than those obtained by the precipitation procedure. The sera of 16 of the 57 subjects showed a sinking pre-$\beta$ lipoprotein band with a mean density of 1.064 ± 0.005 g/ml [calculated as described (7)]. In order to partition this lipoprotein band completely into the LDL-fraction, we used a boundary between HDL and LDL of 1.075 g/ml instead of the usual density of 1.063 g/ml.

*Statistical evaluation*

The response to the test diet was calculated per subject as the change from the end of the control to the end of the test period. Differences in diet effects were examined by comparing the mean responses of the groups by unpaired *t* tests. The effects of sex, initial cholesterol levels, and weight changes were examined by analysis of covariance and by correlation analysis (19).

## Results

*Nutrient intake*

The nutrient intake as measured by food records is shown in **Table 3**. Food records and chemical analysis of double portions indicated that there were essentially no differ-

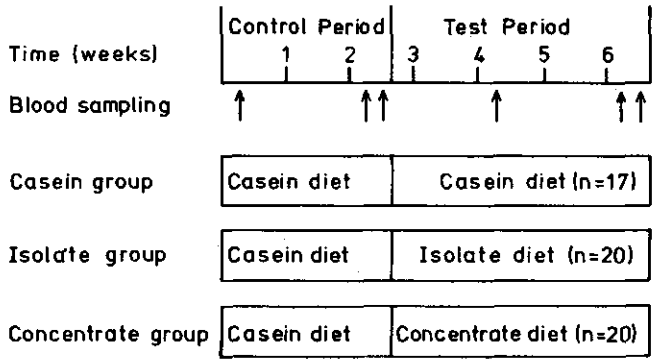



FIG. 1. Experimental design.

TABLE 3
Mean daily intake of nutrients before and during the experiment according to individual food records

| | Habitual intake before experiment* | Control period | Test period | | |
|---|---|---|---|---|---|
| | | Casein diet | Casein diet | Isolate diet | Concentrate diet |
| Energy (kcal/day) | 2265 | 2140 | 2116 | 2166 | 2244 |
| (mJ/day) | 9.5 | 9.0 | 8.9 | 9.1 | 9.4 |
| Protein (% of energy) | 13.8 | 16.2 | 15.9 | 16.2 | 16.1 |
| Casein | † | 9.8 | 9.6 | 0.0 | 0.0 |
| Soy protein | † | 0.0 | 0.0 | 9.8 | 9.8 |
| Other animal proteins | 8.3 | 1.4 | 1.4 | 1.2 | 1.1 |
| Other vegetable proteins | 5.5 | 5.0 | 4.9 | 5.2 | 5.2 |
| Total fat (% of energy) | 36.6 | 34.1 | 34.5 | 34.5 | 34.9 |
| Saturated | 15.9 | 14.8 | 15.3 | 15.0 | 14.9 |
| Monounsaturated | 13.1 | 11.7 | 11.5 | 11.6 | 12.0 |
| Polyunsaturated | 7.6 | 7.6 | 7.7 | 7.9 | 8.0 |
| Carbohydrates (% of energy) | 45.0 | 46.2 | 48.0 | 45.1 | 45.6 |
| Sugars | 21.5 | 21.2 | 22.3 | 20.0 | 20.7 |
| Starch | 23.5 | 25.0 | 25.7 | 25.1 | 24.9 |
| Alcohol (% of energy) | 4.6 | 3.5‡ | 1.6 | 4.2 | 3.4 |
| Cholesterol (mg/day)§ | 297 | 369 | 374 | 381 | 382 |
| Dietary fiber (g/day) | 32 | 36 | 37 | 48‖ | 69¶ |

* 3-day records.
† Not measured.
‡ Percentages of energy during the control period were 2.1, 4.5, and 3.8 for the casein, isolate and concentrate groups, respectively.
§ Mean plant sterol intake was 320 mg/day (β-sitosterol, 50%; campesterol, 22%; stigmasterol, 6%; others, 22%).
‖ Included 6.8 g of unavailable carbohydrates from soy protein preparations.
¶ Included 27.8 g of unavailable carbohydrates from soy protein preparations.

ences between the experimental diets with respect to the amount of protein, fat, carbohydrate, and cholesterol and to the composition of the fatty acids, carbohydrates, and plant sterols. The amount of cholesterol in the experimental diets was about 75 mg/day higher than in the preexperimental diet. The differences between groups in alcohol consumption during the test period were similar to those during the control period. From the food records it could be calculated that about 60% of the total dietary protein consisted of either casein or soy protein. Analysis of the soy isolates and soy concentrates used revealed only negligible differences in amino acid composition. Although the amino acid composition of the whole diet was diluted by those proteins that were common to all diets, the calculated differences between the casein and soy diets were still pronounced and similar to those described previously (7). As expected the test diets differed greatly in dietary fiber content. The mean protein consumption was about 90 g/day, of which about 55 g were derived from the test protein. This corresponded to about 60 g caseinate, 70 g soy isolate, or 100 g soy concentrate. Therefore the isolate and concentrate provided the subjects daily with about 7 and 28 g of dietary fiber (Table 1), including about 0.1 and 0.5 g of polygalacturonic acid (pectin), respectively. The calcium content of the casein diet was slightly higher than that of the soy diets, and the concentrate diet had a lower sodium content and a higher potassium content than the casein and isolate diet. However, it is unlikely that these slight differences in mineral composition influenced the level of serum lipids. Chemical analysis of double portions revealed a slightly higher fat content and a lower oligosaccharide and cholesterol content in all diets than did the calculations based on records plus food tables. This was in agreement with our previous findings (7, 17, 18).

*Serum total cholesterol*

The time course of the mean serum total cholesterol concentration is presented for each group in **Figure 2**. The values of the mean concentrations at the end of the control and test period are given in **Table 4**. During the casein control period of 17 days serum cholesterol concentration decreased in nearly all subjects compared with the uncontrolled preexperimental situation by on average 12 mg/dl (0.30 mmol/l). Changes in serum cholesterol were negatively correlated with the initial levels ($r = -0.40$; $p < 0.001$); thus the largest declines in cholesterol were found in the subjects with the highest initial levels. It is difficult to explain the cholesterol changes by differences between the nutrient composition of the test diets and the preexperimental self-selected diets (Table 3). It may well be that the controlled nutritional conditions and/or the habituation to the new menu- and

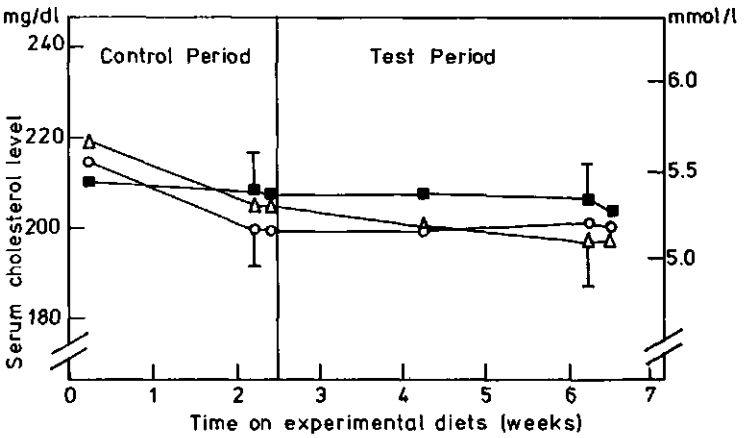



FIG. 2. Effects of casein and soy protein diets on mean serum total cholesterol concentrations in humans throughout the experiment. Vertical bars indicate 1 SEM. ■——■, casein group (17 subjects); △——△, soy isolate group (20 subjects); ○——○, soy concentrate group (20 subjects).

TABLE 4
Effects of casein and soy protein diets on cholesterol concentration in whole serum (mean ± SD); each individual value represents the average of two separate determinations at the end of the respective period

| | Casein group | Isolate group | Concentrate group |
|---|---|---|---|
| | *mg/dl** | *mg/dl* | *mg/dl* |
| | (n = 17) | (n = 20) | (n = 20) |
| All subjects | | | |
| Control period | 207 ± 36 | 205 ± 40 | 199 ± 35 |
| Test period | 205 ± 35 | 197 ± 43 | 200 ± 38 |
| Change | −2.3 ± 10.1 | −7.7 ± 12.0† | +1.2 ± 10.4 |
| Initial cholesterol level | | | |
| Highest 25% | (n = 5) | (n = 5) | (n = 5) |
| Control period | 240 ± 34 | 248 ± 24 | 235 ± 30 |
| Test period | 235 ± 39 | 243 ± 26 | 244 ± 31 |
| Change | −5.3 ± 15.1 | −4.3 ± 18.8 | +9.2 ± 15.7 |
| Lowest 25% | (n = 5) | (n = 5) | (n = 5) |
| Control period | 168 ± 19 | 157 ± 23 | 164 ± 37 |
| Test period | 170 ± 15 | 145 ± 19 | 159 ± 31 |
| Change | +1.6 ± 6.5 | −11.5 ± 8.3‡ | −4.6 ± 7.4 |
| Wt loss during study | | | |
| Less than 3 kg | (n = 10) | (n = 15) | (n = 16) |
| Control period | 214 ± 35 | 205 ± 39 | 204 ± 35 |
| Test period | 214 ± 38 | 199 ± 44 | 205 ± 38 |
| Change | −0.3 ± 7.2 | −6.3 ± 12.5 | +1.5 ± 11.3 |
| More than 3 kg | (n = 7) | (n = 5) | (n = 4) |
| Control period | 197 ± 38 | 202 ± 45 | 180 ± 32 |
| Test period | 192 ± 29 | 189 ± 41 | 179 ± 34 |
| Change | −5.4 ± 13.3 | −12.6 ± 9.0† | −0.8 ± 7.7 |

* 100 mg/dl = 2.59 mmol/l.
† Significantly different from concentrate group ($p < 0.05$).
‡ Significantly different from casein group ($p < 0.05$).

eating-pattern and the special products played a role.

The control group that continued on the casein diet did not show any further change in serum total cholesterol which showed that levels had stabilized at the end of the baseline period of 17 days. The soy concentrate diet also induced hardly any change in average cholesterol concentration, but the soy isolate diet caused a small decline in total cholesterol of about 8 mg/dl (0.20 mmol/l). Analysis of the individual responses during the test period failed to show effects of sex, but revealed a significant diet effect. It appeared that the decline on the soy isolate diet was significantly different from the small increase on the concentrate diet ($p < 0.05$) but not from the small decline on the casein diet. In the casein group changes in serum cholesterol during the test period were negatively correlated with initial levels ($r = -0.37$; $p = 0.07$). However, in the isolate and concentrate group the relationships were positive ($r = +0.25$; $p = 0.14$ and $r = +0.35$; $p = 0.07$, respectively). Thus, in both soy groups the largest declines in serum cholesterol were observed in subjects with the lowest initial levels (see also Table 4).

Although every effort was made to adjust energy intake as required to control body weight, weight losses were observed in nearly all subjects. It has been reported that weight loss can decrease serum total cholesterol (20) and increase HDL-cholesterol (21) but that a considerable amount of weight must be lost (10 to 15%) to produce noticeable effects. Although in our study none of the correlations between changes in either serum total HDL-cholesterol and variation in body weight was significant at the 5% confidence level, Table 4 does suggest some relation between weight loss and decrease of serum cholesterol. Changes in HDL-cholesterol for those subjects who lost less than 3 kg of body weight over the whole experiment are given in a footnote to **Table 5**. Weight loss was

TABLE 5
Effects of casein and soy protein diets on cholesterol concentration in lipoprotein fractions of serum (mean ± SD)

| | Casein group (n = 16) | Soy isolate group (n = 20) | Soy concentrate group (n = 20) |
|---|---|---|---|
| | *mg/dl** | *mg/dl* | *mg/dl* |
| Ultracentrifugation: | | | |
| Very low-density lipoprotein | | | |
| Control period | 14 ± 7 | 15 ± 10 | 15 ± 9 |
| Test period | 14 ± 8 | 14 ± 8 | 16 ± 9 |
| Change | +0.0 ± 5.3 | −0.9 ± 5.4 | +0.3 ± 3.9 |
| LDL | | | |
| Control period | 127 ± 31 | 128 ± 33 | 119 ± 29 |
| Test period | 130 ± 33 | 124 ± 33 | 126 ± 31 |
| Change | +2.8 ± 12.0 | −3.7 ± 15.1† | +7.6 ± 11.5 |
| Total HDL | | | |
| Control period | 62 ± 14 | 58 ± 11 | 58 ± 15 |
| Test period | 58 ± 11 | 59 ± 11 | 56 ± 14 |
| Change | −4.5 ± 9.2 | +1.3 ± 5.9‡ | −1.7 ± 5.5 |
| HDL-2 | | | |
| Control period | 37 ± 13 | 30 ± 9 | 31 ± 12 |
| Test period | 33 ± 10 | 31 ± 9 | 29 ± 12 |
| Change | −4.0 ± 8.4 | +0.3 ± 5.0‡† | −2.8 ± 4.3 |
| Mn-heparin precipitation: | | | |
| HDL§ | | | |
| Control period | 58 ± 11 | 56 ± 10 | 57 ± 13 |
| Test period | 58 ± 10 | 58 ± 10 | 55 ± 13 |
| Change | −0.4 ± 4.6 | +1.9 ± 5.8† | −1.9 ± 3.1 |

* 100 mg/dl = 2.59 mmol/l.
† Significantly different from concentrate group ($p < 0.05$).
‡ Significantly different from casein group ($p < 0.05$).
§ For those subjects who lost less than 3 kg of wt for the whole experiment average changes over the test period amounted to −1.2 mg/dl (n = 10) in the casein group, +2.0 mg/dl (n = 15) in the isolate group, and −1.8 mg/dl (n = 16) in the concentrate group.

most serious in subjects in the casein group. Therefore, it is possible that the effects of soy protein on serum total cholesterol and HDL-cholesterol would have been somewhat larger if there had been no differences between groups in weight loss.

### *Distribution of cholesterol between the lipoprotein fractions*

The mean cholesterol concentrations in the lipoprotein fractions are given in Table 5. In the casein group and soy isolate group there were no appreciable changes in concentrations in any of the fractions. In the soy concentrate group, however, there was an increase in LDL-cholesterol (+7.6 mg/dl or +0.20 mmol/l). Comparison of the changes in LDL-cholesterol concentration on the various diets showed that there had been a decline in the soy isolate group of 11.3 mg/dl ($p < 0.05$) relative to the soy concentrate group, and of 6.5 mg/dl (NS) relative to the casein group. HDL-cholesterol results after ultracentrifugation revealed that the average HDL-cholesterol concentration had increased by 5.8 mg/dl relative to the casein group ($p < 0.5$) and had not changed significantly relative to the concentrate group. Changes in HDL-cholesterol occurred mainly in the HDL-2 fraction. The HDL-cholesterol results after Mn-heparin-precipitation were similar to those found after ultracentrifugation. With the precipitation method there was no noticeable difference in response between the isolate and the casein group, but the difference between the isolate and the concentrate group now reached statistical significance (+3.8 mg/dl or +0.10 mmol/l). No differences in distribution of cholesterol over the serum lipoprotein classes were found between the casein and concentrate group.

TABLE 6
Effects of casein and soy protein diets on serum HDL-cholesterol/total cholesterol ratio (mean ± SD)*

| | Casein group (n = 17) | Isolate group (n = 20) | Concentrate group (n = 20) |
|---|---|---|---|
| Start of control period | 0.27 ± 0.08 | 0.26 ± 0.08 | 0.26 ± 0.06 |
| End of control period | 0.29 ± 0.08 | 0.28 ± 0.07 | 0.29 ± 0.07 |
| End of test period | 0.29 ± 0.07 | 0.30 ± 0.07 | 0.28 ± 0.06 |
| Change over | | | |
| Control period | +0.02 ± 0.02 | +0.02 ± 0.03 | +0.03 ± 0.03 |
| Test period | −0.00 ± 0.02 | +0.02 ± 0.02† | −0.01 ± 0.02 |

* HDL-cholesterol values obtained by the Mn-heparin precipitation method were used for this calculation.
† Significantly different from casein and concentrate group ($p < 0.01$).

Analysis of the individual responses failed to show a sex effect.

The overall changes in the distribution of cholesterol over the lipoproteins can be summarized by the HDL-cholesterol/total-cholesterol ratio. The mean of the individual ratios at the start and at the end of the control period and at the end of the test period are presented for each group in **Table 6**. During the control period there was a significant increase in the ratio in all three groups, mainly because of a decrease in LDL-cholesterol. During the test period the ratio remained unchanged in most subjects from the soy groups and in nearly all subjects from the casein group. However, nine subjects from the isolate group showed an increase of the ratio by more than 10%, and six subjects from the concentrate group showed a similar decline of the ratio. The mean response on the soy isolate diet was significantly different from the response on either the casein or the concentrate diet. No differences were found between the casein and concentrate group.

## Discussion

In our present study with middle-aged volunteers we failed to find a marked effect on serum total cholesterol of diets containing either soy isolate or soy concentrate when compared with a diet containing casein. These results confirm our previous study with younger volunteers (aged 18 to 28 yr) who had lower serum cholesterol concentrations (7). Our results are in marked contrast with those of Sirtori et al. (1, 2), who observed dramatic decreases in serum cholesterol levels in hypercholesterolemic patients on diets containing soy protein. Other studies on hyper- and normocholesterolemic subjects have revealed smaller (22, 23), inconsistent (24), or no effects (25–27) when soy protein diets were fed in comparison with control diets containing animal protein. Consumption of plant proteins other than those from soybean has not been demonstrated to have a hypocholesterolemic effect in humans either (28–31).

In our earlier work (7), we suggested that one explanation for the discrepancy between our results and those of the Italian workers might be that our subjects had comparatively low initial levels of serum cholesterol. However, our present study fails to confirm this explanation. Although clinical hypercholesterolemic subjects were not involved, the average initial cholesterol level was appreciably higher (55 mg/dl) than in our previous study and we still failed to observe dramatic changes in total serum cholesterol concentrations. In fact, those subjects from the soy isolate group with the highest initial levels showed less of a decline than did the subjects with lower initial levels (Table 4). The patients studied by Sirtori et al. (1) had still higher values (mean 342 mg/dl), so we still cannot exclude a specific cholesterol-lowering effect of soy protein in overt type II hypercholesterolemic patients. However, we prefer to think that other properties of the diets used by Sirtori et al. (1) apart from the nature of the protein were responsible for the remarkable efficacy of these diets in combating hypercholesterolemia. A case in point might be the almost complete absence of cholesterol from these diets.

Earlier we suggested a role for the nonprotein components in soy products in lowering cholesterol levels (7). For this reason, our present experiment compared not only a relatively pure soy protein product (soy isolate) with casein, but also a more crude product (soy concentrate) that still contained most of the dietary fiber of the soybean. However,

serum cholesterol levels on the concentrate diet were actually increased when compared with the isolate diet. A cholesterol-lowering action has been described for viscous fiber compounds such as pectin and guar gum but not for those consisting mostly of cellulose and hemicelluloses (32). Earlier work from our laboratory demonstrated that fiber from wheat bran raised cholesterol levels in humans (17). It should be noted that fiber in bran, like that in soy concentrate, consists mostly of hemicelluloses and contains little or no pectin; the main fiber types in soy are arabinogalactans (33, 34), while in bran they are arabinoxylans (32). Decreases of serum cholesterol concentrations after addition of legumes to the diet have been reported (32). However, dietary constituents such as amount and type of fat may also have changed in these experiments (32). Thus the lack of a specific cholesterol-lowering action of the dietary fiber in soy protein preparations is not in conflict with other experimental evidence in humans on this point.

Although we did not find a marked change in total cholesterol concentrations, our data do show a small decline in LDL-cholesterol and a small increase in HDL-cholesterol on the soy isolate diet when compared with the casein control group, but there was no effect with the soy concentrate diet. These effects of the soy isolate diet are identical with our previous findings in young adults with low serum cholesterol levels (7). Changes in HDL were confined mainly to the HDL-2 subfraction. This agrees with the role of HDL-2 as the variable component of HDL (35, 36). Because an increase in the ratio of serum HDL to total cholesterol could be associated with a lower risk of coronary artery disease (37, 38) the significant increase in this ratio on the isolate diet suggests that soy protein could have a small beneficial effect even when the concentration of total cholesterol remains constant. Yet, it must be noted that in our studies these favorable effects were only observed with the rather pure soy protein isolate.

In summary, we believe that there is as yet no evidence that a moderate replacement of animal by vegetable protein in diets of otherwise normal composition will have an effect on the concentration of serum total cholesterol comparable to that observed with changes in the fat composition of the diet. However, a small favorable effect on the distribution of cholesterol over the various lipoprotein classes cannot be excluded. Moreover, one would expect that in practical situations the replacement of animal protein by vegetable protein containing foodstuffs would lead to changes in the concentration of serum lipids because the main sources of animal protein are often also rich in saturated fat and cholesterol.

The authors are most grateful to the volunteers for their invaluable co-operation and keen interest. We wish to express appreciation for the development and preparation of the special products to the following: DMV Milk Industries, 5460 BA Veghel (F. M. W. Visser), Unilever Research, 3130 AC Vlaardingen (P. G. van Stratum), Unimills B. V., 3330 AA Zwijndrecht (A. Visser and W. T. M. de Groot), The Institute for Cereals, Flour and Bread IGMB/TNO, 6701 AN Wageningen (P. Sluimer), Van Den Bergh & Jurgens B. V., 3000 AD Rotterdam (A. P. Ringers), The Dairy Technology Section of the Department for Food Technology of the Agricultural University (H. J. van der Stege and J. H. Jansen), and Purina Protein Europe, Brussels (H. Mesdom). We are indebted to the dieticians Miss J. H. M. de Vries, Miss A. B. M. M. van Kaathoven, Miss J. G. C. van Jeveren, Mrs. G. Sanderink-Hulsink and Miss M. Mager for dietary planning and supervision; to the dieticians Mrs. J. C. M. Barendse-van Leeuwen, Mrs. J. Bos, Mrs. C. Germing-Nouwen, Miss A. E. M. F. Soffers, Mrs. E. A. M. Koot-Gronsveld, F. J. M. Schouten, P. van de Bovenkamp, and H. J. Verweij; to Miss M. Vlassak and P. J. F., de Vries for their excellent assistance; and to all the other workers of our Department. Workers from the Netherlands Institute for Dairy Research (Miss E. C. H. van Beresteijn), Unilever Research, and the Department of Statistics of the Agricultural University gave helpful advice during the planning and performance of the study.

## References

1. Sirtori CR, Agradi E, Conti F, Mantero O, Gatti E. Soybean-protein diet in the treatment of type-II hyperlipoproteinaemia. Lancet 1977;1:275–77.
2. Sirtori CR, Gatti E, Mantero O, et al. Clinical experience with the soybean protein diet in the treatment of hypercholesterolemia. Am J Clin Nutr 1979;32:1645–58.
3. Descovich GC, Gaddi A, Mannino G, et al. Multicentre study of soybean protein diet for outpatient hypercholesterolaemic patients. Lancet 1980;2:709–12.
4. Lewis B. Dietary prevention of ischaemic heart disease—a policy for the '80s. Br Med J 1980;281:177–80.
5. Lewis B, Blankenhorn D, Christian JC, et al. Conference on the health effects of blood lipids: optimal distributions for populations. Prev Med 1979;8:679–714.
6. Secondary prevention in survivors of myocardial

infarction. Joint recommendations by the international society and federations of cardiology scientific councils on arteriosclerosis, epidemiology and prevention, and rehabilitation. Br Med J 1981;282: 894–6.
7. Raaij JMA van, Katan MB, Hautvast JGAJ, Hermus RJJ. Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. Am J Clin Nutr 1981;34:1261–71.
8. Sirtori CR, Descovich G, Noseda G. Textured soy protein and serum cholesterol. Lancet 1980;1:149.
9. Sirtori CR, Descovich G, Noseda G. The soybean protein diet does not lower plasma cholesterol? Atherosclerosis 1981;38:423–5.
10. Helms P. Soybean-protein diet and plasma-cholesterol. Lancet 1977;1:805.
11. Hermus RJJ, Dallinga-Thie GM. Soya, saponins and plasma-cholesterol. Lancet 1979;1:48.
12. Potter JD, Topping DL, Oakenfull D. Soya, saponins and plasma-cholesterol. Lancet 1979;1:223.
13. Oakenfull DG, Fenwick DE, Hood RL, Topping DL, Illman RL, Storer GB. Effects of saponins on bile acids and plasma lipids in the rat. Br J Nutr 1979;42:209–16.
14. Sautier C, Doucet C, Flament C, Lemonnier D. Effects of soy protein and saponins on serum, tissue and feces steroids in rat. Atherosclerosis 1979;34: 233–41.
15. Nederlandse Voedingsmiddelen Tabel. Aanbevolen hoeveelheden energie en voedingsstoffen. 32th ed. 's-Gravenhage: Voorlichtingsburo voor de Voeding, 1979.
16. Hautvast JGAJ. Ontwikkeling van een systeem om gegevens van voedings-enquêtes met behulp van de computer te verwerken. Voeding 1975;36:356–361.
17. Stasse-Wolthuis M, Albers HFF, Jeveren JGC van, et al. Influence of dietary fiber from vegetables and fruits, bran or citrus pectin on serum lipids, fecal lipids and colonic function. Am J Clin Nutr 1980;33:1745–56.
18. Brussaard JH, Dallinga-Thie GM, Groot PHE, Katan MB. Effect of amount and type of dietary fat on serum lipids, lipoproteins and apoprotiens in man: a controlled 8-week trial. Atherosclerosis 1980;36: 515–27.
19. Snedecor GW, Cochran WG. Statistical Methods. 6th ed. Ames: Iowa State University Press, 1967.
20. Anderson JT, Taylor HL, Keys A. Serum cholesterol in subjects on reducing diets high and low in fat. Fed Proc 1956;15;542.
21. Streja DA, Boyko E, Rabkin SW. Changes in plasma high-density lipoprotein cholesterol concentration after weight reduction in grossly obese subjects. Br Med J 1980;281:770–2.
22. Vessby B, Boberg J, Gustafsson IB, Karlström B, Lithell H, Werner I. Substitution with textured soy protein for animal protein in a lipid lowering diet—effects on the serum lipoprotein composition. Eur J Clin Invest 1980;10/211:1823. (abstr).
23. Carroll KK, Giovannetti PM, Huff MW, Moase O, Roberts DCK, Wolfe BM. Hypocholesterolemic effect of substituting soybean protein for animal protein in the diet of healthy young women. Am J Clin Nutr 1978;31:1212–21.
24. Wolfe BM, Giovannetti PM, Cheng DCH, Roberts DCK, Carroll KK. Effects on serum lipids of substituting soy protein for animal protein in the diet of type II hypercholesterolemic patients (Abstr 140). Vär Föda 1979;(suppl 3):223.
25. Holmes WL, Rubel GB, Hood SS. Comparison of the effect of dietary meat versus dietary soybean protein on plasma lipids of hyperlipidemic individuals. Atherosclerosis 1980;36:379–87.
26. Shorey RL, Davis JL. Effects of substituting soy for animal protein in the diets of young mildly hypercholesterolemic males. Fed Proc 1979;38:551(abstr).
27. Bodwell CE, Schuster EM, Steele PS, Judd JT, Smith JC. Effects of dietary soy protein on plasma lipid profiles of adult men. Fed Proc 1980;39:1113 (abstr).
28. Walker GR, Morse EH, Overley VA. The effect of animal protein and vegetable protein diets having the same fat content on the serum lipid levels of young women. J Nutr 1960;72:317–21.
29. Campbell AM, Swendseid ME, Griffith WH, Tuttle SG. Serum lipids of men fed diets differing in protein quality and linoleic acid content. Am J Clin Nutr 1965;17:83–7.
30. Anderson JT, Grande F, Keys A. Effect on man's serum lipids of two proteins with different amino acid composition. Am J Clin Nutr 1971;24:524–30.
31. Milner AN, Jones J, Hume F. Comparative effect of dietary protein (cottonseed vs. beef) on serum lipids of young college women. Fed Proc 1980;39:562 (abstr).
32. Stasse-Wolthuis M. Influence of dietary fibre on cholesterol metabolism and colonic function in healthy subjects. Wld Rev Nutr Diet 1980;36:100–40.
33. Aspinall GO, Begbie R, McKay JE. Polysaccharide components of soybeans. Cereal Sci Today 1967;12: 223–8.
34. Eldridge AC, Black LT, Wolf WJ. Carbohydrate composition of soybean flours, protein concentrates, and isolates. J Agric Food Chem 1979;27:799–802.
35. Knuiman JT, West CE. The concentration of cholesterol in serum and in various serum lipoproteins in macrobiotic, vegetarian and non-vegetarian men and boys. Atherosclerosis (in press).
36. Anderson DW. HDL-cholesterol: the variable components. Lancet 1978;1:819–20.
37. Blackburn H, Chapman J, Dawber TR, et al. Revised data for 1970 ICHD report. Am Heart J 1977;94:539–40.
38. Miller GJ, Miller NE. Plasma-high-density lipoprotein concentration and development of ischaemic heart-disease. Lancet 1975;1:16–19.

# 4. INFLUENCE OF HUMAN DIETS CONTAINING CASEIN AND SOY PROTEIN ON SERUM CHOLESTEROL AND LIPOPROTEINS IN HUMANS, RABBITS AND RATS*

JOOP M.A. VAN RAAIJ, MARTIJN B. KATAN and CLIVE E. WEST

Department of Human Nutrition, Agricultural University, De Dreijen 12, 6703 BC Wageningen, The Netherlands

*Current Topics in Nutrition and Disease: Animal and Vegetable Proteins in Lipid Metabolism. Kritchevsky, D., Gibney, M.J. (eds), in press*

SUMMARY

This paper reports the results of studies in which the effect of casein and soy protein on serum cholesterol and lipoprotein concentrations were compared in humans, rabbits and rats using human diets. In the human studies, no marked effect of diets containing either soy isolate or soy concentrate on serum total cholesterol was observed when compared with a diet containing casein. When rabbits and rats were fed the human diets, lower serum cholesterol levels were found on both the soy diets when compared with the casein diet. The differences being much more pronounced in rabbits than in rats. These results confirm differences in susceptibility between species, and that the normocholesterolemic human appears to be relative insensitive to changes in these dietary proteins. Although in humans no effects on serum total cholesterol level were observed, the soy isolate diet did cause a small decline in cholesterol concentration in the low-density lipoprotein fraction (LDL) and a small increase in cholesterol concentration in the high-density lipoprotein fraction (HDL) when compared with the casein diet. However, there was no effect with the soy concentrate diet. Analysis of apolipoprotein concentrations suggested that the changes in cholesterol concentrations in the lipoprotein fractions resulted mainly from changes in the number of lipoprotein particles, but minor effects on the composition of the LDL could not be excluded. The lack of a cholesterol-lowering effect of the less refined soy concentrate when compared with soy protein isolate in both the human and animal studies suggest that the non-protein part of the soy preparations probably does not have a specific cholesterol-lowering effect. Our studies stress the risk of extrapolating animal data concerning the effect of protein on cholesterol metabolism to man.

* Supported in part by grants from the Netherlands Institute for Dairy Research (NIZO) and the Netherlands Heart Foundation (79.045, 26.003)

## INTRODUCTION

In animal studies, dietary proteins derived from animal sources are generally found to be hypercholesterolemic when compared with proteins from plant sources (Carroll, 1978; Kritchevsky, 1979; Terpstra *et al.*, 1982b). Epidemiological studies (Stamler, 1979) and nutritional studies in vegetarians (Hardinge and Stare, 1954; Sacks *et al.*, 1975; Burslem *et al.* 1978) have also suggested a relation between intake of animal protein and serum cholesterol. Such epidemiological observations, however, should be interpreted with caution as differences in other nutritional factors may be present. In humans, only a few controlled trials relating dietary protein to serum cholesterol have been carried out, and the results are conflicting (Anderson *et al.* , 1971; Carroll *et al.*, 1978; Sirtori *et al.*, 1979; Shorey and Davis, 1979; Descovich *et al.*, 1980; Vessby *et al.*, 1980; Holmes *et al.*, 1980; Bodwell *et al.*, 1980; Wolfe *et al.*, 1981).

We have recently investigated, in strictly controlled dietary studies, the effects of diets containing casein and soy protein on the concentration of serum cholesterol and lipoproteins in large groups of young students and middle-aged subjects. During our studies with humans, duplicate portions of the test diets were collected and were later fed to rabbits and rats. In this paper the results of the human and animal studies are discussed. Some of the results have been published previously (Van Raaij *et al.*, 1981, 1982).

## MATERIALS AND METHODS

### *Human subjects, animals and diets*

In the experiments to be described, three principle sources of protein have been used: casein; soy protein isolate, which is the purest form of soy protein commercially available; and soy protein concentrate, which contains more dietary fiber (Table 1). Six studies have been carried out and they can be divided into two groups on the basis of the diets fed (Table 2 and Figure 1).

The first group of studies (Van Raaij *et al.*, 1981; referred to in this paper as group I) consisted of a study with humans (I-Human) and a study with rabbits (I-Rabbit). In I-Human, 69 young healthy students aged 18 to 28 years were fed for 38 days on diets containing 13% of energy as protein of which 65% was replaced by protein from casein, from soy protein isolate, or from a 2:1 mixture of casein and soy protein isolate. After a control period of 10 days during which all the subjects received the casein-soy diet, 20 subjects

continued on this diet for a test period of 28 days, 25 subjects switched to the casein diet and the remaining 24 subjects switched to the soy diet. Throughout the experiment duplicate portions of the diets were collected, and these were later homogenized in order to carry out a study with rabbits (I-Rabbit). Twelve male New Zealand White rabbits were maintained on a commercial diet (Cunicon I, Trouw and Co., 3881 LP Putten) until 3 months of age (control period-1). Half were transferred to the homogenized casein diet and half were transferred to the soy isolate diet for a test period of 2½ weeks before both groups were transferred back to the commercial diet for 2½ weeks (control period-2). The homogenate was supplied freeze-dried and pelleted during the first 12 days of the test period and as a wet mash during the remaining 6 days.

Table 1
COMPOSITION OF THE PROTEIN PREPARATIONS USED

| | Caseinate[a] | Soy isolate | | Soy concentrate[d] |
|---|---|---|---|---|
| | | UNISOL NH 70[b] | PP500E, PP610[c] | |
| | g/100g | | | |
| Protein[e] | 92.4 | 84.6 | 79.7 | 57.2 |
| Moisture | 3.0 | 3.1 | 5.5 | 5.9 |
| Fat | 0.3 | 0.1 | 0.5 | 1.6 |
| Ash | 4.2 | 3.5 | 4.0 | 6.4 |
| Carbohydrates | 0.2[f] | 8.7[g] | 10.3[g] | 28.9[g] |

a) Calcium and sodium caseinate (spray dried, bland), DMV Milk Industries, 5460 BA Veghel. Data expressed as mean of the values for calcium and sodium caseinate.

b) UNISOL NH 70, UNIMILLS BV, 3330 AA Zwijndrecht (used in I-Human and I-Rabbit).

c) Soy protein isolate PP500E and PP610, Purina Protein Europe, B-1050 Brussels, Belgium (used in II-Human, II-Rabbit, II-Rat and II-Rabbit-SP). Data expressed as mean of the values for PP500E and PP610.

d) Soy protein concentrates Unico (powder) and Dubit (textured, prepared from Unico), UNIMILLS BV. Data expressed as mean of the values for Unico and Dubit.

e) Kjeldahl nitrogen-to-protein factors of 6.38 for casein and 5.70 for soy protein have been used.

f) Lactose; data provided by manufacturer.

g) By difference.

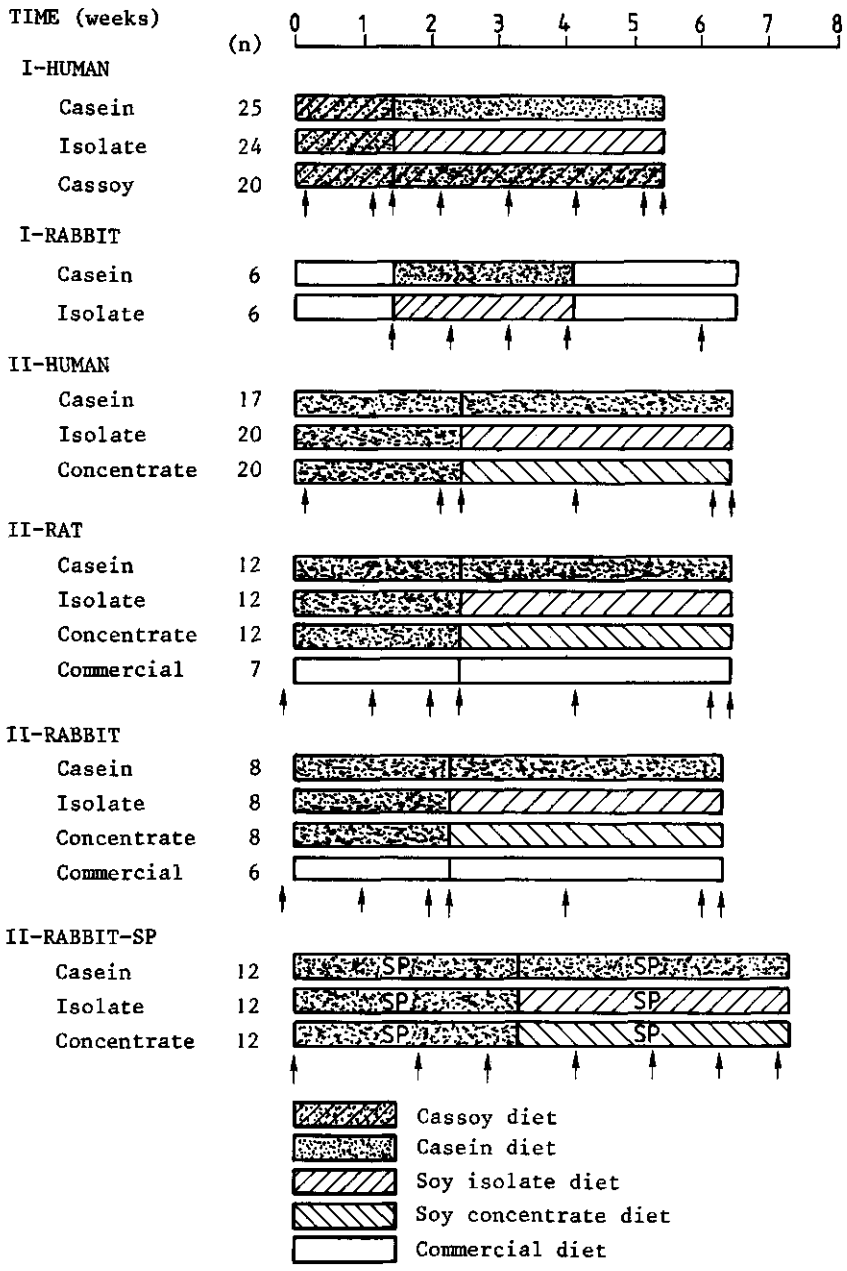



FIG. 1. Experimental designs. Blood sampling is indicated by arrows.

Table 2

COMPOSITION OF THE DIETS USED (G PER 100 G DRY MATTER)[a]

| Experiment | Period and diet | Protein[b] | Fat | | | | Carbohydrates | | Dietary fiber | Ash | Chole-sterol | Fyto-sterols |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | total | satu-rated | mono-unsat. | poly-unsat. | total | sugars | | | | |
| I-Human, I-Rabbit | Control period | | | | | | | | | | | |
| | Cassoy | 18.4[c] | 21.2 | 8.4 | 9.2 | 3.6 | 41.3 | 14.4 | 9.2 | 4.0 | 0.08 | 0.11 |
| | Commercial | 21.3 | 4.8 | e | e | e | 55.4 | e | 10.6[f] | 7.9 | 0 | e |
| | Test Period | | | | | | | | | | | |
| | Cassoy | 17.6[c] | 21.3 | 8.1 | 9.4 | 3.8 | 42.1 | 15.9 | 8.6 | 3.7 | 0.08 | 0.12 |
| | Casein | 18.4[c] | 22.1 | 8.1 | 9.9 | 4.1 | 42.9 | 17.4 | 9.1 | 3.7 | 0.07 | 0.12 |
| | Soy isolate | 17.4[c] | 22.2 | 8.0 | 10.1 | 4.1 | 41.8 | 16.5 | 8.6 | 3.6 | 0.07 | 0.12 |
| II-Human, II-Rat, II-Rabbit | Control period | | | | | | | | | | | |
| | Casein | 20.2[c] | 20.6 | 8.4 | 7.7 | 4.5 | 46.2 | 16.0 | 7.8 | 4.5 | 0.07 | 0.07 |
| | Test Period | | | | | | | | | | | |
| | Casein | 20.1[c] | 20.7 | 8.3 | 7.6 | 4.8 | 45.7 | 15.7 | 8.2 | 4.6 | 0.07 | 0.07 |
| | Soy isolate | 19.7[c] | 19.6 | 7.6 | 7.2 | 4.8 | 45.2 | 15.4 | 10.5 | 4.3 | 0.07 | 0.07 |
| | Soy concentrate | 19.7[c] | 18.4 | 7.2 | 6.6 | 4.6 | 42.9 | 14.0 | 12.8 | 4.6 | 0.06 | 0.07 |
| | Commercial (Rat) | 25.2 | 7.3 | e | e | e | 56.7 | e | 4.7[f] | 6.1 | 0 | e |
| | Commercial(Rabbit) | 21.3 | 4.8 | e | e | e | 55.4 | e | 10.6[f] | 7.9 | 0 | e |
| II-Rabbit-SP | Control period | | | | | | | | | | | |
| | Casein | 21.6[d] | 5.3 | 4.0 | 0.5 | 0.7 | 53.4 | 27.2 | 10.5 | e | 0 | e |
| | Test period | | | | | | | | | | | |
| | Casein | 21.6[d] | 5.3 | 4.0 | 0.5 | 0.7 | 53.4 | 27.2 | 10.5 | e | 0 | e |
| | Soy isolate | 19.1[d] | 5.3 | 4.0 | 0.5 | 0.8 | 53.1 | 27.0 | 12.8 | e | 0 | e |
| | Soy concentrate | 16.8[d] | 5.1 | 3.6 | 0.6 | 0.9 | 47.7 | 24.2 | 17.7 | e | 0 | e |

a) The test diets used in all experiments, except in the control period of I-Rabbit and throughout II-Rabbit-SP, were conventional human diets. These diets were freeze-dried before feeding to the animals. The composition of these diets was determined by chemical analysis (Van Raaij *et al.*, 1981, 1982). In II-Rabbit-SP the protein preparations were incorporated into semipurified pelleted diets. The composition of these diets were calculated on basis of the ingredients used: (in g/1000, 1006 and 1084 g for the casein, isolate and concentrate diet, respectively): casein, 210, or soy protein isolate, 216, or soy protein concentrate, 295; maize starch, 280; dextrose, 210; saw dust, 120; coconut oil, 40; soybean oil, 10; molasses, 50; vitamin premix, 12; mineral premix, 10; $CaHPO_4.2H_2O$, 29; NaCl, 6; $MgCO_3$, 3; MgO, 2; and $KHCO_3$, 18.
As soy protein is deficient in methionine, and as the soy preparations contained more NaCl than casein, 2 g of NaCl was replaced by 2.1 and 1.4 g DL-methionine in the isolate and concentrate diets, respectively.
The composition of the vitamin and mineral premixes has been described earlier (Katan *et al.*, 1982).
In addition to the human diets, pelleted commercial diets were used in I-Rabbit, II-Rabbit and II-Rat.
Data on the composition of these diets were provided by manufacturers.

b) The proportion of protein as test protein was 65% in I-Human and I-Rabbit, 60% in II-Human, II-Rat and II-Rabbit, and 100% in II-Rabbit-SP.

c) Kjeldahl nitrogen-to-protein factor of 6.25 was used.

d) Kjeldahl nitrogen-to-protein factor of 5.70 and 6.38 was used for casein and soy protein, respectively.

e) No data available.

f) Crude fiber.

The second group of studies (group II) included a study with humans (II-Human), one study with rats (II-Rat) and two studies with rabbits (II-Rabbit and II-Rabbit-SP). In II-Human (Van Raaij *et al.*, 1982), 57 healthy subjects aged 29 to 60 years were fed for 45 days on diets containing 16% of energy as protein of which 60% was replaced by protein from casein, from soy protein isolate or from soy protein concentrate. After a control period of 17 days during which all the subjects received the casein diet, 17 subjects continued on this diet for a test period of 28 days, 20 subjects switched to the soy isolate diet, and the remaining 20 subjects switched to the concentrate diet. As during the first study with humans, duplicate portions of the diets were collected, homogenized and freeze-dried in order to carry out a study with male New Zealand White rabbits (II-Rabbit) and also with female lean Zucker strain rats (II-Rat). The experimental design of these two studies was essentially the same as II-Human. Before the control periods, the rats and rabbits were maintained on commercial rat and rabbit pellets respectively (Trouw and Co.). In addition a further experiment with rabbits (II-Rabbit-SP) was carried out according to a similar design, but this time we used semipurified diets in which 100% of the protein was supplied by the same preparations of casein, soy isolate and soy concentrate as used in II-Human, II-Rat and II-Rabbit. At the beginning of the control periods, the rabbits and rats in the second group of experiments were 10-13 and 4 weeks of age, respectively.

For the two human experiments, food records and chemical analysis of the diets indicated that within each study, there were essentially no differences between the experimental diets except for the type of protein and/or the amount of non-protein material derived from the protein preparations used. Apart from 120 kcal per day in I-Human and 240 kcal per day in II-Human, all of the food eaten was daily supplied to the subjects in amounts appropriate to each individual's energy requirements. The composition of the diets is given in Table 2, but more details about the diets have been described previously (Van Raaij *et al.*, 1981, 1982).

In the animal studies water was provided *ad libitum*. In I-Rabbit and II-Rat, the casein and soy diets were provided *ad libitum*, while in II-Rabbit and II-Rabbit-SP, the diets were fed on a restricted basis to supply the rabbits in each experiment with equal amounts of protein.

The animals were maintained as described by Katan *et al.* (1982) except that the rats as well as the rabbits were housed individually. The individual body weights were recorded weekly.

*Sampling of blood and liver and biochemical analysis*

Blood samples were collected throughout the experiments as shown in Figure 1. Blood was taken after an overnight fast as described earlier for humans (Van Raaij *et al.*, 1981), rabbits (Terpstra and Sanchez-Muniz, 1981) and rats (Terpstra *et al.*, 1982a). Serum was obtained by low speed centrifugation, and all the serum samples were assayed for total cholesterol.

At the end of both periods, in both human studies and in II-Rat and II-Rabbit-SP, the lipoproteins of the serum were isolated by density gradient ultracentrifugation using a modification (Terpstra *et al.*, 1981b) of the method described by Redgrave *et al.* (1976). An SW50 rotor (Beckman Inc., Palo Alto Ca. 94304, USA) was used for the separation of lipoprotein classes from 1 ml serum samples, as in II-Rat, and an SW41 rotor was used for the separation from 2 ml samples, as in all other studies. The lipoproteins in the gradient were visualized by prestaining the serum with Sudan Black prior to ultracentrifugation. Lipoprotein fractions were removed either by tube-slicing (I-Human) or by aspiration (all other studies).

In the human studies the following lipoprotein fractions were collected from individual sera; in I-Human: VLDL ($d < 1.015$ g/ml), LDL ($1.015 < d < 1.060$), and HDL ($d > 1.060$ g/ml), and in II-Human: VLDL ($d < 1.006$ g/ml, LDL + sinking pre-bèta lipoproteins ($1.006 < d < 1.075$) and HDL ($d > 1.075$). In I-Rat, the serum samples were pooled per group at the end of the control period and again at the end of the test period. Nine lipoprotein fractions were collected, eight of which floated above the following densities (d, g/ml) respectively, 1.016, 1.032, 1.045, 1.060, 1.078, 1.102, 1.128 and 1.146, while the ninth fraction had a $d > 1.146$ g/ml. In II-Rabbit-SP analyses were carried out on pooled samples with similar cholesterol concentrations. At the end of the control period, 6 pools each of 6 sera were formed and at the end of the test period there were 4 pools within each group with each pool consisting of 3 sera. Three lipoprotein fractions were collected: VLDL ($d < 1.006$), LDL ($1.006 < d < 1.063$) and HDL ($d > 1.063$ g/ml).

At the end of experiment II-Rabbit-SP, that is after 5 weeks on the test diets, the concentration of cholesterol and the proportion present as the ester was measured in the livers of the rabbits. The animals were first stunned by a blow to the head and then killed by severing the major blood vessels in the neck and draining the blood from the body. The livers were removed immediately, dried off and weighed. The liver was then homogenized with a Potter-Elvehjem homogenizer and lipids were extracted from a 10 g aliquot

(Folch *et al.*, 1957). We measured total cholesterol in one part of the extract with Liebermann-Burchard reagent (Huang *et al.*, 1961) after alkaline hydrolysis. Another part of the extract was used for separating and quantitating free and esterified cholesterol by thin-layer chromatography (West and Rowbotham, 1967).

In both human studies serum cholesterol was measured with the reagent of Huang *et al.* (1961) with strict standardization (Katan *et al.*, 1982); animal sera and lipoprotein fractions were assayed for cholesterol by an enzymatic method (Röschlau *et al.*, 1974) using a kit (Catalase kit, no. 124087, Boehringer-Mannheim GmbH, West Germany). Apolipoprotein-B (I-Human and II-Human) and apoprotein-$A_1$ (II-Human) were measured in whole serum by rocket immunoelectrophoresis (Laurell, 1972) largely as described previously (Brussaard *et al.*, 1980). Rabbit antibody against apolipoprotein $A_1$ was kindly donated by Dr. P. Demacker.

The response to the test diet was calculated per subject or animal as the change from the end of the control to the end of the test period. Differences in diet effects were examined by comparing the mean responses of the groups by unpaired two-tailed t-tests (Snedecor and Cochran, 1967).

## RESULTS

### *Human experiments*

The concentrations throughout experiments I-Human and II-Human of cholesterol in whole serum, HDL and LDL and of apoproteins $A_1$ (II-Human only) and B are given in Tables 3 and 4, and in Figure 2. In both studies, there was no clear difference in response with respect to serum total cholesterol between the casein and soy isolate groups. In both studies a decline in LDL cholesterol and an increase in HDL cholesterol was observed on the soy isolate diet when compared with the casein diet, but only in I-Human the difference with respect to LDL cholesterol reached statistical significance (Figure 2, and Van Raaij *et al.*, 1981, 1982).

In I-Human an increase in apolipoprotein-B and a decline in the LDL cholesterol/apo-B ratio was observed on the isolate diet when compared with the casein diet, suggesting that the decline in LDL cholesterol on the isolate diet had not been caused by a decline in the number of LDL particles, but in the composition of the LDL. These findings are confirmed by the changes in the density of LDL (Van Raaij *et al.*, 1981).

Table 3

THE EFFECT OF CASEIN AND SOY PROTEIN DIETS ON SERUM CHOLESTEROL CONCENTRATIONS IN HUMANS, RABBITS AND RATS

| Experiment | Group[a] | n | Serum cholesterol concentration (mg/dl;mean±SD[b]) | | | |
|---|---|---|---|---|---|---|
| | | | Initial | End Control Period | End Test Period | Change over Test Period |
| I- Human | Casein | 25 | 162 ± 32 | 152 ± 27 | 149 ± 24 | - 3 ± 14 |
| | Isolate | 24 | 159 ± 26 | 153 ± 23 | 150 ± 23 | - 3 ± 10 |
| | Cassoy | 20 | 160 ± 27 | 153 ± 24 | 150 ± 25 | - 3 ± 13 |
| I- Rabbit | Casein | 6 | c | 30 ± 7 | 120 ± 65 | + 89 ± 74 |
| | Isolate | 6 | c | 32 ± 12 | 55 ± 18 | + 24 ± 20[e] |
| II-Human | Casein | 17 | 211 ± 38 | 207 ± 36 | 205 ± 35 | - 2 ± 10 |
| | Isolate | 20 | 219 ± 42 | 205 ± 40 | 197 ± 43 | - 8 ± 12[g] |
| | Concentrate | 20 | 215 ± 41 | 199 ± 35 | 200 ± 38 | + 1 ± 10 |
| II-Rat | Casein | 11 | 96 ± 7 | 62 ± 7 | 53 ± 5 | - 9 ± 5 |
| | Isolate | 12 | 95 ± 9 | 63 ± 9 | 51 ± 5 | - 13 ± 6[d] |
| | Concentrate | 12 | 95 ± 10 | 63 ± 7 | 50 ± 6 | - 13 ± 5[e] |
| | Commercial | 7 | 95 ± 10 | 74 ± 12[h] | 60 ± 7[h] | - 14 ± 9 |
| II-Rabbit | Casein | 8 | 45 ± 12 | 160 ± 54 | | |
| | Isolate | 7 | | | | |
| | Concentrate | 8 | | | | |
| | Commercial | 6 | 45 ± 13 | 60 ± 17[h] | 62 ± 26 | + 2 ± 22 |
| II-Rabbit-SP | Casein | 12 | 63 ± 26 | 121 ± 44 | 278 ±119 | +157 ±118 |
| | Isolate | 12 | 54 ± 17 | 117 ± 48 | 92 ± 41[f] | - 26 ± 46[f] |
| | Concentrate | 12 | 65 ± 38 | 120 ± 51 | 95 ± 37[f] | - 26 ± 57[f] |

a) Diets indicated under Group were given in the test period; for diets given in the control period, see Figure 1.

b) 100 mg/dl = 2.59 mmol/l.

c) Not determined.

Statistical comparison with the casein group by Student's t-test:
d) $P < 0.075$; e) $P < 0.05$; f) $P < 0.001$; with the concentrate group:
g) $P < 0.05$; and with all test groups: h) $P < 0.05$.

Table 4

THE EFFECT OF CASEIN AND SOY PROTEIN DIETS ON THE CONCENTRATION OF APOLIPOPROTEINS IN SERUM IN THE STUDIES WITH HUMANS

| | Casein Group | | Soy isolate Group | | Soy concentrate Group | |
|---|---|---|---|---|---|---|
| | End Control Period | Change over Test Period | End Control Period | Change over Test Period | End Control Period | Change over Test Period |
| I-Human | | | | | | |
| Apolipoprotein-B (mg/l) | 488 ± 199 | -47 ± 52 | 462 ± 107 | -15 ± 51[c] | | |
| LDL cholesterol/apo-B[a] (g/g) | 1.63 ± 0.19 | +0.18 ± 0.25 | 1.92 ± 0.17 | -0.08 ± 0.18[c] | | |
| II-Human | | | | | | |
| Apolipoprotein-B (mg/l) | 777 ± 202 | +67 ± 77 | 812 ± 201 | +12 ± 65[c,d] | 783 ± 212 | +52 ± 61 |
| LDL cholesterol/apo-B (g/g) | 1.66 ± 0.25 | -0.10 ± 0.15 | 1.58 ± 0.19 | -0.07 ± 0.21 | 1.54 ± 0.21 | -0.01 ± 0.12 |
| Apolipoprotein-$A_I$ (mg/l) | 1569 ± 242 | -32 ± 100 | 1552 ± 183 | +43 ± 131[c,d] | 1505 ± 276 | -29 ± 99 |
| HDL cholesterol/apo-$A_I$[b] (g/g) | 0.35 ± 0.09 | 0.00 ± 0.02 | 0.36 ± 0.07 | 0.00 ± 0.03 | 0.39 ± 0.06 | -0.01 ± 0.03 |
| Apo-$A_I$/Apo-B (g/g) | 2.15 ± 0.64 | -0.20 ± 0.17 | 2.01 ± 0.46 | +0.04 ± 0.25[c,d] | 2.07 ± 0.73 | -0.19 ± 0.24 |

Results expressed as mean ± SD

a) LDL isolated after ultracentrifugation

b) HDL isolated after Mn-heparin precipitation (Burstein and Samaille, 1960; Van der Haar *et al.*, 1978).

Statistical comparison with the casein group by Student's two-tailed t test:

c) $P < 0.05$; and with the concentrate group: d) $P < 0.05$.

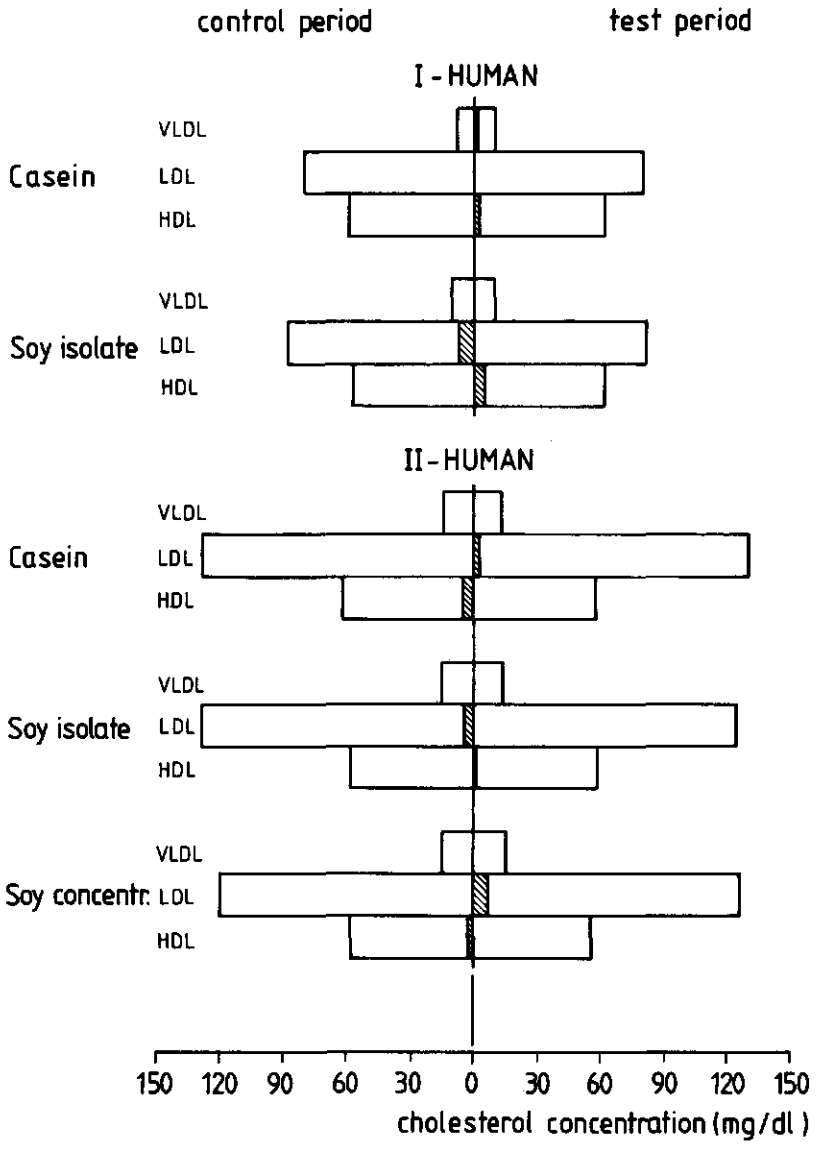



FIG. 2. The effect of casein and soy protein diets on the concentration of cholesterol in lipoproteins in humans (experiments I-Human and II-Human). The shaded areas right of the vertical line represent an increase over the test period, and those left of the vertical line a decrease; changes less than 1 mg/dl are not shown.

In II-Human a decline in apolipoprotein-B, an increase in apolipoprotein-$A_1$, and no clear differences in LDL cholesterol/apo-B and HDL cholesterol/apo-$A_1$ ratios were observed on the soy isolate diet when compared with casein. These results suggest that the decline in LDL cholesterol and the increase in HDL cholesterol were caused by a decline in the number of LDL particles and an increase in the number of HDL particles, rather than by changes in the lipoprotein composition. The correlation coefficients between LDL cholesterol and serum apo-B at the end of the control and test period were $r = 0.86$ ($n=56$) and $r = 0.90$ ($n=56$), respectively. The correlation coefficients between HDL cholesterol and serum apo-$A_1$ were $r = 0.60$ ($n=55$) and $r = 0.67$ ($n=55$), respectively.

Compared with soy protein concentrate, soy protein isolate produced a decrease in serum total cholesterol, LDL cholesterol, apolipoprotein-B, and LDL cholesterol/apo-B ratio, an increase in HDL cholesterol and apolipoprotein-$A_1$, but had no effect on the HDL cholesterol/apo-$A_1$ ratio. These results suggest that the increase in HDL cholesterol has been caused by an increase in the number of HDL particles, and that the decline in LDL cholesterol has been caused by a decline in both the number of LDL particles and in their cholesterol content. No differences in lipoproteins were found between the casein and the soy protein concentrate group.

The overall changes in lipoproteins can be summarized by the HDL cholesterol/total cholesterol ratio and by the apo-$A_1$/apo-B ratio. In experiment II-Human, significant increases in both ratios were observed on the soy isolate diet when compared with the casein or concentrate diet (Table 4 and Van Raaij *et al.*, 1982).

*Feed consumption and growth of the experimental animals*

The feed consumption and growth of the rabbits and rats throughout the experiments are presented in Table 5. In all of the experiments, except in II-Rabbit, feed consumption and growth were satisfactory. In II-Rabbit, the animals consumed all their feed under the restricted feeding regime throughout the control period and for the first two weeks of the test period. However, after that, twelve rabbits failed to eat all the feed provided, their growth faltered and their health deteriorated. Four of the rabbits lost hair. It may well be that the human diets, either before or after freeze-drying, contained insufficient vitamins and minerals for the growth of rabbits. When the experiment was completed, the rabbits were transferred to the commercial rabbit diet, and the growth and health of the less severely affected animals improved

Table 5
FEED INTAKE AND WEIGHT GAIN IN RABBITS AND RATS

| Experiment | Groups | Body weight[a] | Feed intake[b] | | Weight gain | |
|---|---|---|---|---|---|---|
| | | | Control Period | Test Period | Control Period | Test Period |
| | | (g) | (g/day) | (g/day) | (g/day) | (g/day) |
| I- Rabbit[c] | Test (all)[e] | 2322 | f | 75[g] | f | 12.5 |
| II-Rabbit[d] | Test (all)[e] | 1481 | 57 | 57[h,i] | 18.2 | 28.7[i] |
| | Commercial | 1503 | 85 | 85[i] | 22.4 | 25.8[i] |
| II-Rabbit-SP[d] | Test (all)[e] | 1834 | 67 | 67[j] | f | 17.1 |
| II-Rat[c] | Test (all)[e] | 70 | 9.3 | 10.6[k] | 2.6 | 1.8 |
| | Commercial | 68 | 11.8 | 14.0 | 2.1 | 1.7 |

a) At start control period (II-Rabbit and II-Rat) or at start test period (I-Rabbit and II-Rabbit-SP).

b) Dry matter contents of the diets were: 94.5% for the pelleted human diets (I-Rabbit and II-Rabbit); 99.5% for the unpelleted human diets (II-Rat); 91.5% for the semipurified diets (II-Rabbit-SP); and 89.0% for the commercial rabbit and rat diets.

c) Diets were provided *ad libitum*.

d) Diets were provided restricted.

e) Mean data of the test groups are given except for the food intake during the test period in which data for the casein group are given; there were no differences between group means for body weight and weight gain.

f) Not measured

g) Food intake was 83 g per day for the isolate group.

h) Food intake was 58 and 61 g per day for the isolate and concentrate group.

i) During first two weeks of test period.

j) Food intake was 67.5 and 69.5 g per day for the isolate and concentrate group.

k) Food intake was 11.0 and 11.3 g per day for the isolate and concentrate group.

markedly. Because of the problems encountered, data obtaining during the test period of experiment II-Rabbit were deleted in the subsequent analysis of the results. In all other studies, the experimental diets were well accepted, and the feed offered was consumed.

*Serum cholesterol and lipoproteins and liver cholesterol in the experimental animals*

The concentrations of total cholesterol in serum in the experiments with rabbits and rats are shown in Table 3. When rabbits were fed semipurified diets (experiment II-Rabbit-SP), the diet containing casein produced a marked increase in serum cholesterol concentration; most of the increased cholesterol being found in the LDL fraction (Table 6). The declines in concentration of cholesterol after the casein control period seen on the two diets containing soy isolate and soy concentrate, respectively, were identical; these declines were observed in the LDL fraction. The higher concentration of cholesterol in the serum on the casein diet compared with the two soy diets, is reflected in the concentration of cholesterol in the liver (4.5 ± 1.3 mg cholesterol/g liver, mean ± SD) although the difference was only significant ($p < 0.05$) when compared with the soy concentrate group (3.3 ± 1.0 mg/g) and not with the soy isolate group (3.6 ± 1.6 mg/g). Most of the additional cholesterol which is found in the liver at increasing serum cholesterol concentrations is in the form of cholesteryl ester. This is seen from the relationships between serum cholesterol concentration and liver cholesteryl ester concentration (Figure 3) and between liver cholesterol concentration and the proportion of cholesterol in the liver that is esterified (Figure 4).

In the first experiment with rabbits using the human diets (experiment I-Rabbit), the rabbits were transferred straight from the commercial diet to the test diets containing either casein or soy protein isolate. After 2½ weeks of test period, the casein diet produced significantly higher increases in cholesterol than did the soy isolate diet (Table 3). When the rabbits were subsequently returned to the commercial diet, the concentration of cholesterol in the serum of both groups of rabbits fell to a mean value of about 30 mg/dl (Van Raaij *et al.*, 1981). Thus the human diet containing casein has a pronounced hypercholesterolemic effect in rabbits when compared with the soy protein diet.

In the second experiment with rabbits using the human diets (experiment II-Rabbit), the rabbits were transferred from the commercial diet to the human diets for a control period of 2½ weeks before being transferred to the test

Table 6

THE EFFECT OF CASEIN AND SOY PROTEIN DIETS ON THE CONCENTRATION OF CHOLESTEROL IN LIPOPROTEINS IN RABBITS ON SEMIPURIFIED DIETS (EXPERIMENT II-RABBIT-SP)

| Lipoprotein fraction | Control Period | Test Period | | |
|---|---|---|---|---|
| | Casein diet[a] | Casein diet[b] | Soy isolate diet[b] | Soy concentrate diet[b] |
| | n=36 | n=12 | n=12 | n=12 |
| | | mg/100 ml | | |
| VLDL | 17 | 33 | 17 | 18 |
| LDL | 74 | 223 | 48 | 55 |
| HDL | 25 | 41 | 31 | 31 |
| Whole serum[c] | 119 | 278 | 92 | 95 |

a) Mean values of six pools, each of serum from 6 animals.

b) Mean values of four pools, each of serum from 3 animals.

c) Mean values from serum of individual animals.

diets for a subsequent 4 weeks. On the casein control diet, the concentrations of cholesterol in serum increased steeply and significantly when compared with the cholesterol levels of the rabbits that continued on the commercial diet. After 4-5 weeks on the human diets, the rabbits began to show signs of ill--health such as reduced appetite, reduced growth and hair loss. As discussed earlier, this may have been due to a mineral or vitamin deficiency, which did not become manifest within the 2½ week period that the rabbits in experiment I-Rabbit were on the human diets.

In the experiment with rats using the human diets (II-Rat), the rats were transferred from the commercial diet to the human casein diet for a control period of 2½ weeks before being transferred to the human test diets. Concentration of cholesterol in serum was declining in the rats used, as shown by the rats that continued on the commercial diet for the whole experiment. On the casein control diets significant lower concentrations of cholesterol in serum

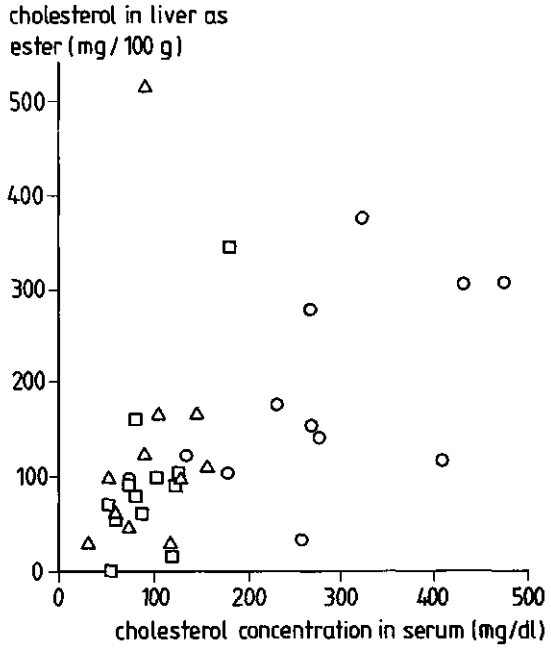



FIG. 3. The relationship between the cholesterol concentration in serum (mg/dl) (x) and the amount of cholesterol as ester in the liver (mg/100 g) (y) in rabbits (experiment II-Rabbit-SP)

$y = 1.22\ x + 63.6$ ; $r = 0.481$, $P < 0.01$

○ , Casein fed rabbits

△ , Soy isolate fed rabbits

□ , Soy concentrate fed rabbits

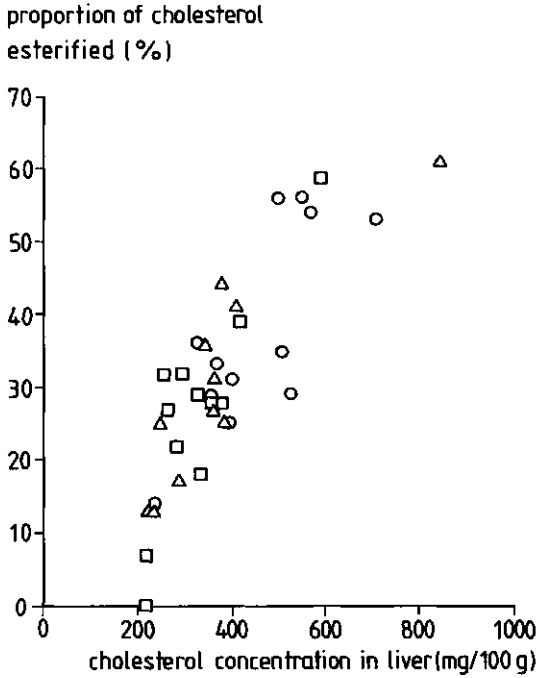



FIG. 4. The relationship between the cholesterol concentration in the liver (mg/100 g) (x) and the proportion of cholesterol esterified (%) (y) in rabbits (II-Rabbit-SP)

$y = 0.071\ x + 2.25$ ; $r = P < 0.001$

○ , Casein fed rabbits

△ , Soy isolate fed rabbits

□ , Soy concentrate fed rabbits

were observed than on the commercial diet. During the test period the declines in serum cholesterol on the human test diets were similar to those on the commercial diet; however, the declines on both soy protein diets were more pronounced than that on the casein diet (Table 3). The larger declines on the soy protein diets occurred mainly in the lipoprotein fractions with densities between 1.060 and 1.128 g/ml (Table 7).

## DISCUSSION

In our studies with young and middle-aged adults we failed to find a marked effect on serum total cholesterol of diets containing either soy isolate or soy concentrate when compared with a diet containing casein. When the soy preparations were incorporated into semipurified diets and fed to rabbits, we obtained results similar to those reported previously (for a review, see Terpstra *et al.*, 1982b): casein was found to be strongly hypercholesterolemic when compared with the soy preparations. Similar results were observed when rabbits were fed the human diets: a human diet containing soy isolate resulted in significantly lower cholesterol concentrations than the human diet containing casein (experiment I-Rabbit). In experiment II-Rabbit, the rabbits also showed a marked increase in serum cholesterol concentration after they had been transferred from the commercial diet to the casein-containing human diet (Table 3). As discussed before, it is unfortunate that in experiment II-Rabbit no comparisons could be made between the casein and the soy protein diets. In rats, the differences between the human diets containing either casein or soy protein were small, but marginally significant.

How can one explain these different results between humans and animals?

### *Species effects*

Animal species differ greatly in their resistance to diet-induced hypercholesterolemia. For example, rabbits are highly susceptible to hypercholesterolemia by dietary means; the replacement of protein in semipurified diets by casein and the addition of small amounts of cholesterol have both been shown to be very effective. Although most studies with rabbits have been carried out with semipurified diets, our studies clearly indicate that human diets give similar results. In our studies the effects with the human diets were less pronounced than with the semipurified diets, but this can be explained, at least partly, by the fact that in the human diets only 65% of the protein

Table 7

THE EFFECT OF CASEIN AND SOY PROTEIN DIETS ON THE CONCENTRATION OF CHOLESTEROL IN LIPOPROTEINS IN RATS (EXPERIMENT II-RAT)

| Lipoprotein fraction | Casein Group | | | Soy isolate Group | | | Soy concentrate Group | | |
|---|---|---|---|---|---|---|---|---|---|
| (density d in g/ml) | Control Period | Test Period | Change | Control Period | Test Period | Change | Control Period | Test Period | Change |
| d < 1.016 | 2.9 | 2.5 | -0.4 | 2.5 | 2.1 | -0.4 | 2.9 | 2.1 | -0.8 |
| 1.016 < d < 1.032 | 1.5 | 1.5 | 0.0 | 1.4 | 1.4 | 0.0 | 1.4 | 1.2 | -0.2 |
| 1.032 < d < 1.045 | 1.7 | 2.1 | +0.4 | 1.9 | 2.1 | +0.2 | 1.9 | 1.9 | 0.0 |
| 1.045 < d < 1.060 | 3.5 | 3.5 | 0.0 | 4.6 | 4.1 | -0.6 | 3.5 | 3.9 | +0.4 |
| 1.060 < d < 1.078 | 8.1 | 7.7 | -0.4 | 10.6 | 9.7 | -1.0 | 8.3 | 6.6 | -1.7 |
| 1.078 < d < 1.102 | 22.4 | 18.6 | -3.9 | 23.4 | 15.3 | -8.1 | 24.6 | 20.5 | -4.1 |
| 1.102 < d < 1.128 | 13.2 | 12.0 | -1.2 | 14.1 | 10.1 | -4.1 | 13.5 | 11.4 | -2.1 |
| 1.128 < d < 1.146 | 2.5 | 2.9 | +0.4 | 2.7 | 3.3 | +0.6 | 2.9 | 3.3 | +0.4 |
| 1.146 < d | 2.1 | 3.5 | +1.4 | 1.9 | 3.7 | +1.7 | 2.1 | 2.9 | +0.8 |
| Whole serum (pools) | 66.0 | 52.6 | -13.4 | 69.1 | 51.5 | -17.6 | 65.8 | 51.7 | -14.1 |

Results expressed in mg/100 ml of serum. At the end of the control and test period, two serum samples were taken at one-day intervals. Serum samples were pooled per group. Each given value represents the average value of the two pools at the end of the respective period.

consisted of test protein compared with 100% in the semipurified diets, while the total proportion of protein in the two types of diets was about the same. The hypercholesterolemic effect of casein increases when the amount of casein in the diet is increased (Terpstra *et al.*, 1981a). The amount of linoleic acid in the diet may also be a contributing factor as the casein-induced hypercholesterolemia in rabbits disappears when increased amounts of linoleic acid are included in the diets (Lambert *et al.*, 1958; Wigand, 1959; Carroll and Hamilton, 1976; Beynen and West, 1981). In our human diets, the proportion of polyunsaturated fat in the diet was about 4% by weight, while in the semipurified diets the proportion was less than 1%. The differences in results between the human and semipurified diets can probably not be explained by difference in duration, because after 2½ weeks on the semipurified diets the difference in effects between the casein and soy protein diets was already greater than on the human diets (data not shown).

In rats fed semipurified diets the effects of dietary proteins are only observed when considerable amounts of cholesterol (approximately 1%) are added to the diets (Terpstra *et al.*, 1982a). In our experiments only 0.07% of cholesterol was present, and this explains why the observed effects in rats were so small. Our studies with humans suggest that normocholesterolemic adults are also relatively insensitive to changes in dietary protein, and one might speculate that in humans, diets have to contain considerable amounts of cholesterol before the differential effect of casein and soy protein on the level of serum cholesterol is expressed. It should be noted, however, that in the experiments of Sirtori *et al.* (1979) the cholesterol-lowering effect of the soy diet in hypercholesterolemic patients was most pronounced against a high-linoleic acid low-cholesterol background, *i.e.* the opposite of the conditions under which the effects of dietary proteins are seen most clearly in animals.

*Age of the animals*

Animal studies in general and our studies in particular, have been carried out with young growing animals, while in most human studies, including those reported here, adults were used. It has been reported that dietary casein-induced hypercholesterolemia in male rabbits occur only in young growing animals (West *et al.*, 1982), and similar results have been observed in rats (McGregor *et al.*, 1971). Such animals spend a considerable proportion of their lives on the experimental diets. Therefore, our results on humans do not rule out the possibility that a long-term intake of animal protein starting at an early age, will cause similar cholesterol-raising effects in normocholesterolemic humans.

*Differences in lipoprotein metabolism*

A point to keep in mind in extrapolating animal data to man concerns the differences in the distribution of cholesterol over the various lipoprotein fractions. In healthy humans, most of the serum cholesterol is transported in the LDL fraction, whereas in normocholesterolemic rabbits and rats the HDL fraction is the main carrier of cholesterol (Terpstra *et al.*, 1982c). Furthermore, hypercholesterolemic diets in animals often cause the appearance of an unusual lipoprotein with the density of VLDL but with a much higher cholesterol content and with a higher proportion of apolipoprotein E (Ross and Zilversmit, 1977; Mahley *et al.*, 1980; Terpstra *et al.*, 1982a; Scholz *et al.*, 1982). In human such a particle is seen only in type III hypercholesterolemia, a fairly rare disease. In healthy humans we did not find any marked abnormalities of the lipoprotein spectrum after 6 weeks on the casein diet.
In comparison, however, the subjects who had received the soy isolate diet showed slightly lower LDL and higher HDL cholesterol levels. Apart from slight changes in the composition of LDL in I-Human, our apoprotein measurements did not point to marked effects of dietary proteins on lipoprotein composition in man. Thus at the level of the separate lipoproteins, casein-fed rabbits and rats appear to be different from humans.

*Possible mechanisms*

The mechanisms underlying the cholesterolemic properties of dietary protein are not clearly understood. Probably several mechanisms are involved. The amino acid composition of the proteins plays a role (Carroll, 1978; Kritchevsky, 1979; Hermus, 1975; Terpstra *et al.*, 1982c), but how amino acids might influence cholesterol metabolism remains to be established. Differences in amino acid composition between casein and soy protein, however, cannot fully explain the different results (Carroll, 1978; Terpstra *et al.*, 1982c). It has been suggested that some substances in the non-protein part of the soy preparations such as fiber or saponins might be partly responsible for the observed effects (Potter *et al.*, 1980; Topping *et al.*, 1980), but our human and animal studies clearly indicate that the non-protein part of soy concentrate did not influence serum cholesterol when compared with soy isolate. Similar results were found by Hamilton and Carroll (1976) in rabbits. As discussed previously (Van Raaij *et al.*, 1982) the type of dietary fiber present in soy concentrate is not likely to exert a marked hypocholesterolemic effect. As casein increases the concentration of cholesterol in both the liver and in serum (experiment II-Rabbit-SP), a redistribution of cholesterol between the serum and liver would not appear to be

involved as is the case with the difference between rabbits which are hypo- or hyperresponsive to cholesterol (West and Roberts, 1974). Perhaps the differential effect of casein and soy protein on serum cholesterol levels in rabbits may be attributible to differences in the digestibility of the two proteins. As has been postulated elsewhere (Terpstra *et al.*, 1982c) soy protein may be less digestible than casein in rabbits and the undigested protein could bind bile acids, thus facilitating their excretion and preventing their reabsorption. It may well be that in humans, soy protein is not significantly less digestible than casein or that the binding of soy protein to bile acids does not have a significant effect on the excretion of bile acids either because of the presence of other materials in the gut or because of the nature of the bile acids involved.

Although we did not find a marked change in serum total cholesterol concentrations in humans on the casein and soy diets, our data do show a small decline in LDL cholesterol and a small increase in HDL cholesterol on the soy isolate diet when compared with the casein diet, but there was no effect with the soy concentrate diet. Apart from small changes in the composition of LDL in I-Human, the apoprotein results suggest that the changes in cholesterol concentrations in the lipoprotein fractions resulted from changes in the number of lipoprotein particles.

An increase in the ratio of HDL cholesterol/total cholesterol has been associated with a lower risk of coronary heart disease (Blackburn *et al.*, 1977; Miller and Miller, 1975), so it may well be that the increase in this ratio on the soy isolate diet could have a beneficial effect even when the concentration of total cholesterol remains constant. Yet it must be noted that in our studies these favorable effects were only observed with the rather pure soy protein isolate and not with the soy concentrate. Thus the dramatic effects observed by Sirtori *et al.* (1979) with textured soy protein not only on HDL cholesterol/total cholesterol ratio but also on the concentration of total cholesterol eluded us when we used the soy concentrate diet.

An expectation has developed that the replacement of animal proteins by vegetable proteins in human diets might aid in lowering serum total cholesterol levels, thereby providing a very useful tool in the prevention of coronary heart disease (Lewis, 1980). This expectation is based on the many studies with animals, particularly the rabbit, and a limited number of studies with humans, particularly hypercholesterolemic patients (Sirtori *et al.*, 1979; Descovich *et al.*, 1980; Wolfe *et al.*, 1981) in which diets containing animal proteins,

particularly casein, have been compared with diets containing vegetable proteins, particularly soy protein. However, the results of most studies with normocholesterolemic humans, including our two studies, do not provide support for this expectation: the normocholesterolemic human appears to be relatively insensitive to changes in the type of protein in the diet. However, a small favorable effect on the distribution of cholesterol over the various lipoprotein classes cannot be excluded.

ACKNOWLEDGEMENTS

The authors are most grateful to the volunteers for their invaluable co-operation. We are indebted to the firms involved in the development and preparation of the special food products for the human studies (see Van Raaij *et al.*, 1981, 1982); to J.B. Schutte and K. Deuring (Institute for Animal Nutrition Research ILOB-TNO, Wageningen) for preparing the animal diets and performing the rabbit experiments; to G. van Tintelen and J. Haas for performing the rat experiment; to H. Koopman for freeze-drying the human diets; to P.H.E. Groot (Department of Biochemistry I, Erasmus University, Rotterdam) and P.N.M. Demacker (Department of Internal Medicine, University of Nijmegen) for providing us with the methodology and materials for apolipoprotein assays; to the technicians Mrs. J.H.M. Barendse-van Leeuwen, Mrs. E.A.M. Koot-Gronsveld, Miss A.E.M.F. Soffers, F.J.M. Schouten and H.J. Verwey; and to A.H.M. Terpstra for helpful advice and discussion.

REFERENCES

Anderson JT, Grande F, Keys A (1971): Effect on man's serum lipids of two proteins with different amino acid composition. Am J Clin Nutr 24: 524-530.

Beynen AC, West CE (1981): The distribution of cholesterol between lipoprotein fractions of serum from rabbits fed semipurified diets containing casein and either coconut oil or corn oil. Z Tierphysiol Tierernährg u Futtermittelkunde 46: 233-239.

Blackburn H, Chapman J, Dawber TR, Doyle JT, Epstein FH, Kannel WB, Keys A, Moore F, Paul O, Stamler J, Taylor HL (1977): Revised data for 1970 ICHD report. Am Heart J 94: 539-540.

Bodwell CE, Schuster EM, Steele PS, Judd JT, Smith JC (1980): Effects of dietary soy protein on plasma lipid profiles of adult men (Abstr 4456). Fed Proc 39: 1113.

Brussaard JH, Dallinga-Thie GM, Groot PHE, Katan MB (1980): Effect of amount and type of dietary fat on serum lipids, lipoproteins and apoproteins in man: a controlled 8-week trial. Atherosclerosis 36: 515-527.

Burslem J, Schonfeld G, Howald MA, Weidman SW, Miller JP (1978): Plasma apoprotein and lipoprotein lipid levels in vegetarians. Metabolism 27: 711-719.

Burstein M, Samaille J (1960): Sur un dosage rapide du cholesterol lié au α- et β-lipoproteins du serum. Clin Chim Acta 5: 609.

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

Carroll KK, Giovannetti PM, Huff MW, Moase O, Roberts DCK, Wolfe BM (1978): Hypocholesterolemic effect of substituting soybean protein for animal protein in the diet of healthy young women. Am J Clin Nutr 31: 1312-1321.

Carroll KK (1978): The role of dietary protein in hypercholesterolemia and atherosclerosis. Lipids 13: 360-365.

Descovich GC, Gaddi A, Mannino G, Cattin L, Senin U, Caruzzo C, Fragiocomo C, Sirtori M, Ceredi C, Benassi MS, Colombo L, Fontana G, Mannarino E, Bertelli E, Noseda G, Sirtori CR (1980): Multicentre study of soybean protein diet for outpatient hypercholesterolaemic patients. Lancet ii: 709-712.

Folch J, Lees M, Stanley GHS (1957): A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497.

McGregor D (1971): The effects of some dietary changes upon the concentrations of serum lipids in rats. Br J Nutr 25: 213-224.

Haar F van der, Gent CM van, Schouten FM, Voort HA van der (1978): Methods for the estimation of high density cholesterol, comparison between two laboratories. Clin Chim Acta 88: 469-481.

Hamilton RMG, Carroll KK (1976): Plasma cholesterol levels in rabbits fed low fat, low cholesterol diets. Effects of dietary proteins, carbohydrates and fibre from different sources. Atherosclerosis 24: 47-62.

Hardinge MG, Store FJ (1954): Nutritional studies of vegetarians. 2. Dietary and serum levels of cholesterol. Am J Clin Nutr 2: 83-88.

Hermus RJJ (1975): "Experimental atherosclerosis in rabbits on diets with milk fat and different proteins". Wageningen, The Netherlands: Centre for Agricultural Publishing and Documentation.

Holmes WL, Rubel GB, Hood SS (1980): Comparison of the effect of dietary meat versus dietary soybean protein on plasma lipids of hyperlipidemic individuals. Atherosclerosis 36: 379-387.

Huang TC, Chen CP, Wefler V, Raftery A (1961): A stable reagent for the Liebermann-Burchard reaction. Application to rapid serum cholesterol determination. Anal Chem 33: 1405-1407.

Katan MB, Vroomen LHM, Hermus RJJ (1982): Reduction of casein-induced hypercholesterolemia and atherosclerosis in rabbits and rats by dietary glycine, arginine and alanine. Atherosclerosis (in press).

Kritchevsky D, Tepper SA, Williams DE, Story JA (1977): Experimental atherosclerosis in rabbits fed cholesterol-free diets. Part 7. Interaction of animal or vegetable protein with fiber. Atherosclerosis 26: 397-403.

Kritchevsky D (1979): Vegetable protein and atherosclerosis. J Am Oil Chemists' Soc 56: 135-140.

Lambert GF, Miller JP, Olsen RT, Frost DV (1958): Hypercholesterolemia and atherosclerosis induced in rabbits by purified high fat rations devoid of cholesterol. Proc Soc Exp Biol Med 97: 544-549.

Laurell CB (1972): Electroimmunoassay. Scand J Clin Lab Invest, Suppl. 124: 21.

Lewis B (1980): Dietary prevention of ischaemic heart disease - a policy for the '80s. Brit Med J 281: 177-180.

Mahley RW, Innerarity TL, Brown MS, Ho WK, Goldstein JL (1980): Cholesteryl ester synthesis in macrophages: Stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. J Lipid Res 21: 970.

Miller GJ, Miller NE (1975): Plasma high-density lipoprotein concentration and development of ischaemic heart-disease. Lancet i: 16-19.

Potter, JD, Illman RJ, Calvert GD, Oakenfull DG, Topping DL (1980): Soya saponins, plasma lipids, lipoproteins and fecal bile acids: a double blind cross-over study. Nutr Rep Int 22: 521-528.

Raaij JMA van, Katan MB, Hautvast JGAJ, Hermus RJJ (1981): Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. Am J Clin Nutr 34: 1261-1271.

Raaij JMA van, Katan MB, West CE, Hautvast JGAJ (1982): Influence of diets containing casein, soy isolate and soy concentrate on serum cholesterol and lipoproteins in middle-aged volunteers. Am J Clin Nutr 35: 925-934.

Redgrave TG, Roberts DCK, West CE (1975): Separation of plasma lipoproteins by density gradient ultracentrifugation. Anal Biochem 65: 42-49.

Röschlau P van, Bernt E, Gruber W (1974): Enzymatische Bestimmung des Gesamt-Cholesterins im Serum. Z Klin Chem Klin Biochem 12: 403-407.

Ross AC, Zilversmit DC (1977): Chylomicron remnant cholesteryl esters as the major constituent of very low density lipoproteins in plasma of cholesterol--fed rabbits. J Lipid Res 18: 169.

Sacks FM, Castelli WP, Donner A, Kass EH (1975): Plasma lipids and lipoproteins in vegetarians and controls. N Engl J Med 292: 1148-51.

Scholz KE, Beynen AC, West CE (1982): Comparison between the hypercholesterolaemia in rabbits induced by semipurified diets containing either cholesterol or casein. Atherosclerosis (in press).

Shorey RL, Davis JL (1979): Effects of substituting soy for animal protein in the diets of young mildly hypercholesterolemic males (Abstr.). Fed Proc 38: 551.

Sirtori CR, Gatti E, Montero O, Conti F, Agradi E, Tremoli E, Sirtori M, Fraterrigo L, Tavazzi L, Kritchevsky D (1979): Clinical experience with the soybean protein diet in the treatment of hypercholesterolemia. Am J Clin Nutr 32: 1645-1658.

Snedecor GW, Cochran WG (1967): "Statistical Methods". Ames IA: Iowa State University Press.

Stamler J (1979): Population studies. In: Levy RI, Rifkind BM, Dennis BH and Ernst ND (eds): "Nutrition, Lipids and Coronary Heart Disease". New York: Raven Press, pp. 25-88.

Terpstra AHM, Sanchez-Muniz FJ (1981): Time course of the development of hypercholesterolemia in rabbits fed semipurified diets containing casein or soybean protein. Atherosclerosis 39: 217-227.

Terpstra AHM, Harkes L, Veen FH van der (1981a): The effect of different proportions of casein in semi-purified diets on the concentration of serum cholesterol and the lipoprotein composition in rabbits. Lipids 16: 114-119.

Terpstra AHM, Woodward CJH, Sanchez-Muniz FJ (1981b): 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. Anal Biochem 111: 149-157.

Terpstra AHM, Tintelen G van, West CE (1982a): The effects of semipurified diets containing different proportions of either casein or soybean protein on the concentration of cholesterol in whole serum, serum lipoproteins and liver in male and female rats. Atherosclerosis 42: 85-95.

Terpstra AHM, Hermus RJJ, West CE (1982b): The role of dietary protein in cholesterol metabolism. World Rev Nutr Dietet (in press).

Terpstra AHM, Hermus RJJ, West CE (1982c): Dietary protein and cholesterol metabolism in rabbits and rats. In: "Current Topics in Nutrition and Disease" : Animal and Vegetable Proteins in Lipid Metabolism (D Kritchevsky and MJ Gibney, eds). New York, Alan R Liss Inc. (in press).

Topping DL, Trimble RP, Illman RJ, Potter JD, Oakenfull DG (1980): Prevention of dietary hypercholesterolemia in the rat by soy flour high and low in saponins. Nutr Rep Int 22: 513-519.

Vessby B, Boberg J, Gustafsson IB, Karlström B, Lithell H, Werner I (1980): Substitution with textured soy protein for animal protein in a lipid lowering diet - effects on the serum lipoprotein composition (Abstr.). Europ J Clin Invest 10/2 11: 1823.

West CE, Roberts DCK (1974): Cholesterol metabolism in two strains of rabbits differing in their cholesterolaemic response to dietary cholesterol. Biochem Soc Trans 2: 1275-1277.

West CE, Rowbotham TR (1967): The use of a computer in the determination by gas-liquid chromatography of the concentrations and identification of individual fatty acids present as free fatty acids, triglycerides and cholesteryl esters. J Chromatog 30: 62-76.

West CE, Deuring K, Schutte JB, Terpstra AHM (1982): The effect of age on the development of hypercholesterolemia in rabbits fed semipurified diets containing casein. J Nutr (in press).

Wigand G (1959): Production of hypercholesterolemia and atherosclerosis in rabbits by feeding different fats without supplementary cholesterol. Acta Med Scand 166 Suppl. 351: 1-91.

Wolfe BM, Giovannetti PM, Cheng DCH, Roberts DCK, Carroll KK (1981): Hypolipidemic effect of substituting soybean protein isolate for all meat and dairy protein in the diets of hypercholesterolemic men. Nutr Rep Int 24: 1187-1198.

# 5. INFLUENCE OF DIETS CONTAINING CASEIN, SOY ISOLATE AND SOY CONCENTRATE ON THE LEVEL OF URATE IN THE SERUM OF HEALTHY HUMANS

J.M.A. VAN RAAIJ and M. VLASSAK

Department of Human Nutrition, Agricultural University, De Dreijen 12, 6703 BC Wageningen, The Netherlands

SUMMARY

Hyperuricemia as a prerequisite for the development of gout is well established, and as a result, prudent advice for hyperuricemic individuals is the avoidance of dietary conditions which elevate serum urate concentration, such as high intakes of purines. Two strictly controlled nutritional studies with large groups of young students and middle-aged subjects have been carried out. The test diets differed only in respect of the type of protein preparation used, that is caseinate, soy protein isolate, or soy protein concentrate. Both studies revealed a significant increase in level of serum urate, between 0.60 and 0.74 mg/dl, in those on the soy protein diets compared to those on the casein diets. The purine content of the soy preparations possibly explains only part of the observed effects. An effect of the difference in amino acid composition between casein and soy protein cannot be excluded. It can be concluded that soy protein preparations may cause a substantial increase in level of serum urate when compared with dairy foods. No substantial difference in level of serum urate may be expected when soy protein preparations are compared with other protein sources such as meat.

## INTRODUCTION

Recently the results were published of two human experiments in which the effects of diets containing either casein or soy protein were compared on serum cholesterol and lipoproteins (1,2). In order to control the diets, the participants were provided with almost complete daily diets. Hot meals were served on weekdays in the Department of Human Nutrition and all other food, including food for the weekend, was provided as package issues. In such studies

it is difficult to ascertain whether or not subjects confine themselves strictly to the diet given or whether they eat other items. It appears however, that individuals who agree to take part in studies of this nature do so with the best intentions. With proper encouragement, the incidence of cheating appears to be rare. Nevertheless, it is always desirable that some type of objective check be made that the participants actually eat all of the diet provided. The experimental diets used differed only in the type of protein preparation: caseinate, or soy protein isolate, or soy protein concentrate (1,2). Caseinates are practically free of purines, but both soy preparations contain considerable amounts of nucleic acids, and also purines. Uric acid is the end product of human purine metabolism (3), and it is well established that dietary purines increase the level of urate in serum (4-8). Thus the level of serum urate may provide a rough check on the adherence of the subjects to the diets.

The medical importance of urate is derived from its low solubility at the pH of body fluids, and its relatively poor excretion by the kidney. If the level of serum urate is elevated, crystals may form in the joints, as occurs in gout, and with excessive renal clearance loads, stones may be deposited in the urinary tract (3). The prevalence of gout is directly related to serum urate concentrations, and most cases occur in association with concentrations over 7.0 mg/dl in men and 6.0 mg/dl in women (9). These borderlines are often used to define hyperuricemia. Hyperuricemia was observed in 14% of white men and women in the Evans County Study (10); a lower percentage, 6%, was reported in the Tecumseh Study (11); but Persky *et al.* (12) in Chicago observed levels of serum urate greater than 7.0 mg/dl in more than 23% of the white men aged 18-64 years. The second reason for studying the level of serum urate is to quantify the hyperuricemic effects of soy protein preparations. It has been recommended that animal protein be replaced by vegetable protein, *e.g.* soy protein, because of the supposed benefical effects on serum cholesterol (13). However, in Western countries levels of serum urate are relatively high, and the effects on levels of serum urate by replacing animal protein with vegetable protein should also be considered.

## SUBJECTS AND METHODS

In the two studies carried out, three principle sources of protein were used: caseinate; soy protein isolate, the purest soy protein preparation available commercially; and soy protein concentrate, a less refined soy protein preparation (1,2). In the first study, 69 healthy students (18-28 yr of age)

were given for 38 days diets containing 13% of energy as protein of which 65% consisted of protein from casein, from soy isolate, or from a 2:1 mixture of casein and soy isolate. After a control period of 10 days during which all the subjects received the casein-soy (cassoy) diet, 20 subjects continued on this diet for a test period of 28 days, 25 subjects changed to the casein diet, and the remaining 24 subjects changed to the soy isolate diet (Figure 1; Ref. 1). In the second study, 57 healthy middle-aged subjects (29-60 yr of age) were fed for 45 days a diet containing 16% of energy as protein of which 60% consisted of protein from casein, from soy protein isolate or from soy protein concentrate. After a control period of 17 days during which all the subjects received the casein diet, 17 subjects continued on this diet for a test period of 28 days, 20 subjects changed to the soy isolate diet, and the remaining 20 subjects changed to the soy concentrate diet (Figure 1; Ref. 2).

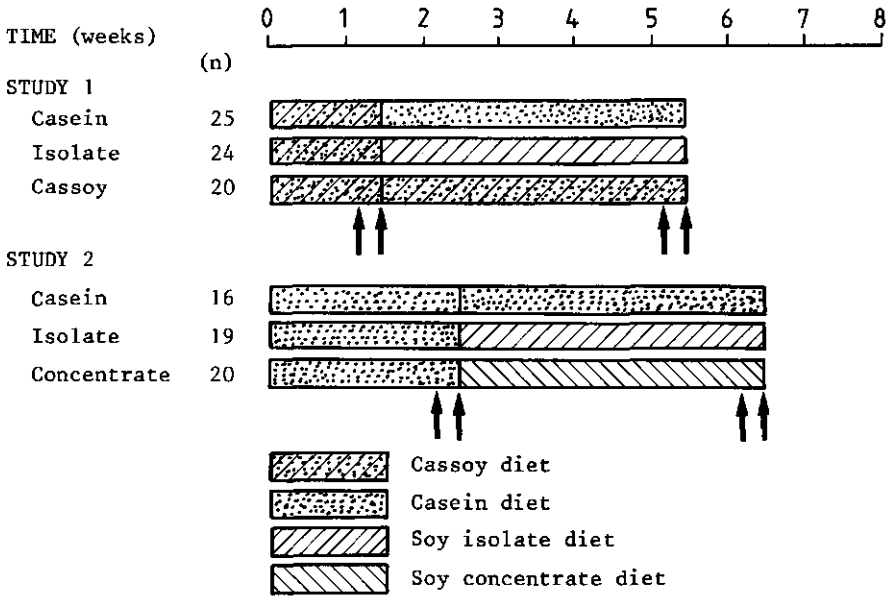



FIG. 1. Experimental designs. Blood sampling for serum urate analysis is indicated by arrows.

Food records and chemical analysis of the diets indicated that within each study there were essentially no differences between the experimental diets except for the type of protein preparation used. Details of the composition of the diet, daily regimen and food supply have been described previously (1,2).

Levels of serum urate were determined at the end of the control and the test period, as indicated in Figure 1. Steady-state levels of serum urate after dietary modifications are usually obtained within 7 to 10 days (8). Specimens of blood were obtained from a vein in the arm after an overnight fast, and serum was obtained by low-speed centrifugation. Serum samples were stored for no longer than two months at -80°C, before assaying for serum urate in duplicate by an enzymatic method (14) using a kit (No 124.761, Boehringer Mannheim GmbH, West Germany). Samples obtained for each person at the end of both the control and test period were analysed within the same run. Equal numbers of sera from subjects from the three diet groups were assayed in each run. Within-run reproducibility for control sera was ±2.6% (C.V.). The response to the test diet was calculated for each subject from the values of the end of the control and the end of the test period. The effect of diet was examined by testing the difference between the mean responses of the groups by unpaired two-tailed t tests (15). In the second study, the data on one of the subjects were eliminated from the analysis because he had been using a hypouricemic drug. The results of another subject were lost. Thus data on only 55 subjects were analysed.

## RESULTS

All levels of serum urate measured were below the hyperuricemia borderlines of 7.0 mg/dl for men and of 6.0 mg/dl for women. The values of the mean urate concentrations in serum at the end of the control and test periods are given in Table 1.

The group means at the end of the control periods were not completely comparable because the groups were not matched for level of serum urate. As expected, women had lower levels of urate in serum than men (10,11,16,17). Values at the end of the control periods were 4.26 ± 0.63 mg/dl and 3.70 ± 0.82 mg/dl (mean ± SD) for women, and 5.26 ± 0.72 mg/dl and 4.54 ± 0.81 mg/dl for men in both studies respectively. Both men and women had higher values in the first study. This was probably because control diets were different in the two studies; the cassoy control diet in which soy protein isolate was incorporated contained more purines than the casein control diet. Serum urate appears not

Table 1

THE EFFECT OF CASEIN AND SOY PROTEIN DIETS ON SERUM URATE CONCENTRATIONS IN HUMANS (MEAN ± SD; mg/dl[a])

| STUDY 1 (young adults) | Casein diet | Cassoy diet | Soy isolate diet |
|---|---|---|---|
| Cassoy control period | 4.57 ± 0.66 | 4.94 ± 1.06 | 5.12 ± 0.82 |
| Test period | 4.37 ± 0.91 | 4.97 ± 1.33 | 5.56 ± 0.99 |
| Change over test period | | | |
| All subjects[b] | -0.19 ± 0.69 | +0.03 ± 0.69 | +0.45 ± 0.71[d] |
| Women | -0.30 ± 0.42 | -0.28 ± 0.65 | +0.07 ± 0.55 |
| Men | -0.12 ± 0.83 | +0.23 ± 0.67 | +0.68 ± 0.70[d] |

| STUDY 2 (middle-aged adults) | Casein diet | Soy isolate diet | Soy concentrate diet |
|---|---|---|---|
| Casein control period | 4.20 ± 0.84 | 3.68 ± 0.82 | 4.42 ± 0.99 |
| Test period | 4.37 ± 0.69 | 4.59 ± 0.81 | 5.19 ± 0.97 |
| Change over test period | | | |
| All subjects[c] | +0.17 ± 0.30 | +0.91 ± 0.40[d] | +0.77 ± 0.52[d] |
| Women | +0.18 ± 0.34 | +0.91 ± 0.44[d] | +0.54 ± 0.45 |
| Men | +0.15 ± 0.29 | +0.89 ± 0.40[d] | +0.96 ± 0.50[d] |

a) 5 mg/dl = 297 µmol/l

b) After deleting the results of the 10 subjects who lost more than 3 kg body weight during the whole experimental period, values were -0.21, +0.03, and +0.29 mg/dl, respectively.

c) After deleting the results of 16 subjects who lost more than 3 kg body weight during the whole experimental period, values were +0.24, +0.99, and +0.72 mg/dl, respectively.

d) Significantly different from casein group (two-tailed t test; $p < 0.01$).

to be age-related in adult men and women, although it may increase in women at the menopause (10,12,16,17). Because the subjects in both studies were adults, it is most unlikely that the difference in age of the subjects in the two studies would explain the observed differences in levels of serum urate.

In both studies the increase in level of serum urate during the test period for those on the soy protein isolate diets was significantly different to the changes observed in those on the casein diets ( +0.64 mg/dl; $p < 0.05$, and +0.74 mg/dl; $p < 0.05$, respectively). The increase in level of serum urate in those on the soy protein concentrate diet in the second study was also statistically significant when compared with the changes on the casein diet (+0.60 mg/dl; $p < 0.05$). As shown in Table 1, these effects were observed both in men and women, although the effects are more pronounced in men. Perhaps, the men were more responsive to the various protein preparations. Another explanation may well be the difference between men and women in absolute intake of protein preparation. Although the composition of the diet expressed in percentages of energy was identical for each subject, the absolute energy intake of men was higher.

Weight loss has been associated with a decline in the level of serum urate (18). In the first study mean weight losses over the test period were 1.3, 1.0 and 1.6 kg for those on the casein, cassoy and soy isolate diets, respectively. In the second study, the weight losses were 1.9, 1.4 and 1.2 kg for those on the casein, soy isolate, and soy concentrate diet, respectively. It is unlikely that these differences in weight loss confound the results on the level of serum urate, and, as shown in Table 1, deleting the results of the subjects who lost more than 3 kg body weight over the whole experimental period does not substantially change the overall results.

## DISCUSSION

In the experiments described, the only difference between the test diets was the protein preparation used. Caseinates are practically free of purines. The soy concentrates used contained about 8.5 mg RNA and about 0.5 mg DNA per gram protein, and thus about 2 mg purines per gram protein (data provided by Dr. A.M. Ledeboer and Drs. P. van Stratum, Unilever Research Laboratory, Vlaardingen). No data are available on the purine content of the soy isolates used. As the subjects in both studies consumed on average about 55 g protein from the protein preparations per day, the difference in nucleic acid intake between the soy and casein fed subjects is estimated to be about 0.5 g per day.

It has been reported that for normal healthy adults, levels of serum urate are increased by 0.6 to 0.9 mg/dl for each gram of dietary RNA (5,7,8). However, extrapolation to the present studies should be done with great caution as yeast RNA was used in those experiments. A difference in nucleic acid intake of about 0.5 g per day may result in a difference in level of serum urate of about 0.3 to 0.45 mg/dl. In both of the present studies with normouricemic adults, an increase in level of serum urate of 0.60 to 0.74 mg/dl was observed in those on the soy protein diets when compared with those on the casein diets. Similar results have also been reported by Rauch-Janssen *et al.* (19). They compared the effects of a soy protein diet (diet contained about 44 mg purines per 1000 kcal) with a formula diet virtually free of purines on level of serum urate in 10 healthy subjects, and observed that levels were about 0.8 mg/dl higher in those on the soy protein diet. These results and those of the present studies suggest that the observed effects with the soy protein preparations may not be fully explained by the nucleic acid content of these preparations.

Increasing dietary protein intake has been reported to increase urate excretion (4,5) and to decrease serum levels (4), but serum levels are not always reported to decline (20). The increased excretion may well reflect a simultaneous increase in urate production and in renal excretion (20). An increased purine synthesis may be caused by the increase in supply of amino acids involved in purine synthesis, *e.g.* glycine and aspartic acid (20,21). The increase in amino acid degradation on increasing protein intake will result in increased urea and keto acids in serum, and these substances may increase renal excretion of uric acids (6). It should be noted that soy protein contains more glycine and aspartic acid than casein, and both of these amino acids are involved in purine synthesis. In addition, in the present studies those on soy protein diets had increased levels of serum urea when compared with those on the casein diets (Van Raaij and De Vries, unpublished results). Therefore, for theoretical reasons, it cannot be excluded that part of the observed effects are caused by differences in amino acid composition of the diets.

Extrapolation of the results of these studies to other dietary conditions should be done with extreme caution. For example, 60-65% of the total protein in the test diets consisted of soy protein, which is not a common situation. In addition, soy protein was compared with casein, which is almost free of purines. It should be noted that the purine content per g protein of soy

concentrate is less than half of that of meat and poultry (8). Therefore, when soy concentrate is compared with meat or poultry, the effect of increasing serum urate may disappear, or the reverse may occur. Van Stratum (22) described an experiment with 82 subjects in which the effects on levels of serum urate by replacing 25% of the animal protein in the diet with soy protein mainly from soy concentrates, were compared. No differences were observed.

In conclusion, soy protein preparations in the diet may cause a substantial increase in level of serum urate compared to dairy foods. This increase is probably no different from the expected increase in level of serum urate with conventional protein sources such as meat. The effect of amino acid composition of proteins on the level of serum urate should be further investigated.

ACKNOWLEDGEMENTS

The authors are indebted to Mrs. J.H.M. Barendse-van Leeuwen, Miss A.E.M.F. Soffers and P.J.F. de Vries, for their technical assistance.

REFERENCES

1. Raaij JMA van, Katan MB, Hautvast JGAJ, Hermus RJJ. Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. Am J Clin Nutr 1981; 34: 1261-1271.
2. Raaij JMA van, Katan MB, West CE, Hautvast JGAJ. Influence of diets containing casein, soy isolate and soy concentrate on serum cholesterol and lipoproteins in middle-aged volunteers. Am J Clin Nutr 1982; 35: 925-934.
3. Boss GR, Seegmiller JE. Hyperuricemia and gout. Classification, complications and management. New Engl J Med 1979; 300: 1459-1468.
4. Leopold JS, Bernhard A, Jacobi HG. Uric acid metabolism of children. Am J Dis Child 1925; 29: 191-199.
5. Waslien CI, Calloway DH, Margen S. Uric acid production of men fed graded amounts of egg protein and yeast nucleic acid. Am J Clin Nutr 1968; 21: 892-897.
6. Bowering J, Calloway DH, Margen S, Kaufmann NA. Dietary protein level and uric acid metabolism in normal man. J Nutr 1969; 100: 249-261.
7. Edozien JC, Udo UU, Young VR, Scrimshaw NS. Effects of high levels of yeast feeding on uric acid metabolism of young men. Nature 1970; 228: 180.
8. Griebsch A, Korfmacher I. Diät bei Hyperurikämie. Münch med Wschr 1975; 117: 1373-1378.
9. Hall AP, Barry PE, Dawber TR, *et al*.Epidemiology of gout and hyperuricemia. A long-term population study. Am J Med 1967; 42: 27-37.

10. Klein R, Klein BE, Cornoni JC, Maready J, Cassel JC, Tyroler HA. Serum uric acid. Its relationship to coronary heart disease risk factors and cardiovascular disease, Evans County, Georgia. Arch Intern Med 1973; 132: 401-410.

11. Myers AR, Epstein FH, Dodge HJ, Mikkelsen WM. The relationship of serum uric acid to risk factors in coronary heart disease. Am J Med 1968; 45: 520-528.

12. Persky VW, Dyer AR, Idris-Soven E, Stamler J, Shekelle RB, Schoenberger JA, Berkson DM, Lindberg HA. Uric acid: a risk factor for coronary heart disease? Circulation 1979; 969-977.

13. Lewis B. Dietary prevention of ischaemic heart disease - a policy for the '80s. Brit Med J 1980; 281: 177-180.

14. Kageyama N. Clin Chim Acta 1971; 31: 421.

15. Snedecor GW, Cochran WG. Statistical methods. 6th ed. Ames: Iowa State University Press, 1967.

16. Gertler MM, Garn SM, Levine SA. Serum uric acid in relation to age and physique in health and in coronary heart disease. Ann Intern Med 1951; 34: 1421-1431.

17. Okada M, Takeshita M, Ueda K, Omae T, Hirota Y. Factors influencing the serum uric acid level. A study based on a population survey in Hisayama Town, Kyushu, Japan. J Chron Dis 1980; 33: 607-612.

18. Nicholls A, Scott JT. Effect of weight-loss on plasma and urinary levels of uric acid. Lancet 1972; 2: 1223-1224.

19. Rauch-Janssen A, Gröbner W, Zöllner N. Comparison of serum uric acid and urinary uric acid excretion under a soy protein diet low in purines and under a formula diet virtually free of purines. Nutr Metab 1977; 21 (suppl 1): 94-97.

20. Bien EJ, Yü TF, Benedict JD, Gutman AB, Stetten D. The relation of dietary nitrogen consumption to the rate of uric acid synthesis in normal and gouty man. J Clin Invest 1953; 32: 778-780.

21. Clifford AJ, Riumallo JA, Baliga BS, Munro HN, Brown PR. Liver nucleotide metabolism in relation to amino acid supply. Biochim Biophys Acta 1972; 277: 443-458.

22. Stratum P van. Physiological effects of a high soybean diet in man. Cereal Fds Wrld 1978; 234-239.

# 6. GENERAL DISCUSSION

In the strictly controlled trials carried out with normocholesterolemic subjects (Chapters 2 and 3) marked effects on levels of serum cholesterol of diets containing either soy isolate or soy concentrate were not observed when compared with diets containing casein. A small decrease in LDL-cholesterol and a small increase in HDL-cholesterol were observed in those on the soy isolate diet when compared with those on the casein diet. No such beneficial effects were observed in those on the soy concentrate diet. However, these effects are small when compared with the effects that can be achieved by changing the fat composition of the diet or the amount of dietary cholesterol. The studies on the animals fed the human diets (Chapter 4) confirm the results reported for semipurified diets. Rabbits appear to be highly susceptible to hypercholesterolemia as a result of dietary protein, whereas rats are much less sensitive. It is clear that extrapolation of the results of animal experiments on the effect of protein on serum cholesterol directly to man should be done with extreme caution.

The results of the studies with humans were in line with other recent studies on normocholesterolemic subjects which revealed small (1) or no effects (2, 3) when soy protein diets when compared with control diets containing animal protein. However, these results are in marked contrast with those reported by Italian and Swiss workers (4, 5), who observed dramatic decreases in serum cholesterol levels in hypercholesterolemic patients on diets containing soy protein. Other studies on hypercholesterolemic subjects have revealed smaller (6, 7), inconsistent (8), or no effects (9).

The results of the various studies are difficult to compare because in each study: different soy protein products have been used; soy protein was compared with other animal proteins; the overall composition of the diets were different; and the studies differed in degree of protein replacement. In addition, the results of some studies are difficult to interpret since the diets compared did not only differ as to the type of protein they contained, but also as to other dietary factors. Furthermore, the experimental design and the number of subjects used did not always allow for an accurate and precise estimation of the diet differences (for a more detailed discussion, see Appendices I and II).

A limitation of most studies cited, including the present studies, is that the experiments were of short-duration and that the subjects were

adult humans. Most animal experiments on the relationship between dietary protein and level of serum cholesterol have been undertaken on growing animals which have been fed on the test diets for the greater part of their lives. It takes longer to reveal effects of protein in adult rabbits than in young rabbits, and the observed effects are much less (10). Thus, the effects of the long-term intake of various proteins, beginning at an early age, have yet to be determined in humans.

Furthermore, as the subjects in most controlled trials seldom form a representative sample of a specified population, extrapolation of the results should be done with extreme caution. The results of studies in which proteins were tested in diets of a specific overall composition should also be extrapolated to other dietary conditions with great care. The "ideal" experiment which takes account of all these difficulties, is difficult to carry out for a number of practical reasons. With all their limitations, human experiments as described in this thesis, remain a valuable adjunct to animal experiments and epidemiological studies.

The present evidence suggest that soy protein when compared with animal protein has a small but real effect on cholesterol metabolism in man. Normocholesterolemic subjects appear to be relatively insensitive to the type of protein in the diet, while hypercholesterolemic subjects seem more responsive. More research is needed to clarify the reasons for this difference in responsiveness. Then it may become possible to characterise people according to their degree of responsiveness which would be of great value in making dietary recommendations. However, this research is hampered by the fact that the mechanisms by which soy protein exerts its effects still remain to be elucidated (Chapters 2-4).

On basis of the results of animal studies and studies on hypercholesterolemic subjects with respect to dietary protein and serum cholesterol, it has been suggested that animal protein in the diet should be replaced by vegetable protein as a beneficial dietary modification in the prevention of atherosclerotic diseases (11). On basis of the present evidence on normocholesterolemic subjects, such suggestions cannot be accepted as general dietary recommendations, although changing the type of protein in the diet may be beneficial to overt hypercholesterolemic subjects. On the other hand, in practice, replacing foods containing animal protein with foods containing vegetable protein may also be beneficial to normocholesterolemic subjects, because the main sources of animal protein are also often rich in

saturated fat and cholesterol. It is well established that these dietary components increase serum lipids.

It should be stressed that dietary modifications may affect several physiological parameters, and not only the parameters under investigation. In making dietary recommendations, all foreseeable consequences should be considered. In the present studies, for example, the test proteins differed in amount of nucleic acids. The consequence was that the subjects on the soy protein diets had higher levels of serum urate than the subjects on the casein diets (Chapter 5). An elevated level of serum urate is a well-established risk factor for gout.

REFERENCES

1. Carroll KK, Giovannetti PM, Huff MW, Moase O, Roberts DCK, Wolfe BM. Hypocholesterolemic effect of substituting soybean protein for animal protein in the diet of healthy young women. Am J Clin Nutr 1978; 31: 1312-1321.
2. Shorey RL, Davis JL. Effects of substituting soy for animal protein in the diets of young mildly hypercholesterolemic males (Abstr.). Fed Proc 1979; 38: 551.
3. Bodwell CE, Schuster EM, Steele PS, Judd JT, Smith JC. Effects of dietary soy protein on plasma lipis profiles of adult men (Abstr 4456). Fed Proc 1980; 39: 1113.
4. Sirtori CR, Gatti E, Montero O, Conti F, Agradi E, Tremoli E, Sirtori M, Fraterrigo L, Tavazzi L, Kritchevsky D. Clinical experience with the soybean protein diet in the treatment of hypercholesterolemia. Am J Clin Nutr 1979; 32: 1645-1658.
5. Descovich GC, Gaddi A, Mannino G, Cattin L, Senin U, Caruzzo C, Fragiocomo C, Sirtori M, Ceredi C, Benassi MS, Colombo L, Fontana G, Mannarino E, Bertelli E, Noseda G, Sirtori CR. Multicentre study of soybean protein diet for outpatient hypercholesterolaemic patients. Lancet 1980; ii: 709-712.
6. Goldberg AP, Lim A, Kolar JB, Grundhauser JJ, Steinke FH, Schonfeld G. Soybean protein independently lowers plasma cholesterol levels in primary hypercholesterolemia. Atherosclerosis 1982; 43: 355-368.

7. Vessby B, Boberg J, Gustafsson IB, Karlström B, Lithell H, Werner I. Substitution with textured soy protein for animal protein in a lipid lowering diet - effects on the serum lipoprotein composition (Abstr.). Europ J Clin Invest 1980; 10/2 11: 1823.
8. Wolfe BM, Giovannetti PM, Cheng DCH, Roberts DCK, Carroll KK. Hypolipidemic effect of substituting soybean protein isolate for all meat and dairy protein in the diets of hypercholesterolemic men. Nutr Rep Int 1981; 24: 1187-1198.
9. Holmes WL, Rubel GB, Hood SS. Comparison of the effect of dietary meat versus dietary soybean protein on plasma lipids of hyperlipidemic individuals. Atherosclerosis 1980; 36: 379-387.
10. West CE, Deuring K, Schutte JB, Terpstra AHM. The effect of age on the development of hypercholesterolemia in rabbits fed semipurified diets containing casein. J Nutr (in press).
11. Lewis B. Dietary prevention of ischaemic heart disease - a policy for the '80s. Brit Med J 1980; 281: 177-180.

# APPENDIX I

# DESIGNS FOR EXPERIMENTS COMPARING THE EFFECTS OF DIFFERENT DIETS

CONTENTS

## 1. INTRODUCTION

In principle, many nutritional experiments with humans and animals are similar. A number of subjects are given one or more diets, the assignment of diets to subjects is controlled, and specified variables in the subjects are measured. The object of the experiment is often simply to compare the effects of various diets.

If the value of a variable is only dependent on the diet, then all the subjects receiving the same diet will have the same value for the variable, and an observed difference in values between two different diets can safely be assumed to be caused by the difference in diets. In reality, however, the value of a variable may not depend on the diet alone, but also on other factors. For example, subjects receiving the same diet may show some variation in values of a variable by reason of variations between subjects and between periods of consumption. Even if these sources of variation did not exist, the values of a variable are not necessarily identical because of laboratory variations and biological variations within subjects. Thus, it would seem that it is almost inevitable that a certain amount of variation may be present in the values of a variable from subjects receiving the same diet. Since these sources of variation are outside the experimenter's control, this variation is often referred to as uncontrolled variation. When such uncontrolled variation is negligible or minor in comparison with the effects of the diets, the observed difference in values obtained on two different diets may still be assumed to be caused by the difference in diets. However, in nutritional experiments the amount of uncontrolled variation may be comparable to the effects of the diets and may have consequences for the experimental design to be used.

Sources of uncontrolled variation can lead to the introduction of systematic errors in the comparison of the effects produced by different diets. This comparison is often referred to as the diet comparison. The situation may arise, for example, in which subjects are given two different diets during separate periods (case 1) or in which two diets are given simultaneously to two groups of subjects (case 2). The diet difference, that is the difference in the effects produced by the two diets, can be estimated by comparing the mean value obtained on each diet. However, comparison of the diets becomes complex because of the possible differences between the two periods in case 1, and in case 2, because of the possible differences between groups of subjects. In other words, factors such as time and between-subject variation may affect the diet comparison.

A further consequence of uncontrolled variation concerns the precision with which a diet difference can be estimated. As mentioned above, subjects receiving the same diets may show some variation in the values for a variable. Thus any estimation of diet difference is also subject to variation and hence, to some degree of uncertainty. The probable magnitude of the variation in the estimate of a particular diet difference can be determined from the data by calculating the standard error for the estimate of the particular diet difference (see Snedecor & Cochran: Ch 2.11 and Ch 4). The size of the standard error is dependent partly on the number of subjects receiving each diet, and partly on the amount of uncontrolled variation affecting the estimate of the diet difference. This uncontrolled variation is the variation in results of all subjects after possible diet effects have been eliminated. When the standard error is calculated, the estimate of the diet difference can be tested for statistical significance (see Snedecor & Cochran: Ch 4). The attained level of significance depends on the size of the standard error and decreases at smaller values of the standard error. The level of significance measures the probability of observing a diet difference equal in magnitude to the estimated diet difference when the true diet difference[1] is zero (or in the opposite direction). If the estimated difference is significant at say the 1% level, then there is little doubt that the true difference is not zero. If, however, the estimated difference is not significant at a low level of significance (in other words, if the estimated difference is consistent with a true zero difference), then it is still possible that a true diet difference of physiological importance exists. However, as a result of a large variation in the estimate of a diet difference (that is, large standard error), even estimated differences of the size of the true diet difference may be considered to be consistent with a true zero difference. It is clear that if the true difference is sufficiently different from zero to be of physiological importance, then the estimated difference must have a good chance of being statistically significant. This means that the standard error of the estimate should be sufficiently small, and thus the diet difference can be estimated with a reasonable degree of precision (for a detailed discussion, see Cox: Ch 8).

[1] An estimate of a diet difference is subject to variation. If the experiment could be repeated many times, then the mean estimate of the diet difference could be considered to be the true value of the diet difference.

It should be clearly recognized that in the foregoing discussion the tests of significance are the basis for statements in respect of the population of experiments which could be carried out under exactly the same conditions, and of which the data are a sample. The purpose of the statistical analysis is to reveal what the results of the experiment can tell about the true diet difference. In such problems the sample is concrete, but the population may appear to be hypothetical. Therefore, the precision of an experiment may give little indication of practicality of reproducing the results. Of course, it is possible to repeat an experiment with similar or the same subjects and diets, but it has to be carried out during another time period. The researcher should consider carefully whether it is reasonable to assume that a repeated experiment can be regarded as belonging to the same population of experiments.

A quite different situation arises when the population is concrete and definite, e.g. all inhabitants of the town of Wageningen, and the problem posed is to obtain specific information about it (for methods for selecting a sample and for estimating population characteristics from the data obtained in the sample, see Snedecor & Cochran: Ch 17).

The following sections show how, in the planning of an experiment, selection of an appropriate design can eliminate the effects of between-subject variation, time trends, and also uncontrolled diets prior to the experiment, firstly, from the diet comparisons in order to avoid systematic errors; and secondly, from the uncontrolled variation in order to increase precision (for the effect of the number of subjects on the precision with which a diet difference can be estimated, see Cochran & Cox: Ch 2.2, Cox: Ch 8, and Snedecor & Cochran: Ch 4.13).

It is not the intention to give complete descriptions of all possible designs and their methods of analysis, but to provide background information on some of the advantages and disadvantages of designs often used in nutritional experiments.

## 2. BASIC ASSUMPTIONS UNDERLYING THE EXPERIMENTAL DESIGNS DESCRIBED

In the designs discussed, the value of a variable obtained when a specified diet is given to a particular subject at a specified time in the experiment is considered as:

(a quantity depending on the particular subject) + (a quantity depending on the specified diet) + (a quantity depending on the specified time)

The following basic assumptions have been made.

a) There is an individual intrinsic level of the variable for each subject on a specified diet under stable conditions. Between-subject variation refers to deviations in the individual intrinsic levels from the average level of subjects receiving the same diet under the same conditions.

b) Each time period has a specific effect on the variable, which is independent of the individual and the diet. This time effect, which includes all environmental influences acting equally upon the level of the variable, is additive to the individual intrinsic level. Between-period variation refers to the deviations of the effects of the various periods from the mean effect of all the periods in the experiment.

c) If a period of sufficient length has been chosen, then each diet has a specific effect on the variable, which is independent of the individual and the time. The diet effect is additive to the individual intrinsic level. Between-diet variation refers to the deviations of the effects of the various diets from the mean effect of all the diets used in the experiment.

d) The difference between the observed and the expected value of the variable, when a specified diet is given to a particular subject at a specified time, is referred to as the residual term. This term comprises all further unexplained variations within a subject on a constant diet under stable conditions. Within-subject variation refers to the dispersion of the individual residual terms, and includes both laboratory and biological within-subject variation.

Any apparent differences in time effects from individual to individual and/or diet to diet, or any apparent differences in diet effects from individual to individual and/or time to time, will be incorporated into the residual term.

e) The deviations of the individual intrinsic level, the time term and the diet term from their respective means, and the residual term are mutually independent and have normal distributions with zero means and finite variances.

According to the model described, the variation in results obtained in an experiment comparing the effects of several diets, will depend on within--subject variation and may depend on variations between subjects, diets and periods. If an experiment is planned to compare the effects of diets, the researcher will be interested in the amount of uncontrolled variation in results of subjects receiving the same diets. This amount of variation depends on within-subject variation and may also extend to that occurring between subjects and between periods. It is clear that the within-subject variation is difficult to eliminate from an experiment, and designs should be sought in which the effects of the other sources of variation on the diet comparisons are absent or balanced out, and in which such variation can be eliminated from the uncontrolled variation. The resulting residual amount of uncontrolled variation is measured by the residual standard deviation.

In the model described, it is assumed that the value of the variable for one individual in one period is independent of the diet given to the same individual in preceding periods. Sometimes it may be preferable to accept the overlap of diet effects and to deal with it in the design of the experiment and in the analysis of the data. This is possible, provided that it is reasonable to introduce a simple modification to the model. In the modified model, when a specified diet is given to a particular subject at a specified time, the value of the variable is considered as:

$$\begin{pmatrix}\text{a quantity}\\ \text{depending on}\\ \text{the subject}\end{pmatrix} + \begin{pmatrix}\text{a quantity}\\ \text{depending on}\\ \text{the diet}\end{pmatrix} + \begin{pmatrix}\text{a quantity}\\ \text{depending on}\\ \text{the time}\end{pmatrix} + \begin{pmatrix}\text{a quantity}\\ \text{depending on the}\\ \text{preceding diets}\end{pmatrix}$$

When such carry-over effects are assumed, experimental designs and the methods of analysing the results may become complicated (see Cox: Ch 13, and Cochran & Cox: Ch 4.6). Some difficulties which may arise in these situations are discussed in the following section.

## 3. DESIGNS FOR EXPERIMENTS COMPARING THE EFFECTS OF DIFFERENT DIETS

### 3.1 Designs for experiments in which each subject receives one only of the test diets and all subjects receive their diets simultaneously

In this type of design, each subject is given one only of the test diets and which all subjects receive precisely at the same time. It is clear that possible time trends have no effect on the experiment. Simultaneous designs have several advantages. Any number of diets may be used and the total duration of the experiment is, in principle, limited to one test period only. Within the simultaneous designs completely randomised designs and randomised blocks can be distinguished.

#### 3.1.1 Completely randomised designs

In these designs the diets are allotted to the subjects completely by chance. An important advantage of such designs is that statistical analysis is relatively simple, even when the results from some subjects are missing (for an lysis of variance of one-way classifications, see Snedecor & Cochran: Ch 4 + Ch 10 In dietary trials it is not uncommon that results on some subjects are lost, or do not become available (*e.g.* because of cessation of participation), or have to be discarded (*e.g.* because of insufficient adherence to the diets). Completely randomised designs can be modified in several ways, depending on whether the value of the variable is measured only at the end of the test period (Figure 1, Model 1), or at both the beginning and end of the test period (Figure 1, Model 2), or, whether in addition to both measurements the test period is preceded by a control period during which all the subjects receive the same control diet (Figure 1, Model 3).

Model 1: The results of each diet are averaged and the difference between means is used as an estimate of the true difference between the diets. Since the diets are allotted to the subjects by chance, an attempt is made to balance out the effect of the variation between subjects from the diet comparisons. For example, it is most unlikely that subjects with high intrinsic levels are concentrated in one group only. The principal objection to the design is that the whole of the between--subject variation falls into the uncontrolled variation, and thus into the standard error of the estimates of diet differences.

Model 2: In this design, the change in value of the variable over the test period is calculated for each subject. The differences between group means of the changes are used as estimates of diet differences. The important advantage of this design when compared to the preceding one

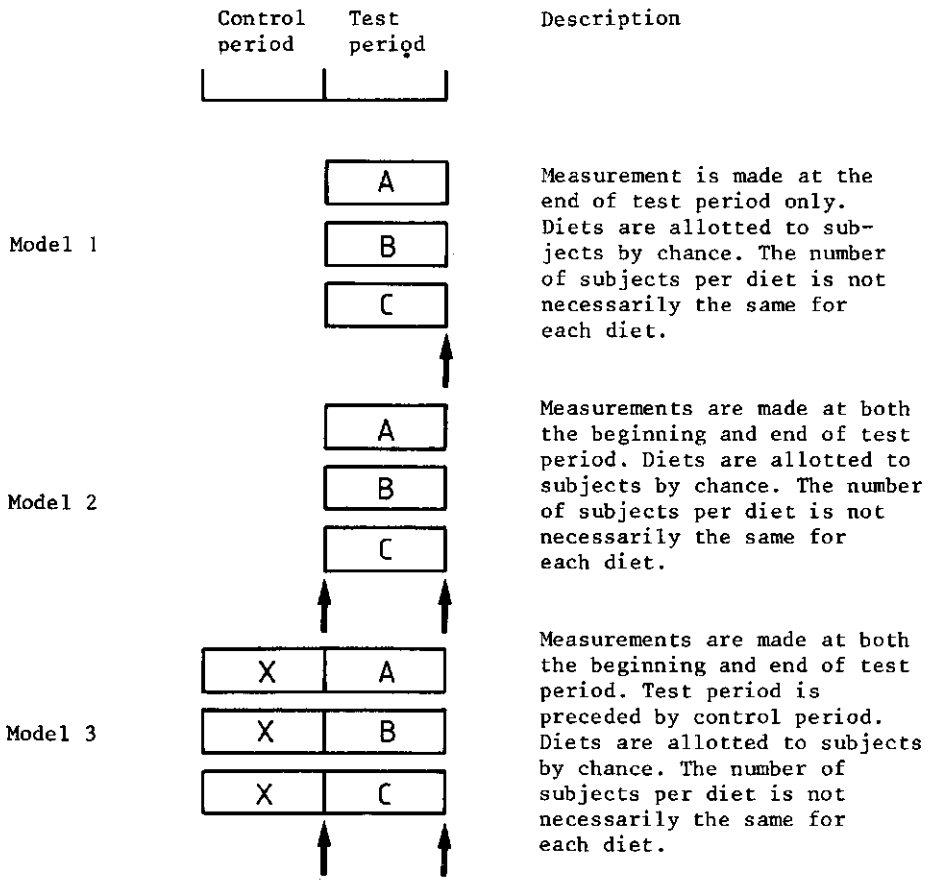



FIG. 1. Examples of completely randomised designs for comparison of three test diets: A, B, and C. The X diet is a control diet. Measuring points are indicated by arrows.

is that between-subject variation is eliminated from the uncontrolled variation. The residual amount of uncontrolled variation is estimated from the observed dispersion of the individual changes over the test period. A disadvantage may be that diets prior to the first measurement are uncontrolled, and accordingly, part of the variation in the results at the beginning of the experiment will be due to the fact that the habitual diets of the subjects differ. This variation will be included within the total uncontrolled variation in the individual changes during the test period. The subjects are allotted to the diets by chance, and therefore an attempt is made to balance out the possible effects of uncontrolled diets from the diet comparisons.

*Example*: Walker *et al.* (1960) compared the effects of a vegetable protein diet and an animal protein diet on the serum cholesterol level in 12 subjects. Six subjects were assigned at random to each diet. The cholesterol level was determined at various times including the commencement of the diet and after five weeks (completely randomised design, Model 2). Mean absolute levels at the commencement and after 5 weeks were (mean ± SD): 189 ± 28 and 161 ± 18 mg/dl for the vegetable protein diet, and 182 ± 43 and 142 ± 19 mg/dl for the animal protein diet. For each subject the change during the test period was calculated and analysis of variance revealed that the observed difference between the vegetable and animal protein diet of 12 mg/dl reached statistical significance ($P < 0.025$). If only final measurements had been made (completely randomised design, Model 1), then the diet difference would have been estimated as +19 mg/dl. In spite of the randomization, the mean initial levels of the groups differed by +7 mg/dl. This would have resulted in an overestimation of the diet difference had only final measurements been made (systematic error). Furthermore, the whole of the between-subject variation would have been included in the uncontrolled variation; the standard deviation obtained from analysis of variance would have been approximately 19 mg/dl, and the standard error of the estimate of the diet difference would have been approximately 11 mg/dl ($SE = SD\sqrt{(1/n_1 + 1/n_2)}$). Only a difference of approximately 29 mg/dl would have reached statistical significance at the 2½% significance level. It should be noted that the variation in

results at the beginning of the experiment is much larger than at the end. This may have been caused by uncontrolled diets prior to the first measurement.

Model 3: This design is similar to Model 2, except that the test period is preceded by a period of controlled diets. Possible effects of uncontrolled diets prior to the test period are thus eliminated from the experiment.

*Example*: Van Raaij *et al.* (1980), compared the effects of a casein, a soy protein and a mixed casein/soy protein diet on the serum cholesterol levels in 69 subjects. All subjects received a control diet (one of the test diets) for 10 days after which the subjects were divided into three groups each receiving one of the test diets. Cholesterol levels were determined at the beginning and at the end of the test period (Model 3). Mean values were (mean ± SD): 152 ± 27 and 149 ± 24 mg/dl for the casein group (n=25); 153 ± 24 and 150 ± 25 mg/dl for the mixed casein/soy group (n=20); and 153 ± 23 and 150 ± 23 mg/dl for the soy group (n=24). The change during the test period was calculated for each subject; mean changes were (mean ± SD) -3 ± 14, -3 ± 13, and -3 ± 10 mg/dl, respectively.

*Analysis of variance of a one-way classification (individual changes in level of serum cholesterol during test period)*

| Source of Variation | Degrees of Freedom | Sum of Squares | Mean Squares | F value | P value |
|---|---|---|---|---|---|
| Diets | t-1= 2 | 2 | 1 | 0.006 | Not significant |
| Residuals | $\Sigma n_i$-t= 66 | 10269 | 156 | | |
| Total | $\Sigma n_i$-1= 68 | 10271 | | | |

The pooled estimate of the residual standard deviation was only 12.5 mg/dl ($=\sqrt{156}$); the standard error of the estimate of the difference between the casein and soy diet has been calculated to be approximately 3.6 mg/dl; this means that in this experiment an observed difference of approximately 7.2 mg/dl would have reached statistical significance at the 5% significance level.

### 3.1.2 Randomised blocks

This type of design with simultaneous application of test diets may be useful when the value of the variable can be measured only at the end of the test period. It should be remembered that the main objection to Model 1 of the completely randomised designs, is that the whole of the between-subject variation falls into the uncontrolled variation. If however, the values of the variable are highly correlated with known characteristics of the subjects, then the subjects can be grouped into blocks on the basis of these characteristics. All the subjects in a block are expected to yield similar values for the variable if the diets are equivalent in effect. The order in which diets are assigned to subjects within each block is randomised (Figure 2). The differences between diet means are used as estimates for the true diet differences. The effect of variation between blocks is eliminated in the diet comparisons because diet comparisons are made within blocks of similar subjects. The variation between blocks can also be eliminated from the uncontrolled variation by an appropriate method of analysis (analysis of variance for two-way classification: diets and blocks, see Snedecor & Cochran: Ch 11). The success of this design depends on skillful grouping of the subjects into similar sets or blocks. When there is no obvious prior knowledge to enable an appropriate grouping, then the completely randomised design (Model 1) should be used.

It should be clearly recognized that between-subject variation may arise from many peculiarities of the individual subjects and that grouping into blocks according to one or more obvious features is unlikely to eliminate all of the between-subject variation. Furthermore, the analysis depends essentially on the balanced nature of the design. Although there are very sophisticated methods available for estimating missing values in this type of design, the requirement of a balanced pattern of results may be a disadvantage.

## 3.2 Designs for experiments in which the subjects receive different diets consecutively

In this type of designs each subject receives all the diets in sequence ( Figures 3 and 4). The design can be constructed so that all subjects receive the diets in the same order or in various orders. The value of the variable is measured at the end of each test period. The most important advantage of these type of designs is that any between-subject variation is automatically eliminated from the experiment because each subject is his own control.

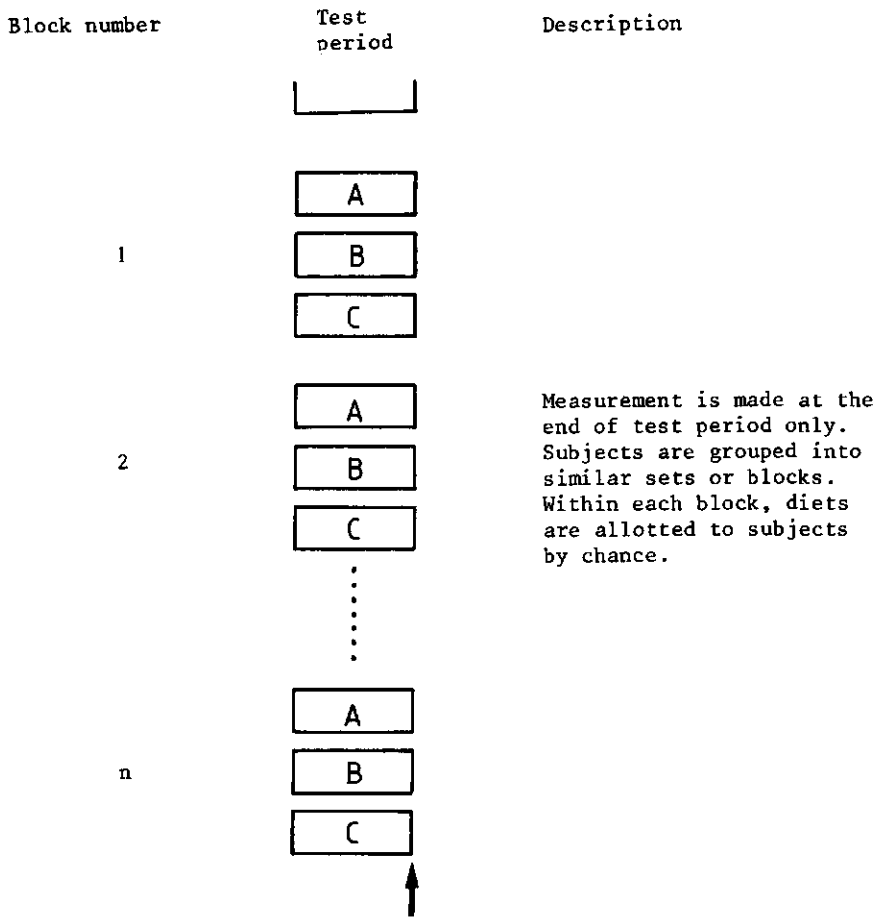



FIG. 2. An example of a design with randomised blocks for comparison of three test diets: A, B, and C. The measuring point is indicated by an arrow.

Less subjects will be needed than with the simultaneous designs because diets are compared within the same subject. A disadvantage may be the greater duration of the total experiment.

### 3.2.1 Single order of diets design

The simplest design for consecutive application of test diets is that in which all subjects receive the diets in the same order (Figures 3 and 4). The differences between diet means are used as estimates of diet differences. The results of the various diets are obtained during different periods. The effects of time trends cannot be separated from diet effects because there is no control group in this design.

> *Example*: Holmes *et al.* (1980) compared the effects of a beef diet, and a soy diet, on the serum cholesterol levels in 12 subjects. All subjects received the beef diet for three weeks followed by the soy diet for a period of four weeks (single order of diets design). Values were (mean ± SD): 241 ± 38 and 235 ± 43 mg/dl for the beef and soy diet, respectively. The estimate of the diet difference of 6 mg/dl did not reach statistical significance. No control group was used in the experiment and time trends were not eliminated from the diet comparison.

### 3.2.2 Single Latin square design (Figure 4)

The disadvantage of the preceding design is that time effects cannot be eliminated from the experiment. It is reasonable to expect some time trends between periods, and it is desirable to balance out not only variation between subjects but also variation arising from time trends.

When both between-subject variation and variation arising from time trends have to be balanced out, then the Latin square principle is indicated.
In general, a txt Latin square is an arrangement of t letters so that each letter occurs once in each row and once in each column. The rows represent subjects, and the columns periods. The diets are randomised over the rows and columns according to a certain procedure (Cochran & Cox: Ch 4.33).

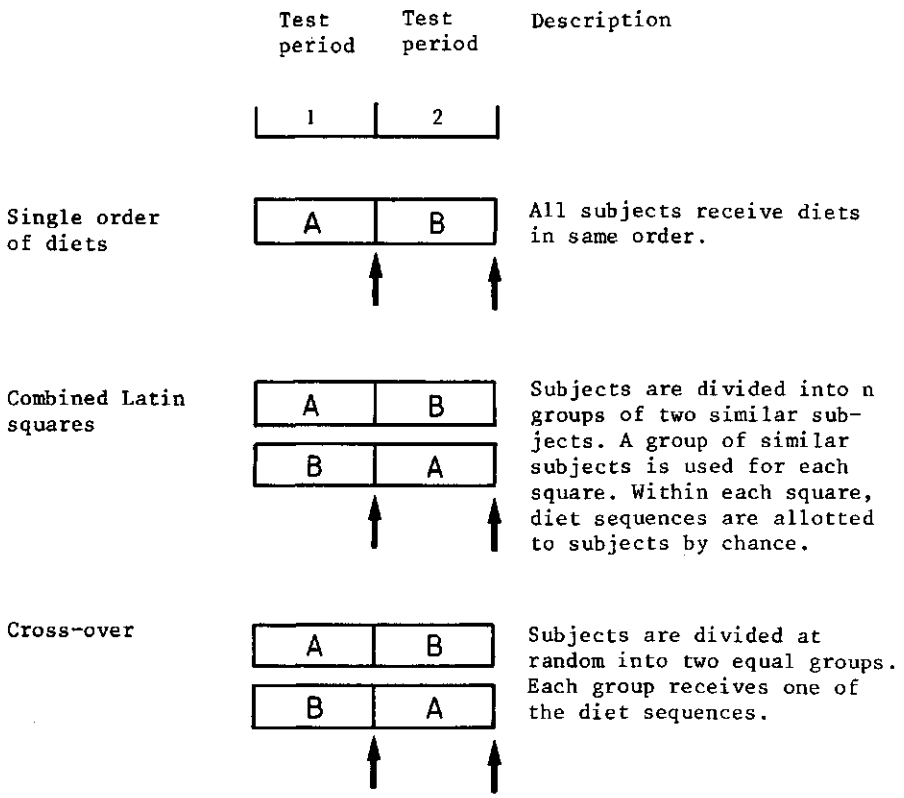



FIG. 3. Examples of consecutive designs for comparison of two test diets: A and B. Measuring points are at the end of each test period and are indicated by arrows.

Diet differences are estimated by comparing the diet means. It is clear that these estimates are free from systematic effects of between-subject variation and that effects of time trends are balanced out from diet comparisons. These sources of variation can also be eliminated from the uncontrolled variation, and the residual standard deviation is usually obtained from analysis of variance (for a three-way classification; see Snedecor & Cochran: Ch 11).

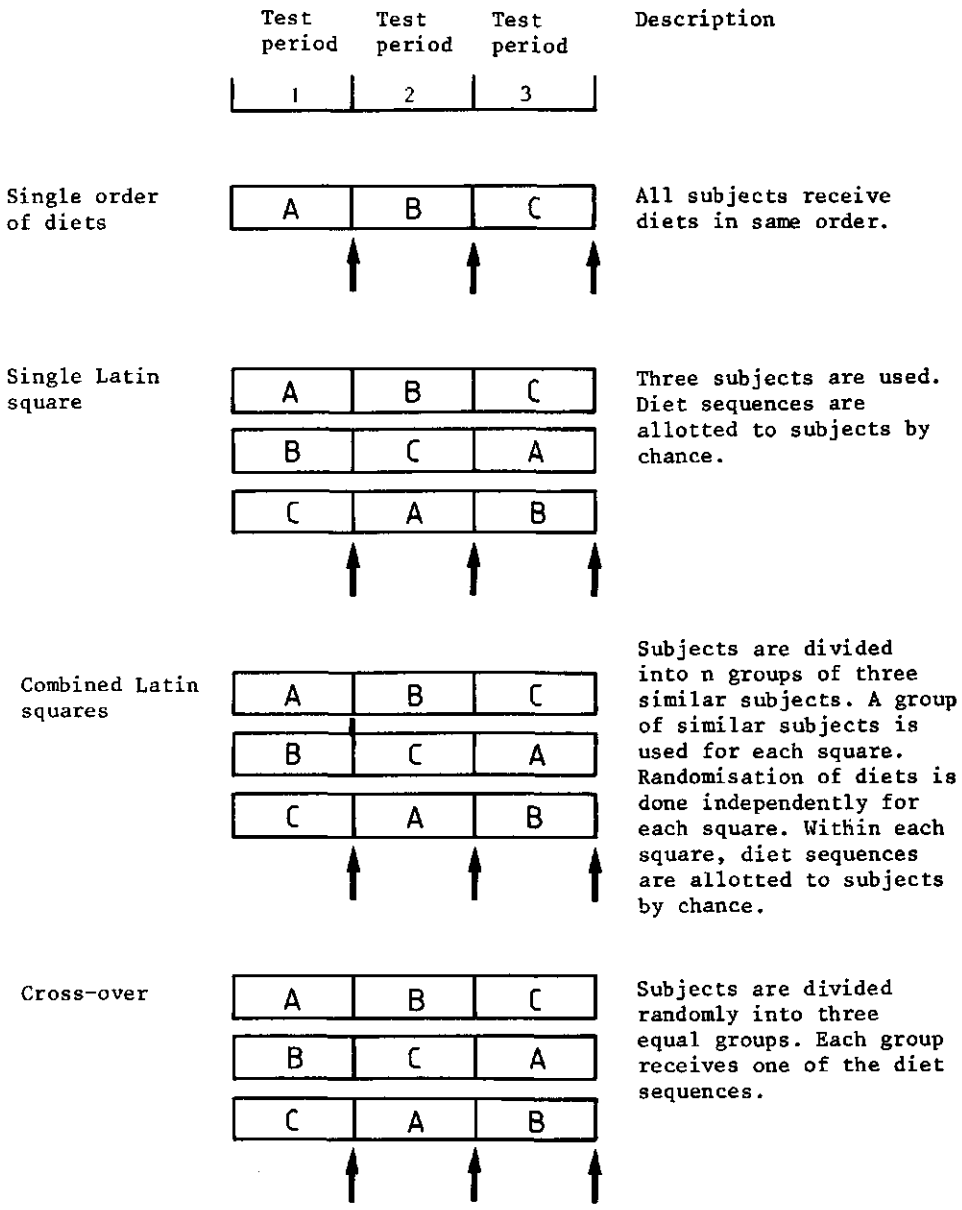



FIG. 4. Examples of consecutive designs for comparison of three test diets: A, B and C. Measuring points are at the end of each test period and are indicated by arrows.

*Example*: Snedecor and Cochran (Ch 11.8) cite an experiment in which the effect of three diets on the milk yield in cows was investigated. Variation in milk yield in cows is normally large and when successive diets are given to the same cows, a change in milk production over time must be taken into account. A Latin square design was used to eliminate both types of variation in this experiment.

*Experimental design*

| | Period | | |
|---|---|---|---|
| | I | II | III |
| Cow 1 | A: 608 | B: 715 | C: 844 |
| Cow 2 | B: 715 | C: 1,087 | A: 766 |
| Cow 3 | C: 940 | A: 766 | B: 832 |

The rows represent cows, and the columns represent the successive periods during lactation. The three animals in the square were chosen as they were most likely to have similar lactation curves and to be at corresponding points on the curve at the commencement of the experiment. In other words, time effects (milk trend effects) would most likely be similar for each animal. The variable was the milk yield in pounds for a 6-week period.

*Analysis of variance of a 3-way classification (milk yield in pounds for a 6-week period)*

| Source of Variation | Degrees of Freedom | Sum of Squares | Mean Squares | F value | P value |
|---|---|---|---|---|---|
| Periods | t-1=2 | 5,900 | 2,950 | | |
| Cows | t-1-2 | 47,214 | 23,607 | | |
| Diets | t-1=2 | 103,436 | 51,718 | 21.4 | $P < 0.05$ |
| Residuals | (t-1)(t-2)=2 | 4,843 | 2,422 | | |
| Total | txt-1=8 | 161,386 | | | |

The mean results were 695, 811 and 957 for the diets A, B and C, respectively. Analysis of variance revealed a significant diet effect. The residual standard deviation (SD) is estimated to be 49 ($=\sqrt{2,422}$)

and is based on 2 degrees of freedom (df). The standard error of the difference between two means, $\sqrt{2SD^2/t}$, is 40. The 5% value of Student's t with 2 df is 4.303. Thus, only the difference between Diet A and C reached statistical significance.

The small squares provide only a few degrees of freedom for the estimation of the residual variance ( df = (t-1)(t-2)). This fact precludes the use of single 2x2 squares, while single 3x3 or 4x4 squares are seldom used for this reason. However, more than one square may be included in the same experiment (combined Latin squares and cross-over designs). Although methods for calculating missing values and modified test procedures are available, the requirement of a balanced pattern of results may be a disadvantage in human trials.

### 3.2.3 Combined Latin squares design

The experiments described using the single Latin square can be extended with additional subjects (but always by a multiple of t) by adding more squares. In the combined Latin squares design each txt square is randomised independently and a group of similar subjects is allocated to each square. The reason for this is that balancing of columns in the Latin square removes the effect of a common time trend. If the time trends are appreciably different for the subjects within the same square, the residual variance is inflated.

*Example*: The experiment cited by Snedecor and Cochran (Ch 11.8) could have been extended by adding more squares to the design. A 3x3 Latin square could have been used for each group of similar cows. It is immaterial if the time effects (common trends in milk yield) are different in the various squares, but it is important that as far as possible any trends that exist are identical for all animals in the same square. Diet differences can be estimated by comparing the diet means. An estimate of the residual variance can be made from the analysis of variance for a four-way classification namely, diets, subjects, periods and squares, and based on (t-1)(rt-r-1) degrees of freedom, where r is the number of squares.

The combined Latin squares design can only be used when the extent of time trends for a given subject can be estimated in advance.

### 3.2.4 Cross-over design

This design is the simplest extention of the Latin square principle. The number of subjects, which must be a multiple of the number of diets (t), are divided at random into t equal groups, each receiving one of the sequences of the diets. The results on each diet are averaged, and diet differences are estimated by comparing diet means. The estimate of the residual variance is usually obtained from analysis of variance.

*Example*: Carroll *et al.* (1978) compared the effects of a mixed protein diet with a plant protein diet on the serum cholesterol levels in 10 healthy women. A cross-over design was used, involving 2 groups of 5 subjects each. The cholesterol level was determined at the end of each period (Figure 3, cross-over design). The results for the first group of subjects were 175 and 161 mg/dl on the mixed protein and plant protein diet respectively, and 161 and 166 mg/dl for the second group on the plant protein and mixed protein diet, respectively. The difference between the plant protein and mixed protein diet was estimated as (161+161-175-166)/2=-9.5 mg/dl. Analysis of variance revealed a significant diet effect.

*Analysis of variance for a three-way classification (levels of serum cholesterol)*

| Source of Variation | Degrees of Freedom | Sum of Squares | Mean Squares | F ratio | P value |
|---|---|---|---|---|---|
| Periods | t-1=1 | 92 | 92 | 1.8 | Not sign. |
| Subjects | rt-1=9 | 13,644 | 1,752 | 34.2 | $<0.01$ |
| Diets | t-1=1 | 433 | 433 | 8.4 | $<0.05$ |
| Residuals | (t-1)(rt-2)=8 | 410 | 51 | | |
| Total | rtt-1=19 | 14,579 | | | |

The residual standard deviation obtained from analysis of variance was 7.1 mg/dl ($\sqrt{51}$) based on 8 df.. The standard error of the estimate of the diet difference is 3.2 mg/dl (SE = $SD\sqrt{2/n}$, where n is the number of subjects on each diet, and the 5% value of Student's t with 8 df is 2.306. Thus, an observed difference of about 7.4 reaches statistical significance.

3.3 Carry-over effects in designs of experiments for consecutive application of diets

In the preceding designs it is assumed that each test diet has no effect in periods subsequent to that in which it is given. Occasionally however, the absence of carry-over effects cannot be assumed, and a diet effect is characterised by a direct effect in the period in which it is given, and by a residual effect in the following period. In this case, a design in which diets follow each other the same number of times should be used (for methods of obtaining suitable arrangements, see Cox Ch 13.3 and Cochran & Cox: Ch 4.6). When, for example, two diets are compared in a cross-over design, this condition is already met (Figure 3); each diet follows the other once only. However, when three diets are to be compared in a cross-over design, the design shown in Figure 4 is not appropriate. It is necessary then, to consider pairs of squares simultaneously (Figure 5, Example 1).

In Example 1 (Figure 5) each diet follows the other except itself the same number of times. However, the values for variables obtained on a specified diet are influenced by the residual effects of all diets except the values obtained in the first period. Assuming that the specified diet has a large positive residual effect and the other diets do not, then the mean value on this diet is suppressed in relation to the other diets. It is possible to overcome this difficulty by introducing a preliminary period in which the same diet is given as in the first period, but in which no measurements are made. Thus each diet is preceded by all the test diets the same number of times (Figure 5, Example 2), and the design is balanced out for carry-over effects (for methods for adjusting diet means for residual effects, see Cox: 13.3). The effects of carry-over can also be eliminated from the uncontrolled variation by using an appropriate method of analysis. An example is given by Cochran and Cox (Ch 4.62a).

Another method of eliminating carry-over effects is to separate each experimental period by a period in which a control or standard diet is given. These additional periods should be of sufficient duration for any effect of the earlier diets to be dissipated. The disadvantage is that the total duration of the experiment is substantially increased.

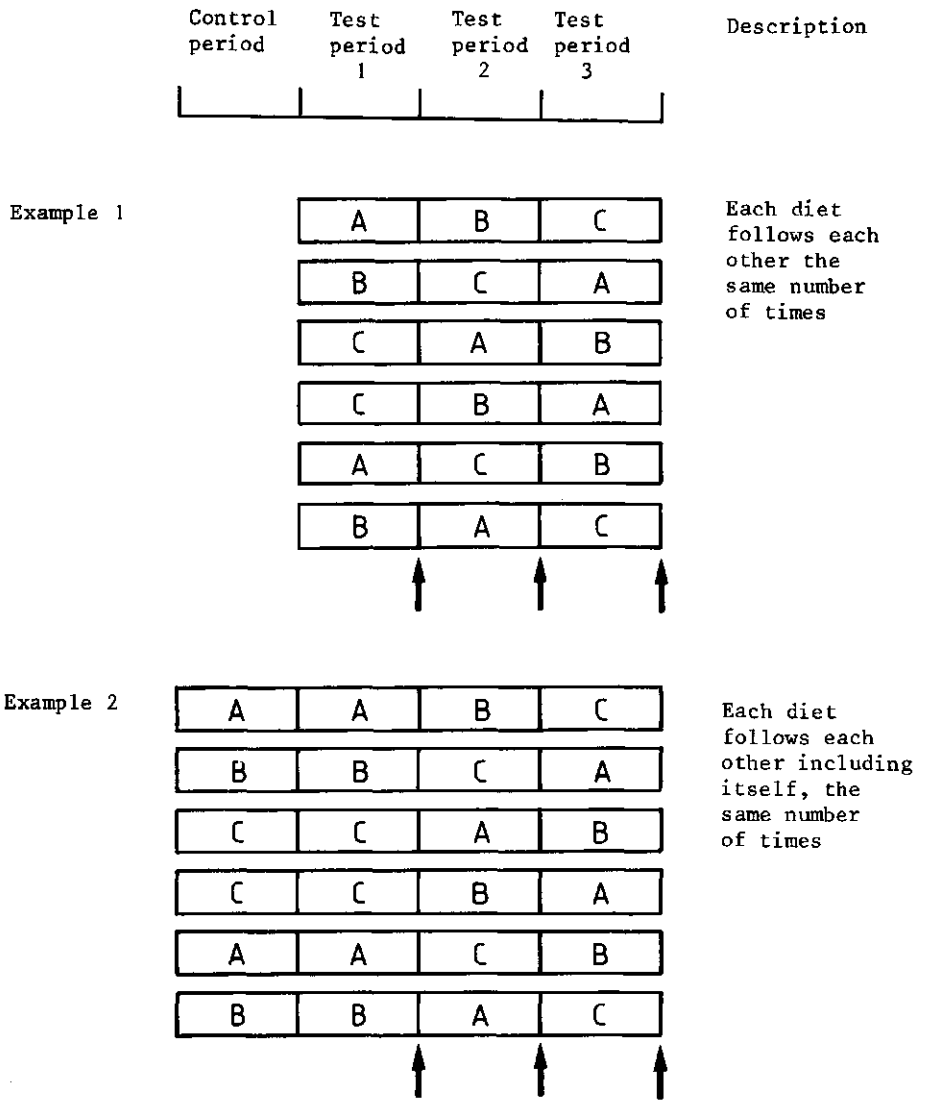



FIG. 5. Examples of consecutive designs for comparison of three test diets: A, B, and C, when carry-over effects cannot be assumed to be absent. Measuring points are indicated by arrows.

## 4. COMPARISON OF THE DESIGNS

In general, most of the uncontrolled variation in the results of a variable in human nutritional studies arises from the peculiarities of individual subjects, that is the between-subject variation. Thus designs should be sought in which this source of variation can be eliminated as far as possible from the experiment. Therefore, designs such as Model 1 of the completely randomised designs are not particularly suitable. When only one measurement can be made on each subject, efforts can be made to eliminate part of the variation between subjects by using a design with randomised blocks. In all other designs discussed, between-subject variation is eliminated by using each subject as its own control but it is necessary to make more than one measurement on each subject.

Since the effects of uncontrolled diets prior to the experiment are eliminated in Model 3 of the completely randomised design when compared with Model 2, the first is the better. However, the total duration of the experiment is longer. The advantage of the simultaneous designs is that the total duration of the experiment is limited to only one test period (Model 2) or to one control plus one test period (Model 3). Many diets can be compared simultaneously and analysis of the results is simple, even when data on subjects are missing. The disadvantage, however, is that a relatively large number of subjects is needed because each subject receives one only test diet.

The consecutive design, in which all subjects receive the diets in the same order (single order of diets design), is not appropriate because time effects cannot be eliminated from the experiment since no control group is involved. Possible effects of habituation on the experimental condition (experimental stress) also belong to time effects. The effects of time trends are balanced out in the other consecutive designs and can be eliminated by means of an appropriate method of analysis. It should be noted firstly that the single Latin square design is appropriate only when a relatively large number of diets are to be compared (in general, more than four). Secondly, the combined Latin squares design is useful only when time trends are dissimilar for each subject, and when the size of the time trends can be predicted for each subject. The advantage of the consecutive designs is that fewer subjects are needed than in simultaneous designs because diet comparisons can be made within subjects. On the other hand, the duration of the experiment may be substantially increased. Furthermore, analysis of the results depends on their balanced pattern, and the design and method of analysis may become complicated when carry-over effects are present.

In summary, the decision as to the final design to be used in an experiment will depend on several factors such as: whether one only or several measurements are to be made on each subject; the availability of subjects; the number of diets to be compared; the possible duration of the whole experiment; whether some values are likely to be missing or not; and whether carry-over effects may be expected. In practice, the decision will also depend on the availability of equipment and staff and on financial resources.

## REFERENCES

Carroll KK, Giovanetti PM, Huff MW, Moase O, Roberts DCK, Wolfe BM. Am J Clin Nutr 1978; 31: 1312-21.

Cochran WG, Cox GM. Experimental designs. 2nd ed. New York: John Wiley & Sons, Inc., 1957.

Cox DR. Planning of experiments. New York: John Wiley & Sons, Inc., 1958.

Holmes WL, Rubel GB, Hood SS. Atherosclerosis 1980; 36: 379-87.

Raaij JMA van, Katan MB, Hautvast JGAJ, Hermus RJJ. Am J Clin Nutr 1981; 34: 1261-71.

Snedecor GW, Cochran WG. Statistical methods. 6th ed. Ames: Iowa State University Press, 1967.

Walker GR, Morse EH, Overley VA. J Nutr 1960; 72: 317-21.

## ACKNOWLEDGEMENTS

This paper relies heavily on the work of Dr R.N. Lussenburg and Dr J. Zaalberg (Unilever Research Laboratory, P.O.Box 114, 3130 AC Vlaardingen, The Netherlands). The author is grateful to them for allowing him to incorporate their ideas in this thesis.

# APPENDIX II

# CARRYING OUT A NUTRITIONAL EXPERIMENT: THE TRIAL WITH MIDDLE-AGED VOLUNTEERS

CONTENTS

In this appendix the planning, preparation and conduct of nutritional trials with human subjects are discussed with special reference to the trial with middle-aged volunteers (Chapter 3). Funding, decisions concerning the experimental protocol, and ethical aspects of carrying out such a study are discussed. An outline is given of the practical work undertaken in the subsequent phases of the experiment, the staff involved and the total cost of the study. Burden and inconvenience to the participants are also discussed.

## 1. FUNDING

The experiment with middle-aged subjects was a part of a larger project which included all the experiments described in this thesis. The protocol for the whole project which was to be of three years duration, contained the broad outline of the planned experiments together with probable total cost and an outline of the scientific and social significance. On the basis of this proposal the Agricultural University provided funds for a Ph.D. student (the author) and allocated space and other facilities. The experiments were financed by a grant from the Netherlands Institute for Dairy Research (NIZO).

## 2. PROTOCOL

In drawing up a protocol for a nutritional experiment with human subjects, a decision has first to be made on the following: the diets to be compared; the types of observations to be made; the required duration of the test periods; and the type of subjects to be used. These issues can be regarded as technical questions peculiar to the subject matter of the experiment. The decisions made for the present study were based on the general information available and on the results of the previous study with young volunteers (Chapter 2). The final choice of the type of subjects to be used, that is healthy middle-aged volunteers, was also influenced by the fact that it was known that a reasonable number of normocholesterolemic subjects were readily available.

An experimental design was chosen in which each subject received one only of the test diets which were to be given simultaneously to all the subjects. The test period was preceded by a control period in which all the subjects received the same control diet. In this type of design more subjects are required than in designs in which each subject receives all test diets consecutively (see Appendix I). However, the design has several advantages: the total duration of the experiment is restricted to one only control and one only test

period, possible carry-over effects of the control diet are balanced out, and the analysis of the results is simple, even when data on subjects are missing (see Appendix I). In regard to one of the important variables under investigation, the concentration of cholesterol in serum, the required number of subjects on each diet was determined on the basis that an appropriate power of the significance test used could be obtained (see Chapter 2).

Other decisions concerned the method of introducing the planned dietary modifications, and the food supply. In order to make the test diets acceptable to the subjects over several months, and that deviation from usual food habits be minimal, it was decided to use common foods wherever possible. Products resembling usual foods were developed in order to introduce the protein preparations under investigation into the diets. It was decided to provide the subjects with almost complete daily diets, so that protein effects would not be masked by effects of uncontrolled dietary components.

All the above issues, together with a detailed description of the conduct of the experiment, the method of analysing the results, the staff and equipment required, and total cost were included in the protocol. The final protocol was produced by the author and two staff members of the Department of Human Nutrition. Helpful advice was also given by experts outside the Department.

## 3. ETHICAL ASPECTS

Certain ethical problems arise in nutritional trials with humans.

*(i) Risks for the subjects*

It is clear that subjects should only participate in a nutritional trial when there are no foreseeable risks. The possible consequences of eating the diets, possible temporary physiological alterations, and the procedures used such as the taking of blood samples, should be considered fully.

*(ii) Informed consent*

The consent of the subjects may be assumed in studies carried out with volunteers, however the ethical value of consent depends on the information provided. The subjects should be given adequate information on: the aims of the study; relevance of the study; the design and methods used; the risks and inconveniences to the subjects; and the right to cease participation at the time desired. Such information should be provided both in written and oral form; it must be correct, complete and comprehensible and should not contain

misleading euphemisms.

*(iii) Choice of subjects*

Are the subjects, for example, recruited from vulnerable groups?

*(iv) Aims, design and methods of the study*

It is unethical to carry out a study of which it is clear that relevant results cannot be obtained, either because of the aims of the study or the design (methods, statistical model).

The scientific and technical acceptability of the present study was evaluated by a number of staff members of the Department of Human Nutrition. The risks to the subjects, the informed consent and the choice of the subjects were evaluated by an external ethical committee which consisted of 2 nutritionists, 2 medical practitioners and a sociologist. The committee approved the proposed protocol.

## 4. CARRYING OUT THE EXPERIMENT

### 4.1. Preparatory period (6 months)

#### 4.1.1. Development of special food products

In the experiment, protein preparations (casein, soy protein isolate, and soy protein concentrate) were to be added to the diets as products resembling usual foods. As such products were not available commercially and as the expertise and equipment to develop these products were not available in the Department, several firms and institutions were approached to develop and prepare these products for the experiment (Chapters 2 and 3). Although stringent sensory demands were not made, the products had to be acceptable for continuous consumption over several months. Therefore, they were tested by taste-panels. They were also chemically analysed in the Department, and their compositions added to a computerised food composition table. It took between 4 to 6 months to develop the products to a satisfactory standard for the experiment.

The products included brown bread, gluten-free bread, and foods similar to milk and yoghurt. Half of the protein in the brown bread consisted of test protein; the protein in the gluten-free bread and in the milk beverages consisted of test protein alone. The milk and yoghurt products were incorporated into soups, sauces, desserts and sandwich spreads. Cheese was used in the casein diet because practically the only protein it contains is casein. A gelated

product was prepared from soy isolate as a counterpart for cheese in the isolate diet, and a commercial textured product was used in the concentrate diet. Butterfat was added to both soy diets to balance the fat in cheese. The gelated and textured soy products were added to sauces and sandwich spreads (see also Chapter 3).

### 4.1.2. Recruitment of subjects

As the success of human studies is largely dependent on the subjects, the selection and motivation of subjects is of the utmost importance. About three months prior to the experiment advertisements were placed in local newspapers. Posters were also placed on bulletin boards in many of the Departments of the Agricultural University, and in institutions and public buildings in and near Wageningen (Figure 1). Prospective participants were sent an extensive information brochure (Figure 2) about the experiment, including the aim and relevance of the study, the protocol and methods to be used, the inconveniences participants might expect, and finally what would be done with the results. It was made clear that the participants were free to cease participating at any time they desired. In the preparation of this information care was taken in regard to the comprehensibility of the contents and to ensure that misleading statements were not included. Those who retained interest in participating after reading the brochure were invited to attend a meeting at which further information was given. Several of these meetings were held at which the information contained in the brochure was discussed, slides of previous experiments were shown, and questions answered. A volunteer was accepted for the experiment only if he/she had attended one of these meetings. By this procedure it was hoped to screen out those who were weakly motivated. Such experiments are too costly to spend time with participants who might leave the study in mid-course because they are bored or disillusioned. In addition, insufficient adherence to the diets invalidates the results.

Although the participants were not offered payment, the diets were free of charge. Since 1974, several controlled nutritional trials have been carried out by the Department, mainly with students from the Agricultural University. The present study was the first study to be carried out with middle-aged volunteers (30 to 60 years). The main motivation for participation in the student experiments was the financial advantage of free meals and the conveniences such as no shopping and no preparation of hot meals on weekdays. These factors played a minor role in the motivation of middle-aged volunteers, although some partici-

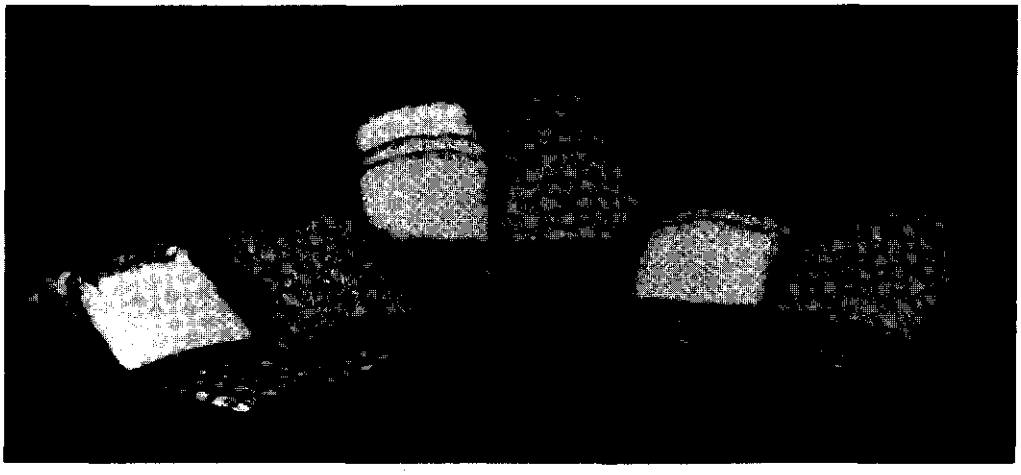


Photo 1. Specially developed brown and gluten-free bread. Half of the protein in the brown bread consisted of protein from caseinate (left), or soy isolate (centre), or soy concentrate (right). The protein in the gluten-free bread consisted of test protein alone.

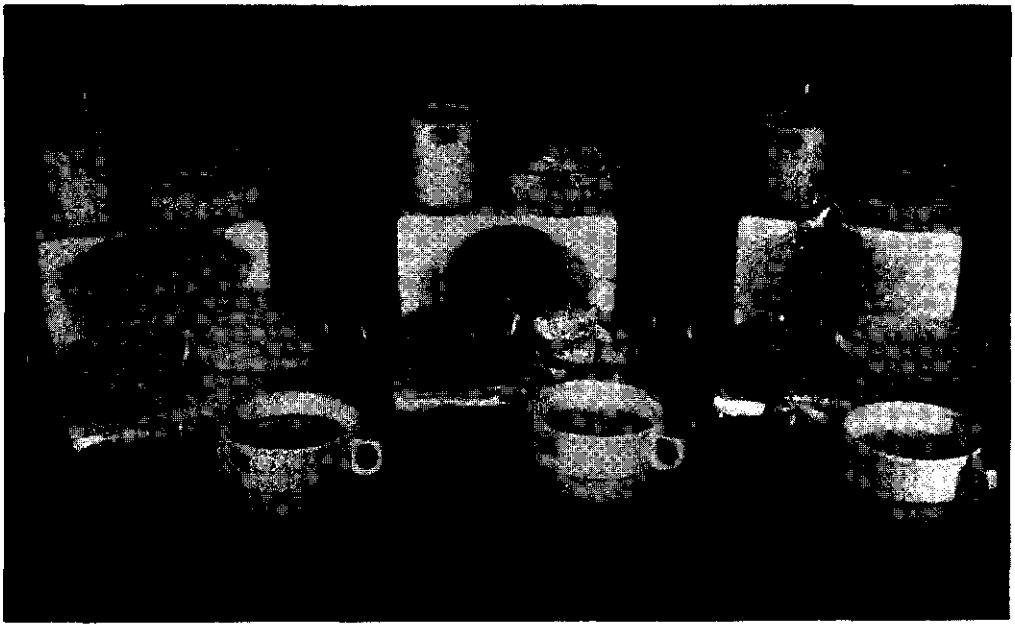


Photo 2. Specially developed analogues of milk and yoghurt. The protein in these products consisted of test protein alone. The analogues of milk and yoghurt were incorporated into sandwich spreads, desserts, sauces and soups. Cheese was used in the casein diet. A gelated soy isolate product and a commercial textured soy concentrate product were used in the isolate and concentrate diet, respectively. These soy products were added to sauces and sandwich spreads.

pants stressed the conveniences. While not systematically investigated, it appeared on inquiry that the main motives in the present study were quite diverse, varying from mere curiosity to desire to learn.


FIG. 1. Poster for recruitment of subjects.


FIG. 2. Front cover of the information brochure. The emblem PROTEINORIX was used throughout the experiment as the logo of the experiment.

Some of the reasons given for participation were: to become more conscious of my own diet; to feel what it means to have a strictly controlled diet, as is the case in some diseases; to learn something about my own body; and to have social contacts. Dedication of one's body to Science was mentioned by almost everyone but only as a second motive.

The subjects were recruited within 6 weeks of placing the advertisements. After a preliminary investigation, 61 subjects were finally accepted for the actual experiment including: 13 married couples, 16 who were married but participated without their spouse, and 19 were unmarried.

4.1.3. Preparatory work for food supply

Apart from 1.0 MJ (240 kcal) per day, all of the food eaten by the subjects was supplied daily in quantities according to individual energy requirement. In order to estimate individual energy intake, the subjects were asked to note and weigh all the foods and drinks consumed during three consecutive days prior to the experiment (see 4.1.4. Preliminary investigation). The subjects were classified into 25 energy groups on the basis of their habitual energy intake, beginning with 4.7 MJ per day, and increasing by intervals of 0.6 MJ. For each energy group the number of grams of each macro-nutrient was calculated to ensure that the overall composition of the diets in terms of percentages of energy was similar for all energy groups, and similar to the planned composition of the diets. The absolute quantities of macronutrients were subsequently transposed into grams of common foods and the specially developed products (see Table 1). The whole procedure necessitated many discussions concerning the feasibility of the planned diet compositions and required much inventiveness on the part of the dieticians. Each day the subjects were required to select 1.0 MJ from a list of foods containing practically no protein and, of which, the fat content and fatty acid composition had been carefully evaluated. These foods are referred to as 'free foods'. This choice was, in fact, the only freedom the subjects were allowed in the composition of their total diet. A point system was devised to rate these foods. With the aid of this system, the participants could easily calculate how much of each food could be eaten in order to achieve 1.0 MJ per day. A show case with some of the free foods was present, and the subjects were free to take out desired foods. Information was prepared on stencils about the diets to be provided, and a booklet on the preparation of hot meals during the weekend was also prepared. (The ingredients were provided for hot meals during the weekend; see 4.2.2. Preparation and supply of daily diets). Guidelines for compiling dietary records and menus were also set out. A stringent coding system was developed for the collection of blood and diet samples.

4.1.4. Preliminary investigation

About 6 to 8 weeks prior to the actual experiment, all subjects underwent a preliminary investigation. They visited the Department of Human Nutrition twice. On the first occasion, a blood sample was taken after an overnight fast, blood pressure was measured and anthropometric measurements were carried out. All subjects were asked to complete at home a brief general (p.128), and a medical questionnaire (p.126-127). They were required to note and weight all the foods and

Table 1

EXAMPLES OF DAILY FOOD SUPPLY FOR SUBJECTS ON THE CASEIN AND SOY PROTEIN DIETS[a]

| | | Casein diet | | Soy protein diets | |
|---|---|---|---|---|---|
| Energy group | | 6 | 10 | 6 | 10 |
| Energy intake (MJ per day) | | 7.7 | 10.1 | 7.7 | 10.1 |
| | Food item | | | | |
| Breakfast and lunch | Brown bread[b] (slices)[f] | 6 | 8 | 6 | 8 |
| | Gluten-free bread[b] (slices)[f] | 2 | 2 | 2 | 2 |
| | Margarine (g) | 20 | 30 | 25 | 40 |
| | Sandwich spread[b] (g) | 50 | 75 | 50 | 75 |
| | Meat (slices)[e,f] | 1 | 2 | 1 | 2 |
| | Sugar (bag)[c] | 2 | 4 | 2 | 4 |
| Dinner | Soup[b] (g) | 175 | 200 | 200 | 200 |
| | Potatoes (g) | 75 | 150 | 75 | 150 |
| | Other vegetables (g) | 150 | 175 | 150 | 175 |
| | Soy sauce[b] (g) | - | - | 125 | 150 |
| | (or Casein sauce[b] (g) | 100 | 125 | - | - |
| | plus cheese (slice))[f] | 2 | 2 | - | - |
| | Egg yolk (g) | 15 | 20 | 15 | 20 |
| | Salad (g) | 30 | 30 | 30 | 30 |
| | Dessert[b] (g) | 100 | 175 | 100 | 175 |
| Snacks | Fruit[d] | | | | |
| | Juice[c] | | | | |

a) Food provided for subjects. In addition to these items, subjects were allowed to choose 1.0 MJ/day of food from a list of low-protein products. Subjects were allowed to consume unlimited quantities of tea, coffee, selected low-calorie beverages, and up to 6 g of coffee whitener per day.

b) This is a special product containing test protein.

c) Sugar was provided as sugar, apple syrup, honey, apple juice or grapefruit juice, as determined by individual preference. Sugar was supplied in bags containing 6 g.

d) The amount of fruit varied with the type of fruit provided, and differed from one energy group to another.

e) For vegetarians, meat was replaced by egg protein and butter.

f) Slices were carefully standardised.

drinks consumed during a three-day period (a small dietary weighing scale was provided for each subject). On the second visit, the participants produced a morning urine specimen and the food records were discussed with a dietician. The blood and urine samples were analysed immediately, and the habitual energy intake of each subject was estimated from the food records. At the completion of the preliminary investigation, each subject received a summary of his/her own measurements together with an explanatory comment. On the basis of the measurements taken (blood Hb, erythrocyte sedimentation rate, blood pressure, no detectable glucose or protein in urine) and the medical questionnaire, a medical practitioner determined whether there were any reasons to prevent any of the subjects participating in this study. Two subjects were requested to withdraw from further participation because of food allergies. A further 4 subjects decided to withdraw for their own reasons. The preliminary investigation was completed about two weeks prior to the experiment. Sixty-one subjects began the actual experiment.

#### 4.1.5. Space

The ground plan of the Department of Human Nutrition given in Figure 3 indicates the amount of space that was available for the experiment. Not all the rooms indicated were occupied continuously throughout the experiment, but they were essential for satisfactory execution of the experiment. Large cooling and freezing rooms for storage of food and the two extra portions of the diets which were set aside for use in the animal experiments (4.2.3), were available in a building adjacent to the Department.

### 4.2. The experiment (2 months)

#### 4.2.1. Food purchases

Most foods were purchased and delivered in bulk every two weeks, with the exception of fresh foods which were purchased from and delivered by the same shopkeepers throughout the experiment. The protein preparations were delivered in bulk, and the required amount of each protein preparation for the whole experiment was derived from the same process batch. Special breads and milk and yoghurt products were prepared and delivered weekly, while the gelated product from soy isolate was prepared and delivered on two occasions during the experiment. One dietician was responsible for all food orders. All financial matters were controlled by the Administrative Officer of the Department.

FIG. 3. Ground plan of the Department of Human Nutrition, indicating the rooms available for the experiment (scale 1 : 400). At the time of the experiment the Department was still housed in the Transitorium Building.

A. Seminar room
- meetings (4.1.2.)
- social events (4.2.8.)

B. Laboratory
- blood sampling (4.1.4., 4.2.4.)

C. Office
- storage packaging materials (4.2.2.)
- collection of double portions of test diets (4.2.3.)

D. Office
- co-ordination centre
- information centre
- preparing food packages (4.2.2.)
- collection of food records (4.2.6.)

E. Dining room
- hot meals and coffee (4.2.2.)
- breakfast (4.2.4.)

F. Kitchen
- preparation of hot meals (4.2.2.)
- supply of hot meals (4.2.2.)

G. Cloackroom
- weight measurement (4.2.5.)
- anthropometric measurements (4.1.4.)
- show case for free foods (4.1.3.)

H. Laboratory
- blood pressure measurement (4.1.4., 4.2.4.)

I. Wall rack for food packages
- prepared food packages (4.2.2.)

J. Information board

K. Notice board for dinner cards (4.2.2.)

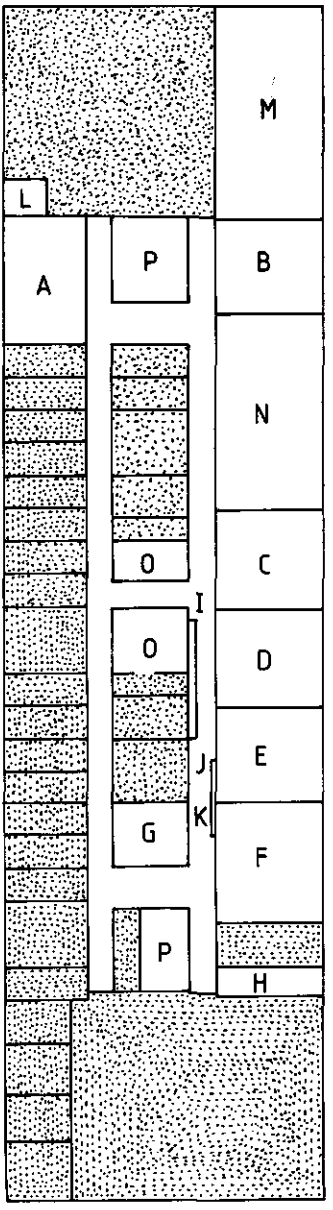
L. Terminal connected to the computer (4.4.)

M. Laboratory for blood analysis (4.3.)

N. Laboratory for food analysis (4.3.)

O. Toilets

P. Entrance/Exit/Stairs



4.2.2. Preparation and supply of daily diets

On weekdays, the hot meals were prepared at the Department by three dieticians, and served in the evening. At the entrance to the kitchen a card for each subject showing the required amounts of soup, potatoes or rice, legumes and dessert was mounted on a notice board. On arrival for dinner, the subject took his/her card, and proceeded along an improvised railed enclosure to where 3 dieticians provided the hot meals. Thus all ingredients of the hot meal were weighed out for each person in quantities appropriate to his or her energy needs.

All other foods were also weighed out for each subject and provided daily as packages. In the weekends, the participants were required to prepare the hot meals themselves and food for the weekend, including ingredients for the hot meals, was provided each Friday. The food was provided in plastic boxes of 30x20x15 cm. Perishable foods were placed in a small styrofoam box with a cooling element. In general, all the food supplied as packages could be placed in one box, except on Fridays, when a second box or an extra bag was required. All foods were packed separately. Various types of packaging materials were used. The prepared food packages were placed in a wall rack in which each subject had a separate compartment. The food packages were taken out by the subjects after dinner.

During the experiment, when a participant considered that he was receiving too much or too little food, or when a subject showed a change in body weight of more than 2 kg during a 2-week period, the subject was placed in another energy group. The estimated habitual intake of energy did not appear to be satisfactory as most subjects changed energy group during the first few weeks of the experiment. However, without a preliminary estimation of the individual energy intakes, changes would have occurred much more frequently, and much practical work is involved in placing individuals in another energy group. Only a few subjects were transferred to another energy group because of large changes in body weight.

4.2.3. Collection of two extra portions of the test diets

Throughout the experiment, two extra portions of each test diet were collected daily and frozen (hot meals and food packages). After completion of the experiment, the food was homogenised, freeze-dried, and analysed,

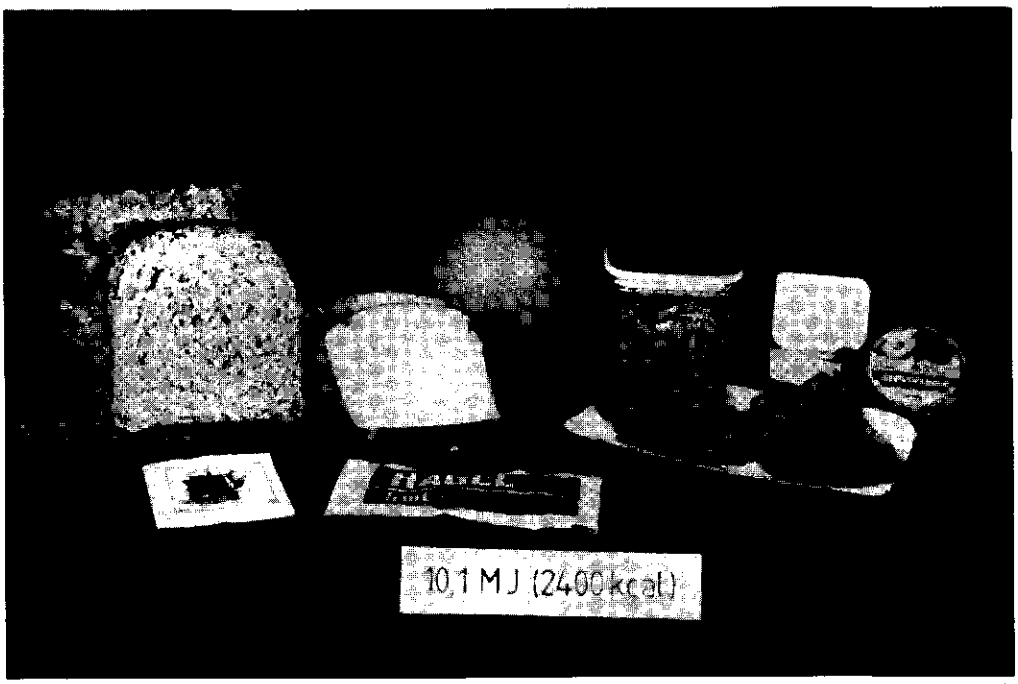



Photo 3. Food daily provided as food package to a participant consuming 10.1 MJ (2400 kcal) per day.

Photo 4. Complete daily diet for a participant consuming 10.1 MJ (2400 kcal) per day.

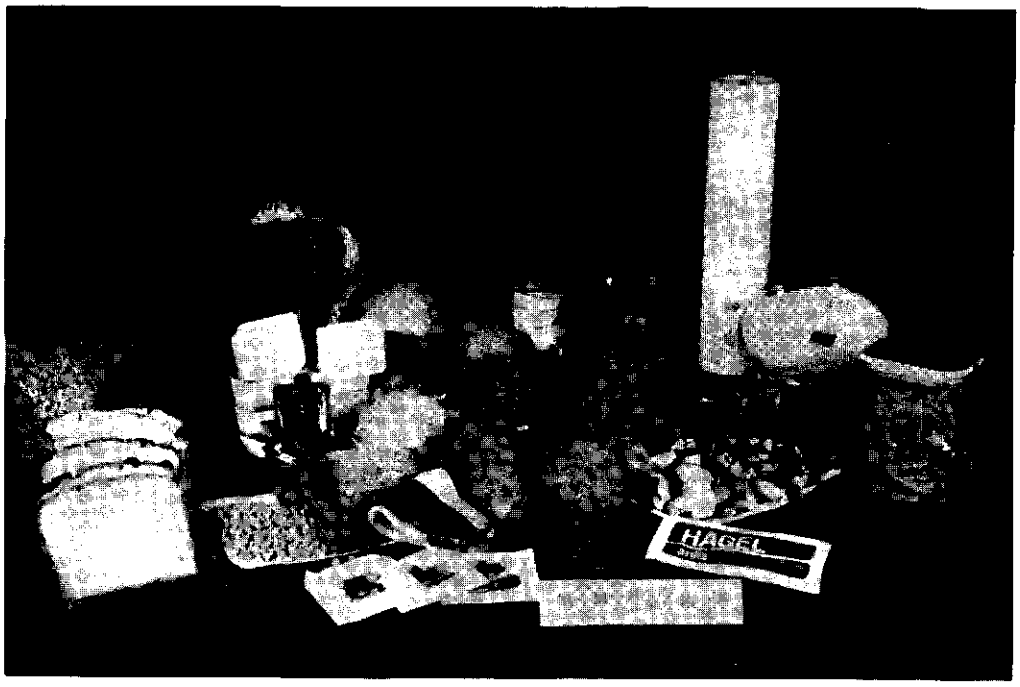



Photo 5. Food for the weekend including ingredients for the hot meals for a participant consuming 10.1 MJ (2400 kcal) per day.

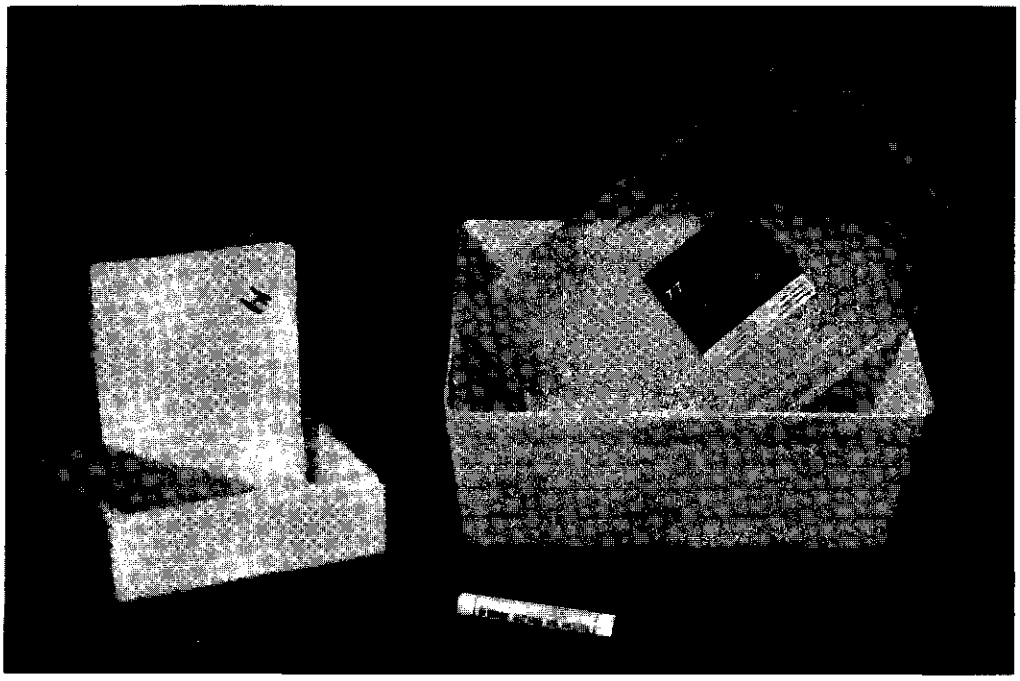


Photo 6. The food was provided in plastic boxes. Perishable foods were placed in a small styrofoam box with a cooling element.

and used in the animal experiments described in Chapter 4.

### 4.2.4. Morning visits

The participants visited the Department on 6 occasions between the hours of 07.00-10.00 a.m. at times nominated by the subjects themselves on a list posted on the notice board. They brought with them morning urine specimens, and blood samples were taken following an overnight fast. Breakfast was provided in the Department. There were no defaulters.

### 4.2.5. Body weight

Each Tuesday immediately prior to dinner, body weight was measured without shoes and heavy clothes.

### 4.2.6. Food records

One day a week chosen at random, each participant weighed and recorded his or her food intake. A dietary weighing scale was provided. The following day the records were discussed with one of the research team.

### 4.2.7. Diaries

At the beginning of the experiment the participants received a diary in which they noted each day: type and amount of free food chosen; whether there had been any non-adherence to the diet; any illness; or intake of medicine. Entries were required as to the days on which blood was collected and days on which the subject was expected to complete a 24 hr food record. Examples of pages of the diary are given on page 129. From the diaries the exact intakes of energy and nutrients from the free foods were calculated. It was decided to eliminate the results of 2 subjects from the analysis on the basis of the information supplied regarding illness and the intake of medicine.

### 4.2.8. Conduct of the subjects

As previously mentioned, the success of a human study depends largely on the interest and conscientious co-operation of the subjects. The motivation of the subjects throughout the experiment is a source of permanent concern for the research team. It is important that the subjects feel that they are continuously well informed and that they are convinced that the research team will do everything possible to present information, is willing to answer

questions and solve problems. One member of the research team at least was freed of other duties to talk with participants during dinner. The author was involved in the experiment as a participant, and was always present in the dining room at that time. In addition, information was posted on the notice board, and there was also a weekly news-sheet (Figure 4). Relationships between subjects and the subjects and the research team are extremely important in the maintenance of morale. Development of rapport, companionship, and loyalty among subjects is vital in the maintenance of determination to carry the study to successful completion. In this respect the day-to-day contacts during the evening meal are very important. It is also essential that members of the research team are able to relate to and sympathise with subjects. It was very much appreciated by the participants that one member was involved in the experiment as a participant. Since most of them were interested in nutrition topics, several evening meetings were organised and guest speakers, selected by the participants, were invited to attend.


WEEKKRANT
voor de
VOEDINGSPROEF
WEEK:

INHOUD:

1. VAN DE REDACTIE
2. WISSELING VAN VOEDINGEN (vervolg)
3. TOELICHTING BIJ KOOKBOEKJE
4. MENU VOOR KOMEND WEEKEND
5. ERGENS WE ONS GEWICHT CONSTANT KUNNEN HOUDEN? (vervolg)
6. KRUISWOORD- PUZZEL
7. MEDEDELINGEN

1. VAN DE REDACTIE

FIG. 4. Front page of the weekly news-sheet

## 4.3. Collection of data (4 months)

The results of blood analyses became available within four months following completion of the experiment. The two extra portions of the test diets were homogenised, freeze-dried and analysed, and used in the animal experiments.

The actual nutrient intake for each individual throughout the experiment was calculated from the food records using a computerised food composition table supplemented with the analyses of the special products. When all the data were available, each participant received his or her own values, together with an explanatory comment. A complete description of the results of the study has been promised to the subjects.

Throughout the experiment erythrocyte sedimentation rate was slightly but persistently elevated (20 to 30 mm/h) in 5 subjects, while another 6 subjects showed a slightly elevated blood pressure (diastolic between 95 and 105 mmHg or systolic between 150 and 160 mmHg). All these subjects were advised to contact their own medical practitioner.

### 4.4. Analysis and interpretation of the data (6 months)

The results were analysed with the DEC-1090 computer system of the Agricultural University using programmes derived from SPSS (Nie NH, et al. Statistical Packages for Social Sciences. 2nd ed. New York: McGraw-Hill Inc., 1975). All the data obtained were stored on computer tape.

## 5. STAFF INVOLVED AND TOTAL COST OF THE STUDY

### 5.1. Staff

In total, the experiment and the analysis of the results took about 18 months. During the last 6 months only one nutritionist (author) was involved full-time in the analysis of the results and writing up the data for publication. During the first 12 months when all the practical work was in hand many people were involved. It is difficult to give a complete list because some people were external to the Department (*e.g.* for developing and preparing the special food products). The following, entirely from within the Department, were involved in the practical work for this particular experiment:

| | |
|---|---|
| 1 nutritionist, | full-time for 12 months (author) |
| 5 dieticians, | 2 full-time for 12 months and 3 part-time (30 hr/week) for 2 months during experiment |
| 8 technicians, | 8 technicians including 3 medical analysts were involved for shorter or longer duration; they undertook the blood sampling and blood analyses (12 months full-time for one technician) and the homogenising, freeze-drying and analyses of the food products and double portions (6 months full-time for one technician). Their scientific guidance was not the responsibility of the nutritionist but of 2 staff members of the Department. |
| 2 doctoral students, | full-time for 6 months, commencing one month prior to experiment; apart from their involvement in all the practical work throughout the experiment, the students |

analysed and interpreted part of the results obtained;
1 medical practitioner, full-time for 2 days during the preliminary investigation, and on standby throughout experiment.

Apart from the staff involved in practical activities, a number of people from within and without the Department acted as advisors both in the planning and execution of the study.

## 5.2. Cost expendable materials

The Department has at its disposal a well-equipped clinical and chemical--analytical laboratory, and all analytical techniques used were already available. At the time of the experiment a research kitchen, and an improvised dining room where about 40 subjects could have dinner simultaneously, were made available. (In the Department's new building, there is a very well-equipped research kitchen complete with dining room, where 60-70 subjects may have dinner at the same time). Furthermore, all the equipment for anthropometric measurements and blood pressure measurements, and dietary weighing scales, were present. Although some new equipment had been purchased prior to the experiment the costs given below are of the expendable materials used in this particular study.

Costs of expendable materials (1980)

| | | |
|---|---|---|
| Food, including special products* | : | Dfl. 41,000 |
| Laboratory materials (test tubes, chemicals, test kits, needles, etc.) | : | Dfl. 8,500 |
| Packaging for food | : | Dfl. 1,500 |
| Computer costs | : | Dfl. 3,000 |
| Postage and stationery | : | Dfl. 1,000 |
| Travelling expenses research team | : | Dfl. 1,000 |
| Miscellaneous | : | Dfl. 2,500 |
| Total | : | Dfl. 58,500 |

*) It should be noted that the cost of the specially developed bread was Dfl. 19,000 and that the other special products were either gratuitous or were delivered at the cost of the ingredients used.

### 5.3. *Financial resources*

The main costs of the study were the salaries of the staff involved. The nutritionist, some of the technicians and the medical practitioner were paid by the Agricultural University. The dieticians were paid by a grant from the Netherlands Institute for Dairy Research (NIZO), while analytical work was partly financed by grants from the Netherlands Heart Foundation. Most of the expendable items in the study were also paid by NIZO.

## 6. BURDEN AND INCONVENIENCE TO THE SUBJECTS

The activities required of the participants are summarised in Table 2. No account is made of travelling time. Furthermore, it is assumed that the daily preparation of the bread meals and that of the hot meals in the weekend, did not take more time than usual. The activities and the inconveniences had been discussed extensively in the information brochures and during the meetings with the participants. Therefore,it is not surprising that almost the total number of the subjects completed the experiment successfully; only 2 out of 61 subjects decided upon early withdrawal. In general, routine activities such as the daily visits to the Department, compilation of diaries or food records, carrying food packages, were not considered onerous. For a few people who lived more than 20 km from the Department,and a few women who also prepared hot meals at home for husband and children, the last weeks of the experiment became burdensome.

A few problems did arise. The participants had to eat all the food supplied, and apart from a relatively small amount of free foods nothing extra was allowed. Many participants found the diets, which were in general well accepted, had become monotonous after a few weeks despite the efforts of the dieticians to vary them as much as possible. Many participants had underrated the disruption of their social activities. The subjects who participated without their partner found it disadvantageous to be separated from the other family members at mealtimes. The married couples and the single people regretted that they could not have dinner with friends for such a long time. Therefore, each participant was allowed to invite a guest several times during the experiment. It is better to recommend that people should participate in an experiment as a group, couple or with friends. Other restrictions on social life are in relation to problems arising when visiting friends;

the participants had to take their own foods with them and had to refuse any food offered. In this respect, it should be clearly recognized that when relatives or friends become irritated by the demands of the experiment, pressures to relax their endeavours will undoubtedly be experienced by the subjects.

Table 2
SUMMARY OF ACTIVITIES REQUIRED OF THE PARTICIPANTS

| Phase of the study and place of activities | Activity | Duration |
|---|---|---|
| Preliminary investigation<br>Department of Human Nutrition | | |
| | FIRST VISIT:<br>blood sample, blood pressure, anthropometric measurements | 1 to 1½ hr |
| | SECOND VISIT:<br>morning urine specimen, discussion of food records | ½ hr |
| Home | | |
| | medical and general questionnaires | ½ hr |
| | 3-day food record | 1 to 1½ hr/day |
| Actual experiment<br>Department of Human Nutrition | | |
| | WEEKDAY EVENINGS:<br>eat dinner*, collect food packages ; discuss food records once a week;<br>body weight measurements once a week | ½ to 1½ hr/day |
| | 6 MORNING VISITS:<br>blood sample;<br>morning urine specimen and measurement of blood pressure on two occassions | ½ hr per visit |
| Home | | |
| | 24 hr food record once a week<br>daily dairy | ½ to 1 hr/record<br>10 minutes/day |

*) Each day the subjects were expected to consume the total amount of food provided plus the amount of 'free foods'.

Wageningen
Vakgroep Humane Voeding

**vertrouwelijk**

# MEDISCHE VRAGENLIJST

Uitsluitend bestemd voor een geschiktheidsverklaring voor deelneming aan voedingsproeven Landbouwhogeschool

Deze vragenlijst wordt vanwege haar vertrouwelijk karakter uitsluitend aan een arts ter inzage gegeven.

---

Naam en voorletters: .....
Man ☐ Vrouw ☐ Geboortedatum: .....

Adres (straat en nr): .....
Postcode: ..... Woonplaats: .....
Telefoonnr: (thuis): .....(werk): .....

---

Wie is Uw huisarts? Naam: .....
Adres: .....
Woonplaats: .....

---

1. Rookt U? Ja ☐ Neen ☐
   Zo ja, wat en hoeveel? .....

2. Gebruikt U alcoholische dranken? Ja ☐ Neen ☐
   Zo ja, welke? en hoeveel glazen per dag? .....
   .....

3. Gebruikt U een medisch voorgeschreven dieet? Ja ☐ Neen ☐
   Zo ja, welk en waarvoor? .....

4. Kunt U bepaalde spijzen of dranken niet verdragen?
   Ja ☐ Neen ☐
   Zo ja, welke? .....



5. Is Uw gewicht de laatste maanden:
   -ongeveer constant gebleven ☐
   -sterk toegenomen (meer dan 5 kg) ☐
   -sterk afgenomen (meer dan 5 kg) ☐

6. Hebt U een normale eetlust? Ja ☐ Neen ☐

7. Lijdt of leed U aan een van hierna genoemde zoekten en/of klachten?
   Vetzucht? Ja ☐ Neen ☐
   Bloedarmoede? Ja ☐ Neen ☐
   Suikerziekte? Ja ☐ Neen ☐
   Overgevoeligheid (allergie) voor bepaalde stoffen?
   Ja ☐ Neen ☐
   Zo ja, waarvoor? .....
   Hepatitis (geelzucht)? Ja ☐ Neen ☐
   Hoge bloeddruk? Ja ☐ Neen ☐
   Hartaandoening? Ja ☐ Neen ☐
   Nieraandoeningen? Ja ☐ Neen ☐

   Hebt U herhaaldelijk maagklachten? Ja ☐ Neen ☐
   Zo ja, welke? .....
   Hebt U chronische darmstoornissen? Ja ☐ Neen ☐
   Zo ja, van welke aard? .....

8. Lijdt of leed U aan niet hierboven genoemde ziekten of klachten? Ja ☐ Neen ☐
   Zo ja, welke? .....

9. Bent U op het moment onder doktersbehandeling? Ja ☐ Neen ☐
   Zo ja, voor welke klachten? .....
   .....

10. Gebruikt U medicijnen? Ja ☐ Neen ☐
    Zo ja, welke? en waarvoor? .....
    .....

11. Hebt U in verband met Uw mogelijke deelneming aan de voedingsproef naar Uw mening nog bijzonderheden te vermelden? Ja ☐ Neen ☐

Zo ja, welke? .....
.....
.....

Alleen voor vrouwen:

12. Bent U thans zwanger? Ja ☐ Neen ☐

13. Gebruikt U orale anti-conceptiva (de 'pil')?
Ja ☐ Neen ☐
Zo ja, welk preparaat gebruikt U? .....

Datum: ..... Handtekening: .....

Wageningen
Vakgroep Humane Voeding



# ALGEMENE VRAGENLIJST

Uitsluitend ten behoeve van het voor-onderzoek voor de voedingsproef van najaar 1980

---

Naam en voorletters: .....
Man ☐ Vrouw ☐ Geboortedatum: .....

Adres (straat en nr): .....
Postcode: ..... Woonplaats: .....
Telefoonnr: (thuis): ..... (werk): .....

---

Wie is Uw huisarts? Naam: .....
Adres: .....
Woonplaats: .....

---

1. Gebruikt U een dieet? Ja ☐ Neen ☐
   Zo ja, welk? .....
   Betreft het een medisch voorgeschreven dieet? Ja ☐ Neen ☐

2. Eet U vegetarisch? Ja ☐ Neen ☐
   Zo ja, hebt U bezwaar tegen het gebruik van 1-3 plakjes vleeswaar per dag tijdens de proef? Ja ☐ Neen ☐

3. Bent U allergisch voor bepaalde voedingsmiddelen?
   Ja ☐ Neen ☐
   Zo ja, voor welke? .....

4. Zijn er bepaalde voedingsmiddelen die U helemaal niet lust? Ja ☐ Neen ☐
   Zo ja, welke? .....

5. Hebt U nog andere bezwaren tegen het gebruik van bepaalde voedingsmiddelen? Ja ☐ Neen ☐
   Zo ja, welke? en waarom? .....
   .....

6. Gebruikt U melk in Uw koffie of thee? Ja ☐ Neen ☐
   Zo ja, is dit: gewone melk ☐
   koffiemelk ☐
   poeder (creamer) ☐
   Hoeveel theelepels in totaal per dag? .....

7. Gebruikt U suiker in koffie of thee? Ja ☐ Neen ☐
   Zo ja, hoeveel theelepels of klontjes in totaal per dag? .....

8. Is Uw lichaamsgewicht de laatste maanden:
   - ongeveer constant gebleven? ☐
   - sterk toegenomen? (meer dan 5kg) ☐
   - sterk afgenomen?(meer dan 5 kg) ☐

9. Hebt U in verband met Uw mogelijke deelneming aan de voedingsproef nog bijzonderheden te vermelden (van welke aard dan ook) die naar Uw mening van belang kunnen zijn voor de proef? Ja ☐ Neen ☐
   Zo ja, welke? .....
   .....
   .....

Datum: .....

10

**Deze week voedingsenquetes op:**

week 3 | MA | DI | WO | DO | VR | ZA | ZO |

**Bloedafname:** dinsdag morgen
(van 7.00-10.00 uur op afspraak)
donderdagmorgen
(van 7.00-10.00 uur op afspraak)

Bloeddruk : dinsdag op afspraak

Urine : eerste ochtendurine van dinsdagmorgen

| datum | niet-verstrekte voeding | afwijking van de verstrekte voeding |
|---|---|---|
| MA 13 | | |
| DI 14 | | |
| WO 15 | | |
| DO 16 | | |
| VR 17 | | |
| ZA 18 | | |
| ZO 19 | | |

15

| | MA 27 OKT | DI 28 OKT | WO 29 OKT | DO 30 OKT | VR 31 OKT | ZA 1 OKT | ZO 2 OKT |
|---|---|---|---|---|---|---|---|
| $Z_1$ klachten | | | | | | | |
| $Z_2$ arts | | | | | | | |
| $Z_3$ medicijnen | | | | | | | |
| $Z_4$ koorts | | | | | | | |
| Sport | | | | | | | |
| Roken | | | | | | | |
| M | | | | | | | |
| $P_a$ | | | | | | | |
| $P_b$ | | | | | | | |

Zie ook gebruiksaanwijzing (blz 3)

ZIEKTE

$Z_1$: Omschrijving klachten: griep, verkoudheid, hoofdpijn, maag/darmklachten, anders nl.:

$Z_2$: Doktershulp ingeroepen?

$Z_3$: Medicijnengebruik: wat en hoeveel?

$Z_4$: Koortsverloop: begin, einde, hoeveel?

SPORT
Intensieve sportbeoefening: wat en hoeveel?

ROKEN
Sterke verandering rookgewoonten.

ALLEEN VOOR VROUWEN

M : Begin menstruatie

$P_a$ : Stoppen pilgebruik voor 1 of meer periodes.

$P_b$ : Begin pilgebruik voor 1 of meer periodes.

# CURRICULUM VITAE

Joop van Raaij werd op 12 april 1951 in Culemborg geboren. In 1968 behaalde hij het diploma HBS-B aan de toenmalige Christelijke Hogere Burgerschool te Culemborg. In datzelfde jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen, en in juni 1976 behaalde hij het doctoraal-examen met Voedingsleer als verzwaard hoofdvak en Dierfysiologie en Wiskundige Statistiek als bijvakken.
Na het vervullen van zijn militaire dienstplicht werd hij in augustus 1978 aangesteld als wetenschappelijk assistent bij de Vakgroep Humane Voeding van de Landbouwhogeschool. Hier verrichtte hij gedurende drie jaar het in dit proefschrift beschreven onderzoek. Met ingang van 1 augustus 1981 trad hij als wetenschappelijk medewerker in dienst van de Vakgroep Humane Voeding.

Joop van Raaij was born on 12 April 1951 in Culemborg, The Netherlands. Between September 1968 and June 1976, he studied at the Agricultural University in Wageningen, The Netherlands. He graduated with a Master of Science degree in Human Nutrition.
From August 1978, after completing his military service, he worked as a research assistant in the Department of Human Nutrition of the Agricultural University. During this time he carried out the research presented in this thesis. In August 1981 he was appointed to the staff of the Department of Human Nutrition as a lecturer.