Experimental atherosclerosis in rabbits on diets with milk fat and different proteins

CENTRALE LANDBOUWCATALOGUS
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Dit proefschrift met stellingen van Rudolphus Josephus Johannes Hermus, landbouwkundig ingenieur, geboren te Klundert op 10 juni 1941, is goedgekeurd door de promotor, dr. C. den Hartog, emeritus hoogleraar in de Voeding en Voedselbereiding.

De rector magnificus van de Landbouwhogeschool,
J. P. H. van der Want

Wageningen, 26 maart 1975

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R. J. J. Hermus

Experimental atherosclerosis in rabbits on diets with milk fat and different proteins

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In this thesis the literature about the pathogenesis of atherosclerosis has been reviewed. The various risk indicators for atherosclerosis are discussed and related to the theory about atherogenesis. A review of the influence of milk fat constituents and dietary proteins on serum lipids and atherosclerosis is presented. Experimental results obtained with rabbits fed on semi-synthetic diets are reported. It is shown that large amounts of butyric acid have the same effect on blood, liver and tissue lipids and fatty acids as starch. Hypercholesterolemic affects of butter oil can be mainly ascribed to its cholesterol content. The hypercholesterolemic effects of semi-synthetic diets containing saturated fats can be mainly ascribed to the use of casein as protein source. The hypercholesterolemia induced by semi-synthetic diets is shown to be the consequence of decreased faecal steroid excretion. It is suggested that the different serum cholesterol levels induced by various dietary proteins are due to a redistribution of serum cholesterol to other body compartments.

Descriptors: protein quality; atherogenesis; epidemiology in man; artificial diets; cholesterol; blood lipids; fatty acids; metabolic pools; dietary interactions.

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Stellingen

1

Bij de interpretatie van zijn uitkomsten heeft Moore onvoldoende rekening gehouden met de verschillende cholesterolgehalten van de verstrekte rantsoenen.

Dit proefschrift.
J. H. Moore, 1969. Brit. J. Nutr. 23: 125-134.

2

De met semi-synthetische rantsoenen verkregen uitkomsten in dier-experimenteel voedingsonderzoek zijn van beperkte waarde.

3

De opmerking van Claude Bernard dat 'Le choix intelligent d'un animal est souvent la condition essentielle du succès d'une expérience et de la solution d'un problème physiologique...' behoeft de aanvulling dat ook kennis omtrent de voedingsbehoeften van het dier voorwaarde moet zijn voor het gebruik ervan.

Claude Bernard, 1865. Introduction à l'étude de la Médecine expérimentale. J. B. Baillière & fils, Paris.

4

Voor een beter begrip van het lipidemetabolisme verdient bestudering van processen op het niveau van het spijsverteringskanaal hoge prioriteit.

5

De nog gebrekkige kennis omtrent de aminozuurbehoeften van de volwassen mens maakt een bepaling van de eiwitbehoefte van de wereldbevolking onmogelijk en een schatting van de ondervoeding tot een subjectieve keuze.

Energy and protein requirements, 1973. W.H.O. techn. rep. ser. No. 522, Geneva.
G. Arroyave, 1975. Proc. 9th int. Congr. Nutrition, Mexico 1972. Vol. 1, pp 43-48. Karger, Basel.

6

Een serum-cholesterolgehalte, onafhankelijk van de leeftijd, lager dan 160 mg per 100 ml (4, 14 mmol per 1) moet als normaal en wenselijk voor de mens worden beschouwd.

7

De resultaten van vele epidemiologische onderzoeken zijn weinig betrouwbaar doordat onvoldoende waarborgen voor juistheid en reproduceerbaarheid waren ingebouwd. Certificering door een referentielaboratorium moet daarom een absolute voorwaarde worden voor deelname aan elk gesubsidiëerd epidemiologisch onderzoek.

8

Het aangeven van tolerantiegrenzen voor sporenelementen heeft een beperkte betekenis indien niet tevens de overige gesteldheid van het diëet, in het bijzonder de aanwezigheid van andere elementen, in de beschouwing wordt betrokken.

W.H.O. Food Additives Series, No. 4, Geneva, 1972.
D. Huisingh & J. Huisingh, 1974. Ecology of Food and Nutrition 3: 263-272.

9

Het verdient aanbeveling voor de leek bestemde voedingspublikaties te voorzien van een waarmerk door een daartoe competente instantie als aanvulling op een in te voeren systeem van voedingswaarde-etikettering.

Nutrition References and Book Reviews, 1972. The Chicago Nutrition Association.

10

De discussies over de wenselijkheid van de dierlijke produktie worden vaak vertroebeld door vaagheid in de uitgangspunten en randvoorwaarden, waarbij de gevolgen van een beperking veelal onvoldoende zijn doordacht.

A. J. H. van Es, 1975. Bedrijfsontwikkeling 6: 136-142.
M. Boserup, 1975. Development Forum (U.N.) III (2) 1-2.

11

De door Scott et al. ontwikkelde methode voor de produktie van linolzuurrijk melkvet en vlees is omslachtig en nauwelijks fysiologisch.

T. W. Scott, L. J. Cook, K. A. Ferguson, I. W. McDonald, R. A. Buchanan & G. Loftus Hills, 1970. Aust. J. Sci. 32: 291-293.

12

De slogan 'Melk moet' is formeel niet, maar praktisch wel juist.

13

Voorstanders van een numerus fixus van studenten op grond van plaatsingsmogelijkheden kunnen steun ontlenen aan de motivering voor het doodvonnis van Lavoisier: 'Nous n'avons plus besoin de savants'.

14

Het nalaten van het toewensen van gezondheid bij het opsteken van een sigaret bevestigt niet dat deze wens niet nodig zou zijn. Het bevestigt slechts de onmogelijkheid ervan.

Proefschrift van R. J. J. Hermus
Experimental atherosclerosis in rabbits on diets with milk fat and different proteins.

Aan Diny en Hanne

Preface

The research described in this thesis, initiated in 1970, was carried out at the Department of Nutrition of the Agricultural University, while the experimental animals were well cared for at the Institute of Agricultural Research of Biochemical Products (ILOB), both at Wageningen. The scope of this research could be considerably enlarged by the financial support obtained from the International Dairy Federation (IDF) and the Netherlands Institute for Dairy Research (NIZO), which is gratefully acknowledged.

Professor den Hartog, I am pleased at finishing this thesis while you are still legally allowed to act as my promotor. I hope you will accept this as proof of my gratitude for introducing me to the vast field of human nutrition and for your continuous interest and your stimulating criticism regarding my investigations. The innumerable personal talks we had, even after your retirement , are evidence that you meant more to me than being my promotor only.

Professor Hautvast, your steady interest in the project, your stimulating optimism if anything went wrong, and last but not least your rapid and critical reading of the manuscript were an invaluable support. Your attempts to exempt me from as many duties as possible in the department while writing my manuscript, which succeeded thanks to the co-operation of my colleagues were very much appreciated.

In the course of years many discussions with experts in the field of nutrition and atherosclerosis have been conducted. I feel much indebted to the members of the group of nutritionists of IDF, Prof. B. Blanc, Prof. W. Halden, Prof. A. Lembke, Prof. J. Trémolières, and Prof. J. Yudkin for their encouragement and the open discussions which broadened my view considerably. The helpful kindness of Dr R. Vles and Dr J. Gottenbos of the Unilever Research Laboratory, Vlaardingen is gratefully mentioned. They initiated me in the secrets of experimentation with rabbits. Without the enthusiasm, skills and perseverance of Ben Schutte many problems would have remained unsolved.

Research of the kind described in this thesis can only be accomplished with the co-operation of numerous people. It was an enriching experience to see how many people felt personally involved in this project, performing their task in more than a routine manner.

The contribution of the following people without forgetting all others, is thankfully acknowledged:

- Dr Ir E. J. van Weerden for the preparation of and supervision over the animal experiments;

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- Mr. E. J. Sprietsma and Mr G. Haak for statistical advice and calculations;
- Bert Gundlach for taking care of the figures;
- Hanny Lamster for typing the preliminary report and mrs A. Peters-Jansen for typing the manuscript.

Curriculum vitae

De auteur werd geboren op 10 juni 1941 te Moerdijk (gemeente Klundert). In 1960 behaalde hij het diploma Gymnasium-β aan het Onze Lieve Vrouwe Lyceum te Breda. In hetzelfde jaar werd de studie aan de Landbouwhogeschool te Wageningen aangevangen. In januari 1968 behaalde hij het ingenieursdiploma met Zuivelbereiding en Melkkunde (verzwaard) als hoofdvak en Fysische en Kolloïdchemie en Bedrijfsleer als bijvakken. Per 1 februari 1968 trad hij in dienst als wetenschappelijk medewerker bij de Landbouwhogeschool, afdeling Voeding en Voedselbereiding. Hij had een werkzaam aandeel in de uitwerking van de nieuwe studierichting Voeding.

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Abbreviations

BO	butter oil
C	cholesterol
Cas	casein
CE	cholesteryl esters
CHD	coronary heart disease
Coc	coconut oil
EC	esterified cholesterol
Gel	gelatin
glc	gas-liquid chromatography
γ-GT	γ-glutamyl transpeptidase
Hb	haemoglobin
HbCO	carboxyhaemoglobin
Hc	haematocrit
HDL	high density lipoproteins
IBO	simulated butter oil
LCAT	lecithin : cholesterol acyltransferase
LDL	low density lipoproteins
MCHC	mean corpuscular haemoglobin concentration
MCR	metabolic clearance rate
MCT	medium-chain triglycerides
MCV	mean corpuscular volume
NEFA	non-esterified (free) fatty acids
PCV	packed-cell volume
PGE_1	prostaglandin E_1
PL	phospholipids
PM	protein mixture
PUFA	poly-unsaturated fatty acids
SBBO	butter oil supplemented with soya bean oil
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
TB	tributyrate
TG	triglyceride
tlc	thin-layer chromatography
VLDL	very low-density lipoproteins

1 Introduction

The goal of human nutritional research is to understand the way food affects man, and to use that understanding to build an environment where man can perform optimally. The outcome of a disease, a threat to optimal performance, is determined by the environmental stimuli that initiate or aggravate the disease process, the etiological agents, and by the reactivity of the individual to etiologic agents, his susceptibility. It is presumed that removing an etiologic agent or reducing susceptibility will reduce the rate and severity of the related disease process (McGill, 1972).

The concept of risk indicators was developed in longitudinal epidemiological studies to indicate any personal or environmental characteristics, associated with a higher-than-average risk of clinical disease (Miettinen, 1973). Risk indicators may include true etiologic – and by consequence causative – factors. They may, however, also include many traits which are only related to the disease, but not proven as its cause. There is much controvery about whether reduction of a risk indicator will also reduce the incidence of disease. More detailed knowledge of pathogenesis will assist in selecting the point, where, when and how a disease should be liable to attack.

Two approaches are available for the prevention of disease. The first, through the conventional physician-patient relationships and supporting para-medical care, directs attention to selected individuals who are at abnormally high risk of disease and attempts to contain or prevent the disease by manipulating the patient's habits (e.g. diet), his physiology (drugs) or anatomy (surgery). The second belongs to the public health system which, by eliminating or reducing one or more causative agents from the environment, aims at reducing disease incidence in the entire population. The two approaches are not mutually exclusive but complementary to each other because of failure of the public health approach or increased individual susceptibility. The contrast is, for example, between a prescription of a low cholesterol diet for an individual and a national campaign through legislation, education and agricultural reform to reduce the cholesterol intake of the entire population (McGill, 1972).

1.1 The need for prevention of cardiovascular disease

Cardiovascular diseases, especially ischaemic heart disease and its underlying cause atherosclerosis are endemic now in the entire Western world. This is firmly established by many national and international studies and statistics (Van den Berg & Straatsma, 1972; De Haas, 1969; Lenègre, 1970; WHO, 1972; Fieren, 1972; Wiklund, 1971; Gordon & Kannel, 1971; see for reviews May, 1974; Gezondheidsraad, 1971;

Van Schaik, 1973; Strong et al., 1972). The high mortality figures for the Netherlands and the picture shown by post-war developments particularly the shift for both sexes towards a higher incidence at younger age groups are alarming. (De Haas, 1969; Van den Berg & Straatsma, 1972). The enormous socio-economic consequences, caused by the loss of thousands of men even in their forties and fifties, estimated to cost more than one billion (10^9) guilders annually in the Netherlands (Gezondheidsraad, 1971; Stuyt, 1973), also urgently demand major action. This action should be based on knowledge of the pathogenesis and proposed regimens should be evaluated in this respect.

1.2 Pathogenesis of cardiovascular disease

Knowledge about the pathogenesis of atherosclerosis and its complications has increased tremendously during recent years (Adams, 1967; Zemplényi, 1968; Schettler & Boyd, 1969; Jones, 1970; Wissler & Geer, 1972; Ciba, 1973; Schettler & Weizel, 1974; Hautvast et al. 1975). In combination with results from large-scale epidemiological studies such as the Seven Countries study directed by Keys (1970), the National Co-operative Pooling Project (Stamler et al., 1972) and the International Atherosclerosis Project (IAP) (McGill, 1968; Strong & Eggen, 1970; Strong et al., 1972), a number of risk indicators, mainly serum cholesterol, blood pressure and cigarette smoking, emerge as real risk factors or etiological agents. The Inter-Society Commission for Heart Disease Resources (1970) designates these three risk factors as major risk factors for premature atherosclerotic disease, especially coronary disease. This is appropriate because of the impact they have on risk, the consistency of the findings and the frequent occurrence of these factors, singly and in combination. All three are potentially amenable to prevention and control. Another important point which evolved from these studies is that no threshold values for risk exist. The higher the risk factor level, the greater the probability for an individual of developing coronary disease.

All the circumstantial evidence about pathogenesis and risk factors has to be verified in a clinical trial to test the hypothesis that intervention can prevent cardiovascular disease. Such trials cost much time, effort and money, the ultimate criterion being a significant reduction in morbidity and mortality. Therefore most trials are limited to intermediate endpoints, selected on the basis of knowledge of pathogenesis, such as lowering of blood lipids or blood pressure. Another approach is by comparative animal experimentation.

1.3 Serum cholesteral and dietary fat

Both from a pathogenetic and from an epidemiological point of view, the cholesterol levels in blood serum are strongly correlated with the disease processes. As serum cholesterol is also correlated between populations with dietary fats, total as well as saturated (e.g. Keys, 1970), it is understandable that the main interest is focused on

this factor. Countless numbers of experiments were performed in animals and men to investigate the relationships between dietary fats or other dietary factors and serum cholesterol. From these experiments two formulae were derived by Keys et al. (1965a, b) and Hegsted et al. (1965), respectively which predict the change in serum cholesterol caused by a change in dietary saturated or poly-unsaturated fat or dietary cholesterol.

According to the majority of the Dutch Nutrition Council (Voedingsraad, 1973), one third of the dietary fat should consist of poly-unsaturated fatty acids. This would imply a doubling of the present consumption. As the total amount of fat should also decrease on average 5% of the calories, it is likely that some fats, containing predominantly saturated fatty acids and cholesterol as well will be reduced in the diet. More detailed knowledge about the properties of individual fats is very necessary either to judge their utility in nutrition or to give advice to modify their composition.

Cow's milk fat is suspect with regards to processes related to atherogenesis, due to its high content of saturated fatty acids, its low linoleic acid and its cholesterol content. Its composition is rather stable and difficult to modify by physiological means. It would have enormous economic and agricultural implications if milk fat should be removed from the milk and dairy products and be replaced by a poly-unsaturated-rich oil.

1.4 The use of experimental animals

In atherosclerosis research the use of experimental animals is necessary. This is partly because in man the disease develops over a period of more than 30 years, which is insurmountable for many and impractical for all investigators. Another problem is presented by the difficult accessibility of the interior of the arteries while alive. The degree of and changes in the degree of atherosclerosis can only reasonably be established post mortem. Both these problems count less in animal experimentation. However, even when the rabbit is used which is known to be the most responsive animal species to atherogenic agents, it is better not to hurry but to stay within physiological limits. As will be discussed in Section 4.1 the rabbit may be a useful experimental animal provided that it is not overloaded with dietary cholesterol and is allowed sufficient time to develop atherosclerotic lesions resembling human ones. The semi-synthetic diet model, without dietary cholesterol, appears to fulfil these criteria (Wigand, 1959). The poor health condition of rabbits on such diets, particularly when saturated fat is fed, may be an indication that these diets are not nutritionally adequate (see Section 4.3). As this inadequacy was thought to be related to the protein component of the diet, and as protein quality and quantity are considered as important factors determining the responsiveness of many animal species, the effect of dietary proteins was studied as well.

1.5 Objectives of this study

For proper understanding of the role of any dietary component in atherogenesis and coronary heart disease (CHD), it is necessary to consider this role in the light of prevailing theories on the pathogenic processes. Chapter 2 will be devoted to a discussion on the risk indicators of atherosclerosis as opposed to CHD. In Chapter 3 they will be related to the theories regarding the development of atherosclerotic changes. Experimental evidence for the role played by milk fat and protein in atherosclerotic processes will be reviewed and discussed in Chapter 4.

There is strong evidence that milk fat is incriminated in some atherogenic processes. One of the two main objectives of the present study was therefore to investigate which of the principle(s) in milk fat might be responsible for its effects on blood lipids and atherogenesis. The role of two characteristic constituents of milk fat, i.e. cholesterol and short-chain fatty acids was particularly envisaged.

From the literature it appeared that the remainder of the diet may profoundly influence the results obtained with various fats in rabbits on semi-synthetic diets. The second main objective of the present study, therefore, was to investigate which factor in the semi-synthetic diet favours the hyperlipidemic and atherosclerosis-promoting effects of dietary saturated fats. Special attention was paid to the source of dietary protein.

2 Atherosclerosis

2.1 General features

2.1.1 Definitions

The usage of the term atherosclerosis, usually attributed to Marchand (1904), is rather confusing. It either denotes the presence of arterial lesions or includes the clinical manifestations. Almost all people have atherosclerosis but only a segment of a population will become ill as a consequence of atherosclerosis. Therefore the inclusion of clinical manifestations is inaccurate.

WHO (1958) defined atherosclerosis as 'a variable combination of changes of the intima of arteries, consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits and associated with medial changes'. Bredt (1969), however, found it impossible to define the disease by a simple predicate. He set out a few elementary phenomena in the following manner:

- a disease of the intima, associated with the metabolic mechanism of the blood-tissue barrier. It depends on the chemistry of the blood but also on the structure and metabolism of those layers of the arterial wall which are supplied by blood from the lumen;
- leading to thickening of the intima; as a consequence the lumen to a large extent narrows considerably;
- fundamentally a localised lesion of the wall. The steadily progressing pathological process is always confined to one focal point;
- punctuated by time. It progresses in phases, called the initial, progressive and quiescent phases.
- a process operating in the metabolism of the intima, as far as the wall is fed from the endothelium;
- showing consistency in its localization.

2.1.2 Morphology

By incorporating most of these features, Adams (1967) described atherosclerosis as a multifocal, proliferative and degenerative condition that affects the tunica intima and inner part of the tunica media of both large elastic arteries and certain muscular arteries in the senescent individual. The proliferative phase is essentially an organizing

or sclerotic reaction of connective tissues in the intima while the degenerative element is manifested by lipid accumulation, by fragmentation and hyalinization of connective tissues, by calcification and by ischaemic necrosis of the centre of the lesion.

The arterial tissue changes, the atherosclerotic lesions are at present the only identifiable parameters of the disease. The lesions appear on gross examination as yellow flat or elevated fatty dots and streaks, gray gelatinous elevations and white or pearly-white fibrous plaques often containing a yellow, amorphous basal core (Haust & More, 1972). The two former are considered to be early, the latter advanced or raised lesions. Small mural thrombi or platelet aggregates are considered to represent the third form of an early lesion (Mustard, 1970). In addition, lesions with ulceration, calcification, haemorrhage or thrombosis are referred to as complicated lesions. The term atheromatous lesion implies that the lesion contains an atheroma, i.e. the central core consists of necrotic proteinaceous and fatty substances.

The thickening of the intima that progresses in certain human arteries throughout life (Löber, 1953; French, 1966) is not considered by Adams (1967) as an atherosclerotic lesion. The shearing stress caused by the pulsatile nature of the blood flow could well be responsible for this progressive diffuse thickening (French, 1966). Because this thickening is more prominent in those arteries that develop severe atherosclerosis, it has been inferred that the diffuse thickening is related pathogenically to the same process that eventually leads to the formation of focal atherosclerotic lesion (Wilens, 1951).

This process of intimal thickening can also by itself contribute to atherogenesis. The outer media of the aorta is nourished by the vasa vasorum, while the inner media relies on direct permeation of nutrients through the intima from the lumen (for review see Adams, 1967). Thus, diffuse intimal thickening would first impair the nourishment of the middle media and in older subjects the inner media.

Previously Adams et al. (1962) suggested that lipids accumulate in the atherosclerotic human aorta because certain energy-producing enzymes reduce activity in the tunica media with advancing age. The metabolic failure of the medial muscle cells would result in reduced synthesis of lipotrophic agents, phospholipids and proteins, which are important agents for transporting or dispersing cholesterol within cells and tissue fluids (Dixon, 1958). Adams & Bayliss (1969) investigated the relationship between this loss of enzyme activity of the media, intimal thickening and lipid accumulation. They concluded that medial enzyme failure is related to the degree of intimal thickening; such thickening would impair the diffusion of nutrients, including oxygen. The accumulation of lipid, in particular cholesterol, appeared subsequently to the enzyme defects and was attributed to an impaired local synthesis of lipothrophic agents.

This hypothesis was further supported by the work of Minick & Murphy (1973) and Hardin et al. (1973). Minick & Murphy (1973) fed rabbits on an almost semi-synthetic diet, a hardened vegetable oil or lard as fat, and gave concomitant injections of foreign protein. The animals developed a mild cholesterolemia (250 mg/100 ml) and in their coronary arteries fibromuscular intimal thickening, closely resembling the

intimal thickening in man, and fatty-proliferative fibromuscular lesions, resembling human coronary atherosclerosis. Hardin et al. (1973) confirmed these findings but observed also that after arterial injury, caused by foreign protein injections into low-cholesterol animals, was allowed to heal for several weeks, the diffuse intimal thickening resembled that in man. When the animals, after this healing period, were placed on a cholesterol-supplemented diet, two-thirds of their lesions were found to contain lipid and closely resembled human coronary atherosclerosis. On this base it is suggested that diffuse intimal thickening, acquired throughout life, can later accumulate lipid preferentially and thus predispose to atherosclerosis.

2.1.3 Natural history of atherosclerosis and geographic pathology

Fatty streaks appear early in the course of both human and animal experimental atherosclerosis. They occur frequently in the aorta of children (Strong & McGill, 1969; Klotz & Manning, 1911). In New Orleans, Holman et al. (1958) found aortic fatty streaks in many children under 3 years and in all children over 3 years. Later Strong & McGill (1962) found that fatty streaks were rare in the coronary arteries before 10 years. They became much more frequent in the second decade of life and were nearly always present after 20 years. Arteries from autopsied young persons from Guatemala, Costa Rica, Columbia, Puerto Rico and South Africa revealed the same pattern (Strong et al., 1958; Restrepo & McGill, 1959; Strong et al., 1959; Galindo et al., 1961). All aortas were involved to some degree, but the average extent of surface involved was small before the age of 10. After the first decade intimal surface involvement increased rapidly in all communities, but there was no consistent difference between different geographic locations. However, incidence and severity of fibrous plaques and other more advanced lesions tended to parallel differences in incidence of CHD in the same populations.

These geographic data were extended and confirmed in a much larger study, the International Atherosclerosis Project (IAP) (McGill, 1968). Aortas, coronary arteries and sometimes cerebral arteries were selected in 14 countries from 23,000 autopsied persons divided into 19 location-race groups and four 10-year age groups (from 25 – 64 years). The extent of involvement varies among these groups at all ages and especially so with regard to raised lesions. Aortic fatty streaks are not strikingly different among the 19 location-race groups whereas mean involvement with fatty streaks in the coronary arteries is more variable. The significance of variation in fatty streaking is discussed by Strong & McGill (1969). They studied the relationship of fatty streak to advanced lesion by three different approaches: by microscopical examination, by comparison of the extent of fatty streaking and fibrous lesions and by comparing the topographical distribution of both types of lesions.

2.1.4 Relation between fatty streak and fibrous plaque

Microscopically no sharp distinction could be made between the pure fatty streak and the typical fibrous plaque. This suggests a gradual transition from one type of lesion to the other. The microscopic appearance of New Orleans coronary arteries was different from that of other populations in which atherosclerosis was less prevalent. In particular more intimal lipid occurred in the New Orleans cases. By comparing groups, populations with extensive coronary artery fatty streaks in childhood tended to have more extensive, raised lesions in middle age. This parallel relationship did not hold for aortic fatty streaks. From this it was concluded that advanced lesions develop by progression and transformation of fatty streaks and that the degree to which the transformation takes place varies among arteries and among racial groups. Also the close topographic association between fatty streaks and fibrous plaques at different ages is consistent with this hypothesis. The fatty streak is therefore considered as a normal evolutionary survival means for lipid storage in the intimal and innermedial smooth muscle cells (McGill, 1974). Only some advanced fatty streaks transform into fibrous plaques for unknown reasons. Programmes to control atherosclerosis and its sequelae should therefore be established before the transition of fatty streak to fibrous plaque occurs, i.e. during the first 2 – 3 decades of life or possibly even earlier. None of the well-known intervention studies fulfilled this criterion (Dayton et al., 1969; Miettinen et al., 1972; Leren, 1970). If, nevertheless, a reduced infarction or mortality rate was obtained, this was more than could be expected from atherogenesis prevention.

2.1.5 Occurrence of raised lesions

The results of the IAP based on the percent surface area of coronary arteries covered with raised lesions were more illustrative (McGill, 1968). These lesions are directly responsible for luminal narrowing of arteries and set the stage for ischaemic complications. An over-all mean extent of raised lesions was computed and the 19 location-race groups were ranked by this mean. The mean extent of raised lesions in coronary arteries of men who died from causes not associated with CHD ranged from a high of 22.3% in New Orleans Whites and 20.2% in men from Oslo to a low of 5.5% in the South African Bantu and in men from Guatemala and Bogota, Columbia.

In each decade there is a large increase in artery involvement in all 19 location-race groups. When the 19 groups were ranked by extent of lesion in all arterial segments together, in coronary arteries alone, in the aorta alone, in each sex alone, and in each decade alone a similar rank was obtained. It was therefore concluded that those populations with more extensive, raised lesions in the aorta also tend to have more extensive lesions in the coronary arteries and that those populations with more extensive lesions in men also tend to have more extensive lesions in women (Strong et al., 1972).

Tejada et al. (1968) compared the mean extent of atherosclerotic involvement with CHD mortality and found a close association. Strong & Eggen (1970) calculated the

rank correlation between lesions and mortality rate from CHD for the age groups from 45 – 54 and 55 – 64 as 0.71 and 0.77. Finland is one of the leading countries with regard to morbidity and mortality rates of CHD. Finnish men under 65 years of age have the highest CHD mortality rate in the world (WHO, 1970; Keys, 1970). In a series of Finnish autopsies the extent of arterial surface area involved with raised lesions was determined by Rissanen & Pyärälä (1974). They used the methodological principles of the IAP study and found a somewhat lower involvement than was obtained for USA whites and Norwegians.

In spite of the many well-known limitations imposed by the nature of autopsy studies, they may be considered to indicate trends in the population (McMahan, 1968). According to vital statistics (WHO, 1970) the CHD mortality rate of middle aged males is twice as high in Finland as in Norway, whereas in the USA the mortality rate is almost as high as in Finland. However, the basic level of atherosclerosis seems to be roughly similar in these three countries. Obviously, the factors responsible for the development of atherosclerosis are not the only ones influencing the onset of CHD and mortality. The same conclusion was drawn by Strong et al. (1972) who found in an autopsy study on New Orleans' deaths of the age group, 30 – 44 years a higher CHD death rate in negro males than in white males. In the IAP-project, however, the mean extent of raised atherosclerotic lesions was much more in white men than in negro men for the corresponding age groups.

If CHD is considered as the outcome of an interplay of a wealth of environmental and host factors only partly working via the process of atherosclerosis one can easily understand that on an individual basis atherosclerosis is poorly correlated with CHD. Also populations with particular nutritional or living habits or genetic properties may differ in incidence of CHD, although achieving the same degree of atherosclerosis (Stamler, 1967). The concept of risk indicators is useful to differentiate between risk of developing CHD or risk associated with atherosclerosis.

2.2 Risk indicators for coronary heart disease and atherosclerosis

2.2.1 Coronary Heart Disease (CHD)

'... denn namentlich leiden oft wohlhabende und reiche Leute an angina pectoris, welche den Genüssen einer reichlichen und luxuriösen Tafel ergeben ohne zugleich die nötige körperliche Bewegung zu haben, zu einer bedeutenden Fettleibigkeit gelangen.'

Th. v. Dusch (1845) (Hort, 1969)

('... for the affluent and wealthy often suffer from angina pectoris. They abandon themselves to a rich and sumptuous cuisine without taking the necessary physical exercise and become obese.')

'Auch fortgesetzte leidenschaftliche Aufregungen und häufiges, lautes Reden sollten zu diesen Ubel disponieren.'

Th. v. Dusch (1845) (Hort, 1969)

('Also continuous emotional excitement and frequent boisterous discussion may increase one's disposition to this evil.')

'...auch ist einem excessiven Tabakrauchen einen Einfluss auf die Entstehung der Angina Pectoris zu zuschreiben...'

Beau & Lancereaux (1868) (Hort, 1969)

('...another incriminating factor in the origin of angina pectoris is excessive tobacco smoking...')

Between 1845 when the observation of Dusch in Hamburg, cited above, was made and 1975, progress on risk indicators has not been very impressive. Epidemiological experiments have identified a large number of characteristics of people or their environment, reflecting the degree of liability to develop the disease. Strasser (1972) reviewed the literature and found 39 factors to be associated with atherosclerosis or its complications. He admitted that his list was far from complete. Even more review articles have appeared during the last years on risk indicators for CHD. A few must be mentioned: Stamler (1967), Heyden (1969), Jones (1970), Simborg (1970), Inter-Society Commission for Heart Disease Resources (1970), Fejfar (1972), Shaper (1972), Miettinen (1973), Dalderup (1974), Stamler et al. (1972), Stamler & Epstein (1972), Hagerup (1974), May (1974), Schettler & Weizel (1974).

Many attempts have been made to divide risk indicators into categories, e.g. controversial and accepted relationships to CHD (Strasser, 1972), personal and environmental characteristics (Miettinen, 1973), avoidable and unavoidable indicators. However, there is no sharp dividing line, but rather a continuum of decreasing certitude and what is a personal characteristic for one person, e.g. hypercholesterolemia, is environmentally determined for another. Stamler et al. (1972) made a useful classification of risk indicators for developing CHD:

- those involving social environment and life style, e.g. habitual diet high in saturated fat, cholesterol, calories; cigarette smoking; sedentary living.
- those involving endogenous biochemical-physiological regulatory mechanisms, but amenable to exogenous influences, e.g. diet, pharmaceuticals: hyperlipoproteinemia, hypertension, hyperglycemia, hyperuricemia and rapid resting heart rate.
- those involving organ pathology, e.g. ECG abnormalities, hypothyroidism and renal disease.
- those involving fundamental biology and not generally amenable to exogenous influences, e.g. age and sex.

This classification is, however, more related to possible treatment than to effects related to the origin of the disease. It would be outside the scope of this thesis to discuss in detail all the relevant data with regard to risk indicators for CHD, as well as all the interrelationships which may exist. In Table 1 therefore a summary is shown of a number of characteristics which were found to be related to CHD in epidemiological, clinico-pathological and experimental investigations. Many references can be easily found in the above mentioned reviews. Such a list does not make much sense as many of these traits are interrelated (Stamler et al., 1972) by cause and effect or by simultaneous occurrence, i.e. calories, total protein, total fat; animal protein, saturated fat, cholesterol; saturated fat, sucrose; obesity, hypertension, blood lipids, hyperglycemia. Others are not important from a public health point of view because of rarity.

Table 1. Risk indicators related to coronary heart disease (CHD).
+: increased risk when characteristic is present.
−: protective effect of the characteristic.
±: effect still controversial, slight or mediated by one or more of the other characteristics.

Age	+
Sex	
male	+
female	−
Blood group other than 0	+
Family history, genetic factors	+
Race	±
Personality type	±
Blood factors	
hypertension	+
lipids and lipoproteins	
cholesterol	+
triglycerides	±
phospholipids	±
uric acid	+
glucose 'intolerance'	+
hyper-insulinism	±
carboxyhaemoglobin	+
Overweight	±
Resting heart rate	+
Organ pathology	
diabetes mellitus	+
hypothyroidism	+
renal disease	+
ECG abnormalities	+
Diet	
calories per day	+
total protein	±
animal protein	±
total fat	+
saturated fat	+
poly-unsaturated fat	−
cholesterol	+
phytosterols	±
sucrose	±
coffee	±
water hardness	−
crude fibre, pectin	−
heavy meals	+
carbon disulphide exposure	+
deficiencies of chromium, manganese, vanadium, ascorbic acid, retinol, pyridoxin, choline	+
hypervitaminosis D	+
Smoking	
cigarettes	+
pipe, cigar	±
Sedentary living	+
Psycho-social factors (stress)	±

Major risk indicators It is essential to establish which indicators are true factors as related to the pathogenesis of the disease, which occur most frequently in a population and which are amenable for change. Evidently the indicators related to fundamentally biological characteristics such as age or sex, or to organ pathology do not fulfil these criteria. In recent years, several statistical methods for multivariate analysis have been applied to this problem (Truett et al., 1967; Walker & Duncan, 1967). With these methods a risk probability can be computed for an individual based on simultaneous evaluation of several findings, e.g. his serum cholesterol, blood pressure, number of cigarettes smoked, age, etc. The key question is, of course, whether the equation which was derived from the Framingham data is generally applicable. Keys (1970) tried to apply the risk function to the data from the Seven Countries Study. With the classification of men in deciles of risk probability values, a strikingly high correlation was found between the number of predicted and observed cases of CHD ($r = 0.83$). When the coefficients estimated for European cohorts were applied to the American group, the relative risk was well predicted ($r = 0.94$) but the absolute number of predicted cases was underestimated considerably, (Keys et al., 1972a), i.e. only 57% of the observed cases. Westlund & Nicolaysen (1972) derived for Oslo about the same coefficients as were found in Framingham, Mass. Obviously, the method grades men and women in regard to risk. The predictive power of the equation may be shown by the ratio of risk for the upper quintile compared with the lower one. This ratio was for Framingham men 40:1 (Truett et al., 1967) and 19:1 for women. The risk ratio and therefore the predictive power was greater for younger age groups. In the Seven Countries Study, Keys et al. (1972a) found a risk ratio for the upper and lower decile of 12:1 for men. By the use of 5 (Keys et al., 1972) or 7 (Truett et al., 1967) risk indicators, a population can be divided into a group at high and a group at low risk. If these methods are more extensively applied, with larger sets of risk indicators and different populations, risk will be better predicted.

Simultaneously it will be possible to decide which indicators contribute most to the risk value and which are of minor importance. Two important points are already clear from the application of the risk equation. Generally no value can be indicated for a risk indicator above or below which a sharp increase in risk appears (Stamler & Epstein, 1972), therefore cut-off points have no sense. The most important risk indicators, as contributors to the overall risk value, are serum cholesterol, blood pressure and cigarette smoking (Inter-Society Commission for Heart Disease Resources, 1970). The question remains whether these epidemiologically derived risk indicators are also true etiological factors causally related to CHD. It would be worthwhile to consider their relationship with the underlying process leading to CHD, i.e. atherosclerosis. A relationship between risk indicators and atherogenesis would give a firmer base to regimens aimed at lowering the level of these characteristics. Together with results from clinico-pathological and experimental studies this might lead to a theory on the origin of atherosclerosis and its complications.

2.2.2 Atherosclerosis

2.2.2.1 Introduction

In comparison with CHD, investigations into risk indicators for the atherosclerotic process are relatively scarce. Since atherosclerotic lesions cannot be visualized directly during life, autopsy studies are necessary to determine the relationship of risk indicators for CHD to atherosclerotic lesions per se. These studies have provided valuable information in spite of the well-known limitations imposed by the very nature of such studies as discussed by McMahan (1968). The data for this discussion are derived from two comprehensive studies, the International Atherosclerosis Project (IAP), reported by McGill (1968) and an autopsy study by Sternby (1968), and many fragmentary studies, frequently taking into account only one or two risk indicators or a few subjects. Raised lesions in the coronary arteries will be the principle type of atherosclerosis considered. Differences in extent of lesions with age, sex, serum lipids and dietary fat, hypertension, diabetes, physical activity, obesity, water hardness, and cigarette smoking will be considered.

2.2.2.2 Age and sex

Age is the strongest and most consistent risk indicator for raised lesions. The average involvement with raised lesions in coronary arteries increases with age in every population sampled by the IAP (Eggen & Solberg, 1968). Sternby (1968) observed the same trend in Malmö. Age, however, has two aspects: age as a time factor and ageing as a process. Accumulation of severe atherosclerosis in the aged is understood as 'cumulative minor episodes' (Pollak & Wadler, 1951). These may fail to occur in which case the vessels of aged people will contain no or very few plaques (Groddeck, 1939). Though ageing cannot be ruled out as a factor leading to changes of the arterial wall which make it more prone to metabolic disturbances (Adams & Bayliss, 1969), it cannot be regarded as sufficient cause. Because lesions vary greatly among individuals of a given population at each age, the association of age with atherosclerosis is probably due to continued exposure to etiological agents (Strong et al., 1972). Moreover the level of a number of other risk indicators, as serum lipids, blood pressure and glucose intolerance tend to rise with age in the coronary-prone populations.

The effect of sex is somewhat confusing. The results of the IAP (Tejada et al., 1968) and of Sternby (1968) indicate that coronary atherosclerosis is more extensive in men than in women in white populations. In negro groups with generally less lesions the sex difference is much less evident.

2.2.2.3 Dietary fat; blood lipids

The populations of the IAP were ranked by serum cholesterol or dietary components (Scrimshaw & Guzmán, 1968). The rank correlation coefficient between rank based on raised lesions and rank by cholesterol was 0.755 while rank by dietary fat gave a coefficient of 0.688. No positive associations within a population were found. Such cross-sectional comparisons at any one time are of limited value.

Because of the very long course of the natural history of atherosclerosis, the true relevance of data from longitudinal studies will be directly related to the duration of follow-up and the numbers of years for which the variable in question, be it diet or serum cholesterol, has been characterized. Careful documentation of habitual diets, thorough serum lipid studies, and standardized autopsies will be required to determine the relationships between diet, serum lipids and extent of lesions, in individual persons. From such a careful study, the Framingham study, evidence was presented (Feinleib et al., 1971) that for male decedents serum cholesterol values, measured 5 and 9 years before death, were significantly correlated with severity of coronary atherosclerosis measured by both intimal involvement and luminal narrowing.

In the study of the relationship between diet, serum cholesterol and atherosclerosis a few points must be kept in mind. The cholesterol value of any individual represents his intrinsic characterization and the effects of his diet. Even on precisely the same diet, in a metabolic unit, the inter-individual standard deviation is of the order of 35 – 40 mg/100 ml, or 15 – 20% (Keys et al., 1957). In comparing two unhospitalized men the dietary component is often the smaller of the two above-mentioned factors that combine to differentiate them in regard to serum cholesterol.

Intra-individual variability is a second problem. On a constant diet and with all other conditions apparantly unchanged, repeated blood samplings show substantial variation (Keys et al., 1957). Again under metabolic ward conditions, the intra-individual standard deviation was found to be about 12 mg/100 ml. For unhospitalized men the variation on a constant diet was about twice as great. Single blood samples obviously, do not reliably characterize individuals. This was pointed out again by Stamler et al. (1972) in their Peoples Gas Company Study. Based on the first, single, cholesterol determination, 102 men had cholesterol values under 175 mg/100 ml, 227 had values of 275 or greater. Based on the mean of 10 repeated determinations of serum cholesterol for each man, only 80 men had values under 175 and only 98 had values of 275 or more. By the same procedure the CHD mortality ratio between these two cholesterol groups increased from 1.74, based on a single determination to 6.84, based on the mean values.

The problem of establishing an individual's habitual diet is still greater. A common criterion for judging the accuracy of any method of measurement is to repeat the measurement under the same conditions. Obviously this allows only limited conclusions about repeatability or reliability. It does not provide any evidence about validity, i.e. true accuracy of the method (Keys, 1968). Dawber et al. (1962) reported their experience of the Framingham Study as disappointing. When the same nutri-

tionist repeated a survey on the same subjects, the correlation between data of the first and second occasions was generally very good. The correlation for grams of total fat was $r = 0.88$, for animal fat $r = 0.79$, but for calories from saturated fatty acids, $r = 0.58$ only. Thus for the factor most often correlated with serum cholesterol and atherogenesis, two-thirds of the variance remained unexplained. When two different nutritionists did the interviews, the correlations were even less: $r = 0.27$ for total fats and $r = 0.44$ for calories from animal fats.

Extensive research about methods to assess the habitual diet of individuals was reported by Den Hartog et al. (1968) from data of the Seven Countries Study. For every cohort the intra-individual variance for percentage of calories from fats proved to be similar to or greater than the inter-individual variance. For saturated fats the intra-individual variance was even greater. But the averages for the population samples were stable. Other sources of variability, leading to inaccuracy, should be added to this individual variance, as differences in food composition, food preparation, seasonal factors. Finally atherosclerosis develops over thirty years or more and therefore the diet consumed during the last one or two years cannot predict completely and reliably the status of the vessels.

From all these data it can easily be concluded that relationships between diet, serum cholesterol and extent of atherosclerosis are easily obscured by the substantial intra-individual variability in food intake and serum cholesterol and by the large inter-individual variability, reflecting a different response to exogenous stimuli. On the same base it is easily understood that within culturally homogeneous populations it would be unreasonable to expect to find significant relationships between estimates of diet, cholesterol values and extent of atherosclerotic lesions for individuals. There would be no reason to expect anything but negative results from a search for such relationships (Keys, 1970; Stamler et al., 1972).

From the foregoing the erroneous deduction may not be made that the abundant inter-population and animal-experimental data may be forgotten. Stamler et al. (1972) summarized the state of knowledge as follows:

- a relationship has been demonstrated between populations for mean intake of certain nutrients (saturated fat and cholesterol) and mean serum cholesterol level.
- there is a relationship, both between populations and within populations, between serum cholesterol level and risk of atherosclerotic disease.
- there is an association between change in dietary composition (amount and type of fat and cholesterol) and serum cholesterol level.
- it is not possible to account for the large inter-individual variability in serum cholesterol by inter-individual variability in intake of nutrients, specifically saturated fat and cholesterol.

There is even less information about whether the level of serum triglycerides is related to the extent of atherosclerosis than about its relation to CHD. In the Framingham study a raised serum triglyceride level was only indicative for increased risk for CHD when superimposed upon a raised serum cholesterol level (Kannel et al.,

1971). Wilhelmsen et al. (1973) found no predictive value for serum triglycerides in a prospective study in Göteborg on acute myocardial infarction, when other indicators were taken into account. In the Stockholm prospective study, serum triglyceride concentration appeared as a risk indicator independent of cholesterol (Carlson & Böttiger, 1972), without taking into account the other known risk indicators. Moreover, in this study the population was divided into 'normal' and 'high' with a cut-off point for cholesterol at 280 mg/100 ml. In view of the continuous rise in risk with a concomitant rise in serum cholesterol, this value is already high. It is more reasonable to think of risk due to the high triglycerides superimposed on an already existing risk due to the high cholesterol values.

Data from the IAP confirm the epidemiological data mentioned above that serum triglycerides are not a major indicator. Malcolm et al. (1968) compared serum cholesterol and triglyceride levels in samples from male blood donors at hospitals contributing to the autopsy samples in the IAP, with extent of atherosclerotic lesions in males from these autopsy samples. The data suggest that current triglyceride levels do not parallel differences between populations in atherosclerotic lesions. These obviously weak findings need firm confirmation.

2.2.2.4 Blood pressure

There is probably no risk indicator, so unanimously agreed upon, as arterial hypertension. Data from the IAP (Robertson & Strong, 1968), confirm the many previous studies that, on the average, persons with hypertension have consistently more coronary and aortic atherosclerosis than persons without hypertension for all sex, age, race or location groups. The same trend has been reported by Sternby (1968). However, hypertension effectively increases risk for CHD only in communities already susceptible to CHD (Shaper, 1972). Though hypertension increased the extent of raised lesions in all populations of the IAP, the increase in populations with a low incidence of CHD, is superimposed on a low base resulting in a severity which is still not sufficiently great to produce frequent CHD (Strong et al., 1972). Hypertension does not appear to be a primary cause of atherosclerosis however, because atherosclerosis may be severe and extensive in persons without the disease (Strong & Eggen, 1970). On the other hand, hypertension is common in population groups in Africa and Asia, where atherosclerosis and CHD is uncommon (Shaper, 1970; Fejfar, 1972).

Many experiments in many animal species unequivocally demonstrate that hypertension per se never induces atherosclerotic lesions as long as the diet remains low in cholesterol and fat. However, in the presence of even a mild hypercholesterolemia hypertension aggravates the atherosclerotic process (Katz & Stamler, 1958; Bronte-Stewart & Heptinstall, 1954; Heptinstall et al., 1958; Koletsky et al., 1968; Wakerlin et al., 1957; Campbell et al., 1973; Fisher et al., 1973).

Though the etiology of hypertension is not well understood, epidemiological and experimental evidence suggests that the dietary sodium intake may play a critical role,

although genetic factors are apparently of considerable importance in determining the individual response to sodium intake (Dahl, 1972). However, Filer (1971) concluded his summary of the evidence on this issue as: 'the evidence associating salt intake and hypertension in man is inconclusive'. Therefore the issue remains questionable (Lowe, 1972). With the exception of body weight, an important correlation between elevated blood pressure and a number of environmental factors has not been convincingly demonstrated (Dawber & Thomas, 1968). These factors included physical activity, serum cholesterol, coffee, alcohol, cigarettes. The role of cadmium in the etiology of hypertension has aroused much interest during the last years (Masironi, 1974).

2.2.2.5 Physical (in)activity

Physical inactivity has become the most clear-cut characteristic of the civilized creature in the industrialized nations. It is reasonable that information on activity is taken into account in establishing risk (see for review: Fox et al., 1972). In the past mainly occupational activity has been considered. However, since working hours are reduced and the range of occupational activities narrows due to mechanization and automation, it might be more worthwhile to consider activity in leisure time as well (Morris et al., 1973). Initially evidence was presented indicating that the incidence of, particularly acutely, fatal events tended to be lower in men engaged in physically active, compared with sedentary work (see e.g. Morris et al., 1953). Later it appeared that most of the difference could be ascribed to differences in blood pressure, serum cholesterol, etc. (Shaper et al., 1971). Stamler et al. (1972) reviewed the results of a large number of studies in the US and found their results to be inconsistent, some reporting a positive association, others none. They observed that stratification by job did not represent division into groups with different energy expenditure at work. The basic hypothesis obviously needs reformulation.

Vigorous physical activity should produce measurable objective effects in the organism, such as enhanced cardiopulmonary fitness, possibly decreases in serum lipids, blood pressure, thrombogenic tendency etc. In the Framingham Study tests related to cardiopulmonary fitness (Kannel, 1967) were carried out. CHD mortality rate was consistently higher for the group classified as less active and less fit. The findings of Morris et al. (1973) in almost 17,000 civil servants of middle age are in agreement with such a concept. They found that the relative risk of developing CHD in men reporting vigorous exercise in leisure time was about a third of that in comparable men who did not exercise. Lighter exercise and estimates of overall activity showed no such advantage.

The effects of physical (in)activity on the condition of the arterial wall are less studied. Morris & Crawford (1958) could not differentiate formerly active from inactive people, in 3,800 autopsies, regarding severe atherosclerosis of the coronary vessels. The same results were obtained by Spain & Bradess (1960). Also a study in New Orleans by the group of Strong et al. (1969) did not reveal a consistent relation-

ship of occupational activity and coronary atherosclerosis. The coronary vessels of Masai, an African tribe with a habitual high physical activity, appeared to have the same degree of atherosclerosis as American men (Mann et al., 1972).

It is in conclusion very unlikely that positive effects of heavy physical activity manifest themselves in the degree of coronary artery involvement. Two alternatives have been hypothesized. Luminal enlargement was reported for very active people (Mann et al., 1972; Currens & White, 1961) and this was sufficient to more than compensate for the increase of the intimal and muscular layers of the vessel wall. Another hypothesis by Kannel (1970) was derived from the findings of Morris & Crawford (1958) and Spain & Bradess (1960) that sedentary people have demonstrated more occlusions and myocardial scars but not more uncomplicated atherosclerosis. He hypothesized that activity benefits the cardiovascular apparatus primarily by promoting collaterals to compensate for a compromised coronary circulation.

Physical inactivity thus cannot be regarded as a factor in atherogenesis. The protective effect of activity on the fatal complications appears only temporarily and activity of more than five years ago may not diminish current mortality risk (Kahn, 1963).

2.2.2.6 Smoking

A considerable number of epidemiological studies has been made in the last decade in populations differing in geographical location and socio-economic background and with different techniques, to determine the relationship of smoking to CHD. They have shown with remarkable consistency a significant association between cigarette smoking and an increased incidence of and mortality from CHD (for review see: Kershbaum & Bellet, 1966). International data demonstrate a high order correlation ($r = 0.64 - 0.79$) between average per capita consumption of cigarettes and CHD mortality for both male and female populations of the developed countries (Stamler et al., 1972). In several retrospective and prospective studies, risk for attracting CHD was invariably related to the amount of cigarettes consumed (Spain & Nathan, 1961; Mulcahy et al., 1963; Paul et al., 1963; Doll & Hill, 1964; Hammond, 1964; Hammond et al., 1964; Doyle et al., 1964; Inter-Society Commission for Heart Disease Resources, 1970). Risk generally increased stepwise with the number of cigarettes used daily. For users of pipe or cigar tobacco the incidence rate is only slightly and not significantly higher than for those who have never smoked (Spain & Nathan, 1961; Paul et al., 1963; Doll & Hill, 1964; Hammond et al., 1964; Doyle et al., 1964; Stamler et al., 1972).

The availability of a rather large group in every population who quit smoking makes it possible to study the effect of a reduction of a risk indicator on the incidence of CHD. Russek (1965) found an even higher prevalence for CHD among nonsmokers than former smokers. In the Framingham Study Doyle et al. (1964) reported a nearly equal risk of CHD for nonsmokers and ex-smokers. In two extensive studies the rates for ex-smokers were intermediate between present smokers and those who

never smoked (Doll & Hill, 1964; Hammond, 1964). Size of the population studied, different techniques and criteria used may easily explain these different results. The benefits of reducing cigarette smoking remain quite obvious.

The susceptibility for heavy cigarette-smokers to develop CHD can, at least in part, be ascribed to the more extensive lesions observed in heavy smokers. While Wilens & Plair (1962) found only slightly more myocardial infarctions at autopsy in heavy smokers than non-smokers, the degree of aortic sclerosis was much more frequently above average in heavy and moderate smokers than in non-smokers. This study lacked age matching of smokers and non-smokers. In their autopsy study of 1372 men, dying of causes other than CHD, Auerbach et al. (1965) determined the degree of atherosclerosis in the coronary arteries in relation to former smoking habits. Advanced coronary atherosclerosis was two to three times more frequent among heavy smokers than among non-smokers. In pipe and cigar smokers the degree of atherosclerosis was about the same as in non-smokers, and definitely less than in cigarette smokers. Former smokers had less severe atherosclerosis than current smokers but more than non-smokers. Roughly the same results were obtained by Strong et al. (1969) in an autopsy study in New Orleans. This applies to raised lesions in the coronary arteries as well as in the aorta of whites and blacks. A similar relationship between cigarette smoking and degree of aortic involvement was reported earlier by Sackett et al. (1968).

Many experiments have been done to test the relationship between smoking and the production and progression of experimental atherosclerosis and the effects of smoking on various biological processes involved in atherogenesis. These studies have centered around two points of interest: the effects of nicotine and of carbon monoxide.

Nicotine In the rabbit, Wenzel et al. (1959), using graded doses of nicotine in drinking water, failed to discern any effect of this agent on aortic atherosclerosis. Using a dose of nicotine, equivalent to 175 cigarettes a day in man, Stefanovich et al. (1969) found slightly greater aortic atherosclerosis and serum cholesterol in cholesterol -fed rabbits. Nicotine failed to influence aortic acid mucopolysaccharide content. This component is often increased when the atherosclerotic process is augmented (Fisher, 1961). Most of the studies might be regarded as acute and therefore unrevealing with respect to such a chronic disorder as atherosclerosis. A more realistic daily dose of nicotine equivalent to 35 cigarettes failed to affect, quantitatively or qualitatively, atherosclerosis of aorta and extramural or intramural coronary arteries or serum lipids in normotensive or hypertensive rabbits with and without dietary cholesterol supplement (Fisher et al., 1973).

The effects of nicotine on various biological processes supposedly involved in atherogenesis and its complications are more clear (Isaac & Rand, 1969). These effects, tachycardia and an increase in cardiac output, are attributed to the stimulating effect of nicotine on the sympathetic nervous system and to catecholamine release (Burn, 1960). The net effects of these actions have been interpreted to represent an adverse increased oxygen demand by the heart. Increases in free fatty acid (FFA)

mobilization after smoking (Kershbaum et al., 1961), higher serum cholesterol and triglyceride values (Jenkins et al., 1968) in heavy smokers, a shorter coagulation time (Donner & Billimoria, 1970) an increased thrombosis tendency (Hawkins, 1972), a delayed fibrinolysis (Dalderup et al., 1970, 1971) are all effects which are related to smoking and probably to nicotine. These effects, however, may contribute more to the occlusive episode and the fatal outcome of the disease than to atherosclerosis. Nicotine may provoke arrhytmias in smokers with a damaged myocardium and it might further impair oxygen supply to already deprived sites by its vasoconstrictory effects.

Carbon monoxide In animal experimental studies a very potent atherogenic role has been demonstrated for another component of smoke: carbon monoxide. Astrup et al. (1967) demonstrated that exposure of cholesterol-fed rabbits to a carbon monoxide atmosphere led to an increase of aortic cholesterol to 5 times during 10 weeks. The carboxyhaemoglobin saturation values were 16 - 18% (HbCO). When rabbits were exposed to carbon monoxide, leading to HbCO values of about 10%, subintimal oedema occurred followed by increased formation of mucopolysaccharides and collagen and formation of fibrotic plaques (Wanstrup et al., 1969). The aggravation of cholesterol induced atherosclerosis in the coronary arteries of Squirrel monkeys (*Saimiri sciureus*) by carbon monoxide was demonstrated by Webster et al. (1970). The primary effect of carbon monoxide on the vessel wall seems to be an increased endothelial permeability, leading to subendothelial oedema (Kjeldsen et al., 1972), an increased influx of plasma constituents and subsequent repair processes. About the same HbCO values as were induced in rabbits are common in cigarette smokers (Kjeldsen, 1970; Stewart et al., 1974).

The last link in this chain, the association between HbCO level and risk for CHD has been filled in by Wald et al. (1973). It was concluded that smokers with a HbCO level of 5% or higher had a 21 times higher risk for CHD than smokers with values below 3%. Therefore the inhalation of tobacco smoke, containing carbon monoxide, may very well be the main determinant of the risk inferred by smoking.

The similarity of effects of carbon monoxide and hypoxia was stressed by Astrup et al. (1970), while hyperoxia may be useful in the regressive phase of atherosclerosis. If carbon monoxide is of major importance in atherogenesis it might be advisable to measure HbCO values in epidemiological studies instead of asking for smoking habits. Differences in inhalation may cause a lot of variation in the relationship between smoking and CHD.

Though it will not be discussed in detail here, it should be mentioned that heavy atmospheric carbon monoxide pollution may also lead to increased carboxyhaemoglobin levels (Stewart et al., 1974; Aronow, 1973; Borst, 1967). This carbon monoxide may be present in motor vehicle exhaust, at large airports and in public and private places where smoking is not restricted (Russell et al., 1973). This carbon monoxide source is in general much less important, however, compared with inhalation of tobacco smoke.

2.2.2.7 Diabetes, obesity, water hardness

Diabetes In clinical medicine and pathology, it has been recognized for years that the main problem particularly in maturity onset diabetes is the enhanced risk for atherosclerotic disease (Katz et al., 1958; McGill, 1968; Epstein, 1967). Diabetics have atherosclerotic disease more frequently, more severely and more prematurely than non-diabetics. Persons with atherosclerotic disease exhibit more frequently abnormalities in glucose tolerance than controls (Epstein, 1967). It is still a matter of debate whether diabetes or glucose intolerance are independent risk indicators. In many studies (Epstein, 1967) a relationship was found between hyperglycemia, serum lipids, particularly triglycerides, and hypertension. Also in the Framingham study this question is left open after treatment of the data by multivariate analysis (Gordon et al., 1971). From the data of the Peoples Gas Company study (Stamler et al., 1972), it is inferred that hyperglycemia is an especially clear-cut risk indicator for men with concomitant hypertension.

It is highly unlikely that raised blood glucose per se enhances atherosclerotic changes. It may very well serve as an indicator for some derangement of carbohydrate or lipid metabolism, i.e. insulin level or action. The literature concerning insulin levels in patients with coronary disease is confusing. Increased as well as decreased levels have been reported. If it could be proven that the precursor of diabetes is characterized by an exaggerated insulin response, changing later to a diminished insulin output, the finding of an increased or decreased insulin response in a patient with CHD merely reflects at what point in time he happens to be examined (Epstein, 1974). There is also other evidence that diabetes does not inevitably lead to atherosclerotic disease as an integral part of the syndrome. Keen (1972) reported Japanese evidence, where CHD in the general population is very low (Keys, 1970), that CHD as a cause of death in Japanese diabetics is extremely low (5% of 865) compared with American diabetics (54% of 1283). It is tempting to conclude that the diabetic is a particularly sensitive indicator of some atherogenic factor in the environment.

In the autopsy studies of the IAP (Robertson & Strong, 1968) and of Sternby (1968) more coronary artery and aortic atherosclerosis was present in the diabetics than in persons without known diabetes. This observation holds for all sex, age, race or location groups. The relationships which obviously exist between diabetes and other risk indicators for CHD evidently obscure the true relationship between diabetes and extent of lesions. Diabetes aggravates atherosclerosis in populations generally not seriously affected by CHD. What is said in this respect about hypertension clearly applies to diabetes as well: the increase in atherosclerosis is superimposed on the low base and the result is not sufficiently great to produce frequent CHD.

Obesity Obesity, more often measured as overweight, is always a strong risk indicator for CHD when not taking into consideration the accompanying risk indicators (see e.g.: Doyle et al., 1957). Even when hypertension is absent overweight remains a significant risk indicator (Stamler et al., 1972). In their review on risk indicators they

found a strong positive correlation between relative weight and blood pressure, serum uric acid, plasma glucose, and serum lipids and a negative correlation with smoking. In the Seven Countries study, Keys et al. (1972b) concluded that overweight does not increase risk when correcting for age, blood pressure, cholesterol and smoking. In the Framingham study (Truett et al., 1967) overweight attributed only a small risk after correction for cholesterol and blood pressure.

Though the other accompanying risk indicators may confound the picture also in this case no association between obesity, as measured in different ways, and atherosclerotic lesions in coronary arteries and aorta was observed in the IAP (Montenegro & Solberg, 1968).

Water hardness Since Kobayashi (1957) showed in Japan a relationship between cerebral haemorrhage (apoplexy) and the sulphate-bicarbonate ratio of the water supply, much interest has been aroused in possible protective or noxious factors in tap water. Schroeder (1960) demonstrated a higher mortality from CHD in areas with a soft water supply in the US. Particularly in England this trace was further investigated by Crawford et al. (1968) (see review: Crawford, 1972). They not only confirmed the findings of Schroeder (Crawford et al., 1968) but demonstrated also that a change in water hardness was accompanied by a change in the death rate from CHD (Crawford et al., 1971). To find out whether the 'water factor' was related to the known risk indicators, a group of men from soft and hard water areas was examined (Stitt et al., 1973). Mean values for blood pressure, plasma cholesterol and heart-rate were slightly higher in people from the soft water area. Calculation of risk with the formula of Truett et al. (1967) resulted in a 14% higher risk in the soft water group, which is small in comparison with the observed difference in mortality (50%).

With regard to the pathogenic/protective role of the 'water factor' two possibilities remain. It might either affect the atherosclerotic process or its complications. The first appears to be ruled out by two studies, one in Glasgow (Crawford & Crawford, 1967) and the other in the IAP (Strong et al., 1968), where no differences in coronary lesions were found between men from areas with hard and soft water. Results were negative from experiments in pigs on the extent of atherosclerosis or the level of serum lipids when given water of different hardness (Howard et al., 1967; Püschner et al., 1969). The second possibility might be related to an increased susceptibility of the myocardium in the soft-water area. This is supported by several findings indicating that the excess mortality in the soft-water areas is entirely due to an excess of 'sudden deaths' (Masironi et al., 1972).

Which factor in the water might be held responsible for the effects of hardness and softness remains to be elucidated. Protective effects of some trace elements, for example vanadium, manganese, chromium, and lithium and harmful effects of others, such as lead, cadmium and cobalt are implicated (Masironi et al., 1972; Masironi, 1974). Crawford (1972), however, emphasizes the importance of the relative amounts of calcium, magnesium and sodium, in their mutual relationships and in relation to some of the trace elements.

3 Atherogenesis

3.1 Introduction

The many proposed theories on the pathogenesis of atherosclerosis point to the impossibility to mark one factor or pathway as the cause or mechanism of atherogenesis. It has been realized that there are most probably numerous operating factors culminating in atherosclerosis. These factors are not exclusive of each other but may also augment or potentiate one another (Haust & More, 1972). This also makes it understandable why so many theories have been developed in the past and why they were all insufficient to deal with all aspects of the disease.

Only a brief description will be given of the most important theories which were developed during the last 100 years. After this I will try to place the known data in the perspective of the complex dynamic process that at any moment represents the interaction between the blood components, the haemodynamic forces and the structure and function of the arterial wall (Duff & McMillan, 1951).

3.2 The thrombogenic theory

The thrombogenic or encrustration theory was originally proposed in the mid-19th Century by Von Rokitansky (see: Adams, 1967), extended by Mallory in 1913 (see: Haust & More, 1972) and revived by Duguid in 1946. In its first version it was proposed that fibrinous substances are being deposited from the lumen upon the arterial intimal surface. These deposits than undergo a metamorphosis into a pulpy mass composed of a large number of cholesterol crystals and fatty globules. There exists conclusive evidence for the presence of fibrin deposits within atherosclerotic arteries (Haust et al., 1964) but the origin of this fibrin still remains to be determined. In particular it is not clear whether it is incorporated as such or whether it enters in the form of fibrinogen and is transformed into fibrin within the wall (Studer, 1970).

It appears very difficult to prove this hypothesis experimentally. Injection of large thrombi, like emboli, will lead to organization into lesions resembling atherosclerosis (Chandler, 1967) but is hardly physiological. After administration of thrombin in rats or rabbits fibrin layers on the intima are rarely detected. In fact, formed fibrin disappeared within hours in rats and within days in rabbits (see Studer, 1970). Duguid (1955) described the occurrence of numerous small thrombi, microthrombi, attached to the aorta wall of even young people. These mural thrombi may start at least two processes.

The vessel wall reacts to a thrombus or a platelet aggregate by organizing (con-

verting) it to living tissue, or from the platelets are constituents released. Thrombin, collagen, antigen-antibody complexes, some bacteria and some viruses cause this release as well as platelet aggregation (Packham et al., 1968). Among the released constituents are the nucleotides ATP and ADP, serotonin, potassium, epinephrine, histamine and some lysosomal enzymes (Mustard, 1974). The release of ADP, serotonin and epinephrine from the platelets may be important in causing more platelets to adhere to those which have already clumped. There are also substances released which increase vessel permeability (Mustard et al., 1965; Packham et al., 1968). In addition, the released lysosomal enzymes can cause tissue injury (Mills et al., 1968). It is therefore possible that when platelets interact with the vessel wall they may icrease the permeability of the endothelium and thus contribute to the local accumulation of protein and lipoproteins from the plasma in the arterial wall (Packham et al., 1967). In fact, Jörgensen et al. (1972) did correlate the presence of aortic microthrombi with focal intimal oedema and suggested that the oedematous lesions resulted from injury to the vascular lining by the thrombi.

Organization takes place by an ingrowth of connective tissue while endothelium grows over the thrombus to restore continuity of the vascular lining (Duguid, 1946). The connective tissue is derived from modified smooth muscle cells, capable of synthesizing collagen (Haust et al, 1960) and present in the intima. Variations in the rate of endothelial overgrowth and organization relative to thrombolysis or fibrinolysis can significantly influence the amount of thrombotic material incorporated into the arterial wall (Davies et al., 1969). A study of Crawford (1956) indicated a fairly slow endothelial regeneration to cover a needle puncture wound, i.e. 6 – 8 days.

It has been possible to relate all kinds of advanced lesions, from the sclerotic and largely fibrous ones till the atheroma containing ones to the variable composition of the thrombi (Chandler, 1970) i.e. relative proportions of fibrin, cellular elements and plasma. Whole blood clot emboli do not form fatty plaques (Chandler, 1970). Red blood cells are a potential source of lipid in thrombotic plaques (Barnard & Thompson, 1966) but platelets contain considerably more lipid than red blood cells (Chandler, 1970). When whole blood clots are injected into systemic veins of rabbits, fibrous intimal plaques are formed in pulmonary arteries (Harrison, 1948). When platelet-rich thrombi are injected, typical fatty atherosclerotic plaques containing many foam cells develop (Hand & Chandler, 1962). Also significant is the observation of Huant et al. (1972) that aortic thrombi in normolipemic rabbits develop into lesions remarkably similar to fibro-fatty atheromatous lesions in man. Whether the cholesterol in the plaques is directly related to the cholesterol in the platelets is, however, very unlikely. Smith (1967) made a quantitative comparison of the amount of cholesterol present in platelets and lesions, which showed that the platelet cholesterol is too low to make any significant contribution to the cholesterol content of the lesions. She also found no evidence that thrombi could trap or accumulate plasma lipid.

All the evidence in favour or disfavour of this hypothesis has been reviewed by many experts in this field (Adams, 1967; Chandler, 1967; Chandler & Pope 1975; Haust et al., 1964; Studer, 1970; Mustard, 1970, 1974; Haust & More, 1972).

Although there is much evidence in support of the thrombogenic hypothesis of atherosclerosis, exact knowledge of its importance is still incomplete. In particular it is not yet known at what stage in the development of the disease, thrombosis begins to play a significant role (McMillan, 1965). The thrombotic origin of a plaque is obscured as a result of metamorphosis of the thrombus. The true incidence of thrombus-derived plaques is therefore difficult to ascertain. In their survey on the frequency with which thrombi can be identified in plaques of aortas, Chandler and Pope (1975) calculated this to be in 41 to 45 percent of the lesions. They are most often identified within fibrous and fibro-fatty plaques. In fatty streaks, Woolf & Carstairs (1967) noted a diffuse pattern of immunofluorescence, specific for fibrin/fibrinogen antigen, but not platelet antigen. On the base of its diffuse pattern it was thought to represent fibrinogen that had infiltrated along with plasma from the lumen. The presence of thrombotic components in atherosclerotic plaques of the coronary arteries was reported by Hudson & McCaughey recently (1974).

So far no reasonable explanation has been offered as to why platelets adhere to the arterial wall. Occasional small thrombi have been observed on apparently healthy walls (Duguid, 1955) but Movat et al. (1959) cautioned that undetected and pre-existent microscopic changes could be present in a seemingly normal artery. Nevertheless, exposed subendothelial tissue is commonly present (French, 1970), even though with minimal injury this may occupy no more than a gap between two separated endothelial cells. The exposed components of the wall consist of collagen and elastic fibres, intimal ground substance and basement membrane. Collagen fibres induce adherence of platelets in vivo and in vitro and are therefore considered as the major stimulus for platelet adhesion during thrombus formation (see: French, 1970; Mustard et al., 1974).

Evidently, not all features of atherogenesis can be accounted for by the thrombogenic theory. The majority of fatty streaks and dots is probably not derived from mural microthrombi, though they may be a consequence of oedema, caused by increased permeability of the endothelium underneath a mural thrombus. If it appears correct that endothelial injury is a prerequisite for thrombogenesis, the factors causing such an injury should be the primary study object. Also the factors regulating platelets to aggregate and des-aggregate are of main interest as well as the process of fibrin formation and fibrinolysis.

3.3 The lipid theory

3.3.1 Introduction

A central place in the lipid theory is given to lipids, which invade the arterial wall from the blood stream. The accumulating lipids act as irritating foreign bodies on the arterial tissue, provoking a proliferative reaction around them. This hypothesis appears to have been first proposed by Marchand (1904) and almost monopolized the field of atherosclerosis.

Evidence that lipids have something to do with this disease is overwhelming (see: Constantinides, 1965; Adams, 1967). Tremendous amounts of lipid are found in atherosclerotic arteries. Much evidence is available that most of this lipid, particularly cholesterol, is not generated locally in the wall, but comes from the blood (Hirsch & Weinhouse, 1943; Zilversmit, 1970). A second argument comes from the extensive epidemiologic studies which claim that there is a definite correlation between blood lipid levels, atherosclerosis and CHD. This disease is found much more often among communities or nations with higher blood lipid levels than among those with lower levels (see 2.2.2.3). A third argument comes from animal experimentation.

Since the first two decades of the 20th Century, it is known that atherosclerotic changes can be induced by diet. Ignatowski (1909) produced aortic atherosclerosis by feeding rabbits on a diet of milk, meat and eggs. Egg yolk was the most important atherogenic component of this diet. Cholesterol was identified by Anitschkow (1913) as the atherogenic factor. Since those days numerous experiments have been carried out, feeding many animal species special diets under variable conditions, which invariably lead to hyperlipemia and atheroma formation (see: Constantinides, 1965; Adams, 1967).

Atheroma is at first expressed as a generalized lipid storage in the arterial tissue, but becomes subsequently fibrous. It will closely resemble human atherosclerosis if enough time is allowed to progress from its initial appearance to the more advanced forms, and if the hypercholesterolemia is mild or intermittent (Constantinides, 1965). The whole lipid filtration theory is based on the concept that the metabolic needs of the arterial wall, i.e. food supply and waste disposal, are served by a continuous diffusion of blood fluid through it from the lumen to the lymphatic channels at the adventitial side. Under normal conditions, all of the lipid molecules carried in that percolating fluid go straight through the wall, except for a small proportion of them that are picked up by the cells of the vessel wall (Kellner, 1954; Watts, 1963; Adams & Morgan, 1966). If the filtration of lipoproteins and other blood constituents is a normal process, then the main problem becomes: what causes the accumulation of lipid in atherosclerotic lesions. This may arise from excessive filtration, from failure of outward transport or from selective binding underway (Adams, 1967). Clearly a combination of these mechanisms will exaggerate the response.

3.3.2 Sclerogenic properties of lipids

An important feature of some lipids is their sclerogenic action (Adams, 1967). The subcutaneous implantation of free cholesterol or desmosterol was found by Spain (1961) to provoke fibrosis. Later Spain & Aristizabal (1962) showed that tripalmitin, tristearin and cholesterol esters were fibrogenic, while triolein induced less fibrosis. This was confirmed by Adams et al. (1963) in the rat while the addition of phospholipid inhibited this response. It was also found that the degree of unsaturation of the phospholipids markedly influenced the resorption rate of the cholesterol implants (Adams & Morgan, 1967). Hirsch (1938) had already demonstrated that emulsified

cholesterol ester is inert, but that the non-emulsified ester is intensely sclerogenic. Cholesterol in lipoprotein form is also non-sclerogenic (Byers & Friedman, 1963). These observations suggest that cholesterol is more readily absorbed from connective tissue and is less fibrogenic when dispersed. Abdulla et al. (1967) graded many purified lipids for sclerogenic activity and slowness in resorption as follows: free cholesterol > cholesterol stearate > cholesterol myristate > cholesterol oleate > cholesterol palmitate > cholesterol elaidate > linoleic acid > linolenic acid > arachidonic acid > cholesterol linoleate > cholesterol linolenate > triolein > stearic acid > oleic acid > cholesterol arachidonate > lecithins.

Many investigators have concluded that the arterial tissue reaction to lipids may be an important atherogenic mechanism (e.g. McGill et al., 1963). Lipids do, in fact, appear to be more sclerogenic than serum proteins. Waters (1965) reported a fibroblastic proliferation in the cornea of rabbits after injection of homologous hypercholesterolemic serum, but only a negligible reaction is seen after lipid was extracted from the serum.

Smith & Slater (1972) concluded that the major part of the cholesteryl esters in the advanced lesion originates directly from plasma lipoproteins. The fatty acid composition of these esters resembles that of plasma, with a preponderance of linoleic acid (Smith et al., 1967). Most of this lipid is extracellular. However, fatty streaks contain predominantly intracellular lipid, mainly cholesteryl esters, with oleic acid as the principal fatty acid (Smith et al., 1967). When the disease progresses the arterial wall becomes more permeable to allow also influx of intact lipoproteins and thus cholesteryl esters (see 3.3.3 and 3.3.4).

Fatty streaks appear to be actively concerned with esterification of cholesterol (Chobanian & Manzur, 1972). The CoA-dependent esterifying system in the intima preferentially forms cholesteryl oleate (Bowyer et al., 1967) which is consistent with the fatty acid pattern of early lesions (Smith et al., 1967). In this sense the arterial wall is its own worst enemy (Adams, 1973). After lipoproteins have invaded, their apo-protein part is split off and metabolized, and their lipid part is deposited. Extracellular lipid clearly reflects the fatty acid composition of the serum lipids. It might be inferred that a high amount of cholesteryl linoleate in the serum lipoproteins would be less sclerogenic and therefore less harmful than saturated or mono-unsaturated esters. In most animal experiments there is always a combined effect of a high serum cholesteryl linoleate proportion and a low serum cholesterol level, making interpretation of the results difficult. For an extensive discussion of this problem see: Gottenbos (1975).

3.3.3 Lipid accumulation

During recent years an enormous amount of research effort has been spent to elucidate the mechanisms underlying the accumulation of lipid, and particularly cholesterol in arteries. Excellent reviews have been published by Adams (1967, 1970, 1973); Zilversmit (1970); Stein & Stein (1973); Walton (1975); Bondjers (1975). It

would be far beyond the scope of this thesis to discuss all the relevant data on lipid uptake, transfer, removal, biosynthesis and accumulation. A few points, however, deserve attention.

Simple lipid or lipoprotein infiltration cannot be the sole mechanism for lipid entry into normal artery (Bondjers, 1975). The uptake of free cholesterol far exceeds that of cholesterol esters while the ratio in serum is just the opposite (Dayton & Hashimoto, 1970). In addition Adams et al. (1968) observed a differing distribution of cholesterol and protein entering the normal rabbit aortic wall. Cholesterol entered from the luminal side, the proteins from the adventitial side, probably via the vasa vasorum. In the atheromatous aorta both entered from the luminal side, indicating an increased permeability. With different techniques the presence of serum lipoproteins in atherosclerotic lesions has been demonstrated, together with fibrinogen (Walton, 1975).

Though the influx of intact lipoproteins into an atheromatous intima is now well established, it is still a matter of debate how cholesterol enters the 'normal' artery. From his experiments in vitro, Jensen (1969) concluded that the unidirectional transport of cholesterol from serum to intima-media under normal conditions, is a process that requires energy. Active glycolysis probably provides the immediate energy required. He proposed a pinocytotic mechanism involved in the cholesterol transport across the endothelial cell membrane. Alternatively, Rothblat & Kritchevsky (1968) suggested that active transport could also involve binding of free and esterified cholesterol at the cell membrane, rapid hydrolysis of the ester, and intracellular transport in its free form. Bondjers & Björkerud (1973) observed an inverse relationship between transfer of labeled free cholesterol and free cholesterol content in intact aortas. This suggests an adjustment of cholesterol transfer by the cholesterol present in the tissue in the normal state.

Zilversmit (1968), Jensen (1969) and Constantinides (1969) postulated the existence of a metabolic barrier against the influx of cholesterol in arterial tissue. The gradual break-down of this barrier during atherogenesis might allow cholesterol to increase exponentially during experimental atherosclerosis (Zilversmit, 1968). However, such increased influx is probably localized to areas with endothelial defects (Bondjers & Björkerud, 1973). The rate of entry by filtration may exceed active transport when such defects are present, even in rabbits with cholesterol levels of about 50 mg/100 ml.

In contrast with these findings, Smith & Slater (1972) could identify low-density lipoproteins (LDL) in the 'normal' human aorta, together with fibrin/fibrinogen antigen. However, in the aortic intima of children none of this material or much less than expected could be detected. These young children's aortas may be the only normal ones and 'normal' areas of elsewhere affected arteries of older people may not be very normal. This hypothesis on normality would agree with the findings of Adams et al. (1968), mentioned above, that proteins enter the normal rabbit aorta from the adventitial side but atheromatous aortas from the lumen. It can therefore be hypothesized that cholesterol enters the normal aorta by an active process but that lipoproteins, probably together with other plasma constituents as fibrinogen and

fibrin, enter the intima after some injury. The finding of greatly increased amounts of lipoproteins, expressed as μl serum/100 mg dry tissue, in the intima of hypertensive patients compared with normotensives by Smith & Slater (1973) illustrates this theory. They also showed that the amount of LDL in the intima again expressed as μl serum was independent of the subject's serum cholesterol but the absolute amount of intimal LDL was strongly correlated with the serum cholesterol. This suggests that a rather constant amount of plasma is retained in the intima.

3.3.4 Intimal injury

If the arteries are injured experimental atherosclerosis can be produced very quickly and with very little lipemia. However it takes an extremely high lipemia or a very long time to accomplish the same result if the arteries are normal, apparently because injury produces gaps in the endothelial lining (Constantinides, 1965). The endothelium of normal arteries is proposed to represent a barrier that prevents the crossing of large protein and lipoprotein molecules. Normally only small molecules, such as water, electrolytes, glucose, small proteins and hydrolysis products like amino acids and fatty acids are transported across the endothelium to serve the metabolic needs of the artery (Constantinides, 1969).

When an artery is injured, the permeability of the endothelium increases, gaps are created through which also the large molecules pour into the wall in great quantities, such that they cannot be metabolized quickly enough and therefore accumulate. There may be also a mechanism by which some large molecules are selectively trapped. The most likely mechanism for this selective entrapment is the interaction between LDL and fibrinogen and the sulphated glycosaminoglycans (S-GAGs) of the arterial wall (Walton, 1974).

Many of the stimuli giving rise to increased vascular permeability have been shown also to produce a mesenchymal reaction in the subendothelial tissues of arteries, associated with increased production of S-GAGs (Hauss et al., 1969; Wissler, 1968). Matthes et al. (1969) could confirm that acute arterial hypertension in rats induced a rapid and significant increase in sulphate incorporation in S-GAGs. Half an hour of hypertension was already sufficient for this effect. Lipid deposition occurred several hours later and should be considered as a secondary phenomenon. Matthes et al. (1969) proposed the following sequence: injurious agents → non-specific mesenchymal reaction → mesenchyme proliferation → transport disturbance → lipid accumulation. About the same process was described for hypoxia as injurious agent (Garbarsch et al., 1969).

In experiments, in vitro, only LDL and fibrinogen from the serum proteins form insoluble complexes with S-GAGs derived from the arterial wall (Walton, 1952; Gerö et al., 1960). In the intact artery histochemical localization of LDL and fibrinogen and radioactive sulphate uptake show a significant correlation between the distribution of S-GAGs and these proteins (Curran & Crane, 1962). Adams & Bayliss (1973) were able to show that underneath focal lipid deposits in tendons and athero-

sclerotic plaques GAGs were present, supporting the idea that they act as a trap in the arterial wall for LDL. Their presence in a similar situation in tendons also indicates that the lipid infiltration and binding in atherogenesis are not unique phenomena confined to the arterial wall but may in fact be general characteristics in avascular sites.

Injurious agents Many agents produce gaps in the arterial endothelium, some by opening intercellular junctions and others by destroying cells and causing them to exfoliate into the lumen (Constantinides, 1969): angiotensin, serotonin, tyramine and other amines, proteolytic enzymes, bile acids, calciferol, lysophosphatides and antigen-antibody complexes. Also hyperlipemia itself can be an injuring agent (Shimamoto et al., 1966; Constantinides, 1973). Some diabetes metabolites, e.g. β-hydroxybutyric acid and acetoacetic acid tended to cause dilatation of the intercellular endothelial junctions. In addition acetoacetic acid caused marked oedema and rupture of endothelial cells.

Nicotine by intra-arterial perfusion caused injury only in the form of endothelial cell oedema in rabbits, not in rats. Marked species differences were also present towards norepinephrine and epinephrine. The rat being slightly sensitive to both, the rabbit is very sensitive to all (Constantinides, 1973).

These results are very difficult to interpret, considering all variables which may interfere. The species, the dosage of the component under investigation, the route of application are all factors which may modify the outcome. The general principle, however, remains that some physiological or 'normal' components may injure the endothelial lining, causing increased permeability and thus a possibility for larger molecules to penetrate the arterial wall.

Walton (1975), in his review of the literature on factors affecting lipoprotein deposition in the arterial wall, concluded that there was evidence that vascular permeability increases with age. Smoking precociously leads to the same changes as characteristic for advanced ages. High levels of LDL and VLDL and altered vascular permeability are interdependent and hypertension always accelerates lipid infiltration in areas exposed to the maximal effects of such pressure. Injury to the outer coats of arteries, inhibiting the drainage of the vascular fluid, causes localization of plaques in the area overlying the traumatized area.

3.3.5 Lipids, thrombosis, coagulation and fibrinolysis

The discovery of an effect of dietary fats and blood lipids on processes linked with thrombosis enhanced the importance of lipids in atherosclerosis and atherogenesis. A thrombus is the end-product of primary platelet aggregation, secondary deposition of fibrin and thirdly failure of fibrinolysis.

A raised serum non-esterified fatty acid (NEFA) level in man is associated with increased platelet adhesiveness (Murchison & Fyfe, 1966). Also the kind of fatty acid or lipid class plays an important role. Kerr et al. (1965), observed in vitro the formation

of reversible aggregates under influence of oleic acid, phosphatidyl serine, phosphatidyl ethanolamine and irreversible aggregates with stearic, palmitic and myristic acid, and sphingomylin. Lecithin, linoleic acid and linolenic acid were without effect.

The ingestion of saturated triglycerides appears both to increase platelet stickiness (Rowsell et al., 1965) and to promote thrombosis in experimental animals. The addition of saturated triglycerides to diets containing cholesterol, cholic acid and thiouracil leads to the formation of arterial thrombi in the dog (Hartroft et al., 1962) and the rat (Thomas et al., 1959; Renaud, 1966). A reduction of the protein level of the diet from 22 till 10% by weight makes the application of thiouracil superfluous (Renaud, 1966). The thrombogenic properties of this kind of diet are ascribed by Bizzi et al. (1963) to an increased serum triglyceride level.

The concentration and the composition of β-lipoproteins also appear to affect platelets. Farbiszewski & Worowski (1969) observed an increased platelet adhesiveness and enhanced aggregation induced by ADP after the addition of isolated β-lipoproteins obtained from cholesterol-fed rabbits. This increase is greater than when lipoproteins from normally fed rabbits were added. The lipoproteins of the cholesterol-fed rabbits had an increased proportion of lipids compared with protein, containing twice as much cholesterol as the controls.

Hornstra & Vendelmans-Starrenburg (1973) developed a useful method to measure the thrombus-formation rate, inserting an 'aorta-loop' into the abdominal aorta of rats and measuring the time before complete occlusion takes place with platelet-rich thrombi. With this method they were able to show a significant delay in obstruction time by intra-arterial and to a less extent by intravenous infusion of prostaglandin E_1 (PGE_1). The same authors also reported a positive linear relationship between obstruction time and different amounts of dietary sunflower-seed oil substituted at the expense of saccharose. That fibrin has something to do with thrombus formation was confirmed in this model by intravenous heparin infusion. This procedure lengthened the obstruction time significantly. From this and other experiments with mixtures of sunflower-seed oil, coconut oil and butterfat, Hornstra (1975) concluded that linoleic acid was the antithrombotic fraction of sunflower-seed oil while the role of the fat level was not significant.

In another series of experiments Hornstra (1975) compared several dietary fats with differing fatty acid composition. He found only a weak relationship between the linoleic acid content of the dietary fats and their thrombogenicity. The main determining factor appeared to be the content of saturated fatty acids with 14 or more carbon atoms. Myristic acid had only half the activity of longer-chain fatty acids. Two fats did not fit this relationship: medium chain triglycerides (MCT) and palm oil. The MCT fat is metabolized quite differently and behaves more as a carbohydrate (Hermus, 1970). The different behaviour of palm oil cannot be explained yet. From still other experiments Hornstra (1975) concluded that oleic acid is neutral, linoleic and probably linolenic acid are anti-thrombotic and trans-fatty acids behave as their cis-isomers.

With the filtragometer (Hornstra, 1975) the aggregatability and disaggregatability

of the blood platelets of men from both experimental groups in the Helsinki dietary prevention trial were measured (Hornstra et al., 1973). In the high linoleic acid group the aggregation time was longer, the aggregation slope was flatter and the disaggregation induction time shorter as compared with the subjects on the low linoleic acid diet. It is tempting to speculate that the observed reduced mortality from CHD on the high linoleic acid diet (Miettinen et al., 1972) can at least partly be attributed to the reduced aggregation tendency of the blood platelets.

The role of dietary fat and blood lipids in blood coagulation and fibrinolysis has probably received less attention than the properties of the platelets during recent years. The effects of lipids, however, result in almost the same picture as do platelet effects. Alimentary lipemia in man is commonly associated with hypercoagulability of the blood (Keys, 1963). Increasing the proportion of unsaturated fats in the diet lengthens the clotting time (Buzina et al., 1961). Several serum phospholipids, particularly of the cephalin group and containing much oleic acid, have clotting activity (Billimoria, 1964). Also serum triglycerides are considered as important determinants for blood coagulation (Bizzi et al., 1963; Rifkind et al., 1966). In the Helsinki trial, Karvonen et al. (1974) found no clear changes in coagulation tests when the dietary fat was reversed from saturated to poly-unsaturated and vice versa. However, whole blood clotting time was positively correlated with cholesterol and lipid phosphorus. The Stypven time (measured with Russel's snake venom) was inversely related with cholesterol, triglycerides and lipid phosphorus, while fibrinolysis time was positively correlated with cholesterol and triglycerides.

Fibrinolysis may be defined as the enzymatic liquefaction of blood or fibrin clot and its postulated function in vivo is the removal of unwanted fibrin deposits (Fearnly, 1971). It may be regarded as the physiological antithesis of blood coagulation. A measurement of the blood coagulation activity should always accompany measurement of the fibrinolysis time as the coagulation/fibrinolysis equilibrium is what really matters. Much of the disagreement in the literature about the precise relationship between serum lipid levels and fibrinolytic activity (Adams, 1967) and effects of dietary constituents on fibrinolytic activity (Ollendorf et al., 1964) may be explained by such an unilateral determination of an equilibrium.

Ollendorf et al. (1964), observed a hypercoagulability of the blood after all kinds of acute fat loading, but animal fats diminish only the fibrinolytic activity (Thomas & Scott, 1957; Gajewski, 1961; Blix & Bjørkheim, 1965). Although fats apparently do have acute effects, Dalderup et al. (1971) could not find a relation between fibrinolytic activity and habitual sugar, carbohydrate or fat consumption within a group of normal people. Karvonen et al. (1974) could also find no consistent differences between two groups of people on a 'normal' fat diet or on a diet rich in linoleic acid. A summary of known effects of dietary components on fibrinolytic activity was given by Dalderup & Van Haard (1971a). Beer, butter, cream, egg yolk, a fatty meal containing bacon, eggs and butter, peanuts, wine and cider were all reported as fibrinolysis inhibitors. Glucose, nicotinic acid, onions and vegetable fats as coconut oil, sunflower-seed oil and arachis oil were promotors of fibrinolysis.

3.3.6 Conclusion

The evidence in favour of the lipid theory is strong. In epidemiological studies serum cholesterol invariably was the best predictive risk indicator for CHD and also atherosclerosis. Hypertension and cigarette smoking came next. With the help of the known facts about the pathogenesis of atherosclerosis it should be possible to distinguish among the risk indicators in terms of a primary or essential role versus a secondary or contributory, adjuvant role (Stamler et al., 1972). Obviously many non-smokers and non-hypertensives develop premature CHD. Neither cigarette smoking nor hypertension are therefore necessary causes. Since the reverse is equally true neither of these risk indicators can be regarded as sufficient causes. This conclusion is based both on epidemiological studies and for hypertension also on animal experiments (Inter-Society Commission for Heart Disease Resources, 1970; McGill, 1968; Keys, 1970; see 2.2.2.4).

Hypertension and cigarette smoking are thus neither sufficient nor necessary causes for CHD. Evidence on cholesterolemia shows that this is also not a sufficient cause as many individuals with considerably elevated cholesterol levels do not appear to develop CHD. The reverse is also true. However, as indicated in 2.2.2.3 most relationships are calculated on the base of one or two cholesterol determinations. As this parameter has a considerable intra-individual variation, this procedure is rather critical. Moreover, the disease develops over a period of 30 or more years and in fact the cholesterol levels should be known over this entire period. The predictive power from an individual's cholesterol level of his blood vessel's condition is even less known. From animal experiments, however, a straightforward relationship is known between an animal's serum cholesterol level and its atherosclerosis grade. Also the prognosis of a patient, suffering from essential hypercholesterolemia, is very bad, and he can die from premature CHD, with extensive atherosclerosis as the underlying cause.

From the fact that even normocholesterolemic people develop atherosclerosis and CHD it cannot be argued that cholesterol is not a necessary factor. Data from populations with virtually no atherosclerosis (McGill, 1967) suggest that 140 or 150 mg/100 ml may be the upper limit for normal cholesterol levels. The knowledge about the sclerogenic properties of lipids is still scanty. If it is proven to be correct, it may have important consequences. Firstly it would be possible to explain the presence of large amounts of fibrotic tissue in plaques. Secondly it would explain why sometimes a poor correlation is observed between serum cholesterol and CHD because not only the absolute level would be of importance but also the kind of cholesteryl esters. Though still poorly understood, the role of lipids in thrombogenesis might be of great significance in the genesis of mural thrombi as well as in the occlusive episode.

Based on all this evidence it is tempting to attribute to the lipids and particularly to cholesterol a central role. It would, however, go too far to ascribe the whole process to this factor only. It appears particularly important to know how the cholesterol-bearing lipoproteins get into the arterial wall and become trapped. It seems necessary

that some noxious factor injures the integrity of the endothelial lining or provokes a non-specific mesenchyme reaction. The first mechanism may lead to an increased influx, the second to an accumulation of lipid. It is unlikely, that influxed material will be limited to lipoproteins. Fibrinogen/fibrin will penetrate as well and will also lead to a fibrotic tissue reaction.

Effects of lipids should therefore always be considered in view of the complex dynamic interrelationships between blood and its constituents, the haemodynamic forces and the arterial wall structure and metabolism.

3.4 The insudation theory

The insudation theory, originally proposed by Rössle (1944) was elaborated by Doerr (1970) and extended by Haust (1970). This theory proposed that the action of injurious agents, noxious agents, on either the endothelium or subendothelial connective tissue is followed by a local serous inflammation of the intima. This manifests itself initially in the presence of an insudate derived from the blood. When the injury is mild, the insudate is serous and can be reabsorbed easily. Alternatively, it may become organized by an avascular connective tissue.

In the extended form, discussed by Haust (1970), the theory becomes all-embracing with respect to the initiation of all three forms of lesions as well as their progression. It can accomodate all the valid points of the thrombogenic and lipid theories. Thus Walton (1975) stated that the relationship of the major risk indicators to the disease can be satisfactorily explained on the basis of an insudative hypothesis, in terms of the insudation of lipoproteins and fibrinogen into the arterial wall. The findings of Smith & Slater (1972) also agree with such a mechanism. They observed a rather constant volume of the subject's own plasma in normal intima in the age group of 49 – 69 years, while this volume appears to increase in hypertension. Expressed in absolute terms they found similar concentrations of albumin and lipoproteins. They therefore support the idea that plasma enters as a unit, presumably through endothelial cell junctions by leakage (Smith & Slater, 1973). In areas with 'gelatinous thickening', corresponding to focal intimal oedema, a twofold till fourfold increase of lipoproteins was present compared with the normal intima, while the 'residual' cholesterol was only slightly increased. Thus, if an agent injures the endothelium in a given area, this may result in increased permeability, leading to an enhanced influx of plasma with its constituents.

Also a microthrombus may be deposited (see 3.2) which may be organized subsequently or which may itself cause some injury to the vascular lining leading to an oedematous reaction and increased permeability (Jörgensen et al., 1972). The properties, composition and amount of the insudate will be determined by the severity of the injury and by the composition of the blood (Haust & More, 1972). The more severe the injury, the more fibrin will enter the intima in the insudate; the higher the lipid content, the more will be present in the entering fluid.

The organization of the proteins in the insudate contributes to the fibrous com-

ponent of the emerging lesion, the lipid content will contribute to the lipid component. The reactivity of the arterial wall is, however, a determining factor in the final outcome of an insudate. Minute injuries may be followed by a complete restoration. The small amount of probably serous oedema may separate, but not damage the local tissues, and be reabsorbed entirely because it contains only small molecules which are readily diffusible, as albumin. But fibrinogen and low-density lipoproteins, present in serofibrous insudates, may present more problems. Fibrinogen, after conversion to fibrin, and LDL are precipitated as they are not amenable for reabsorption because of their size. Fibrin contributes to the local increase in connective tissue; the lipid must be phagocytized if it is to be transported out of the intima (Haust, 1970).

Besides disposing of substances that have become insudated, the repair should also replace the original damaged tissues. The avascular reparative connective tissue which results from these processes should be minimal to restore integrity of structure and function maximally. As a once repaired area is more susceptible to subsequent injury, this will lead to an accumulation of connective tissue. This area will be more liable to local hypoxia, to arrest and precipitation of traversing substances. Metabolic derangements and degenerative changes will ensue, enhancing in turn repair processes and further connective tissue accumulation.

Though the insudation theory is very attractive and fits all known data, the most crucial question about the identification of the injurious agents remains unanswered. In the past many factors have been shown experimentally to cause injury to the endothelium and intima and to produce lesions resembling in some or more aspects human atherosclerosis. Apart from those, already mentioned (3.3.4), Haust (1970) gave an impressive enumeration of injurious factors, including: various microorganisms and their toxins; electric stimulation; ionizing radiation; heat; cold; direct trauma; forces applied to the body as a whole, such as rapid deceleration, negative and positive gravitational forces, mechanical vibration; neurovascular disturbances; hypo- and hypervitaminotic states; manipulation of endocrine organs or their active principles; enzymes; conditions of stress; changes in haemodynamics; quantitative or qualitative alterations of normal plasma components either by diet, drugs or other means; generally altered metabolic state; numerous chemicals and poisons; hypoxic states (for literature, see: Constantinides, 1965; Roberts & Straus, 1965; Adams, 1967; Hueper, 1944, 1945; Duff & McMillan, 1951).

As the arterial lesion in atherosclerosis is mainly limited to the intima, the injury cannot be very severe, but must rather be subtle. Avascular tissue, continuously stretched by the pulsatile nature of the blood flow imposing constant tension is one of the peculiarities of arteries. Therefore it is likely that susceptibility of the intima to subtle injury far exceeds other tissues' susceptibility. Among the injurious factors are many agents which are also risk indicators. It is, however, evident that the point of attack of all these factors may vary. It is therefore more useful to group them according to point of attack, i.e. blood factors, factors pertaining to structure and metabolism of the arterial wall and factors related to haemodynamics (Haust, 1970).

3.5 The arterial wall, blood components and haemodynamic forces in atherogenesis

From the foregoing it can be concluded that none of the proposed theories explains all features of atherosclerosis exclusively. There are a lot of interrelationships possible between the theories e.g. arterial injury and lipid influx; dietary fats, serum lipids, thrombosis tendency, arterial injury, intimal oedema etc. The theories are therefore not mutually exclusive but rather mutually complementary. Haust (1970) and Haust & More (1972) reformulated the existing theories into a universal concept, based on morphological and chemical knowledge about atherogenesis and taking into account the concept of the risk indicators.

The atherosclerotic process is of an episodic nature. It starts with any form of injury to either the endothelium or intima. These forms of injury include physical, chemical, metabolic and biological factors (see 3.4). Fatty streaks, gelatinous elevations and mural thrombi are all forms of initial or early lesions. Increased focal permeability with consequent insudation of blood components into the intima or microthrombi deposition or both may be consequences of local injury. Fatty metamorphosis of local smooth muscle cells following insudation or mural thrombosis may be the origin of fatty streaks. Local hypoxia, following, for example, intimal thickening, may be another route for developing fatty dots and streaks. Growth of all three kinds of lesions to advanced lesions may be the result of repeated episodes of insudation, mural thrombosis or haemorrhage, or a combination of these. All are every time followed by a process of partially or entirely succesfull organization. Degeneration of the connective tissue of the lesion promotes calcification, fissuring and secondary thrombosis. The thinning of the fibrous cap may result in ulceration and again a thrombus may be formed over the ulcerated lesion. All these processes lead to the conversion of advanced into complicated lesions and set the stage for the precipitation of clinical manifestations.

Each of the known risk indicators can now be envisaged as to the role it plays in the chain of events that ultimately ends in CHD. Any factor may act at one or more processes, involving the initiation, progression or complication of lesions or in the precipitation of clinical manifestations. Evidence for this is compiled in the preceding chapters.

Hypertension may act at the very beginning of the lesion as a mechanical injurious factor to the endothelium and intima. It may consequently provoke mural thrombosis and enhance the influx of blood constituents by the process of insudation. Hypertension not only initiates the lesion but also intensifies the insudative processes leading to the progression of lesions. By causing intimal capillary haemorrhages and plaque fissuring it may provoke complicated lesions. Finally by the same processes it may be a precipitating factor of clinical disease.

The plasma lipids may also operate at several levels. The insudate will be richer in lipids, the richer the plasma is. It will also be more difficult for the intimal cells to discharge from these lipids when present in lipid-rich insudates. Lipids may influence the progression of the lesion by the same mechanism. Also the quality of the lipids

may be a determining factor. By their influence on blood platelets and the thrombotic-fibrinolytic system they may also play an indirect role, via the formation of mural thrombi, in the initiating, progressive and terminating processes of atherogenesis.

The carbon monoxide of cigarette smoke and other sources probably directly injures the arterial wall, followed by an insudate. A continuously elevated carboxyhaemoglobin level is a continuous threat to the structural integrity of the endothelial lining, promoting recurrent episodes of insudation. The combined effect of hypoxia and carbon monoxide aggravates the injury and its consequences. The relationship of cigarette smoking to thrombosis tendency is another way by which smoking may act as a pathological factor. The role of nicotine is probably more important in the precipitation of the complications.

The three major risk indicators, blood cholesterol, hypertension, and cigarette smoking, can now be considered from a pathogenetic point of view as real risk factors implying a causative role in atherogenesis. From animal and epidemiological studies it is known that there is a continuum of risk increase by increasing one of these factors. An attempt should therefore be made to lower all risk factors till the lowest level possible, which is for cholesterol certainly below the average value of so-called normal, apparently-healthy people in the Western industrialized nations. But also a moderate decrease of the level of several risk factors in combination will decrease risk considerably. This forms the base for the coronary risk profile, as constructed by Gordon & Kannel (1973).

4 Nutrition and atherosclerosis

4.1 Arguments for and against several rabbit models

4.1.1 Cholesterol feeding

Following the early pioneer studies of Ignatowsky (1909), Starokadomsky (1905), Stuckey (1912) and, particularly the famous studies of Anitschkow (1913, 1914, 1933), cholesterol-induced atherosclerosis in the rabbit has become the most commonly used experimental model of this disease but at the same time the most criticized one (Prior et al., 1961; Clarkson, 1972; Scott et al., 1972). The main disadvantages may be shortly summarized as follows: the arterial lesions have generally been predominantly 'foam cell' lesions, with little or no necrotic gruel accumulating; complicated lesions, as characterized by ulceration, thrombosis, calcification, etc. have been reported only rarely; the lesions have frequently been limited to the intima, with little or no extension to the media, constituting an important difference from the human lesion; there has been a widespread deposition of lipid in the reticuloendothelial system, resembling a fat storage disease; after approximately 5 months of cholesterol feeding, rabbits begin to lose weight and have lipid deposits in kidneys, adrenal cortex, bowel mucosa, lymphatic nodules of the viscera, and the footpads; the distribution of lesions is slightly different from the human one, occurring more in the proximal aorta than in the abdominal part. Most strikingly different from the human situation is the tremendous hypercholesterolemia, roughly 40 times the normal level, amounting to 1000 - 2000 mg/100 ml. For a more detailed review and discussion on this subject, the reader is referred to the many reviews in the literature (e.g. Roberts & Straus, 1965; Constantinides, 1965; Adams, 1967; Zemplényi, 1968; Wissler & Vesselinovitch, 1968).

It has been found that most workers in this area employ a diet containing between 1 and 3% of cholesterol and can expect a good arterial response in about 60 days (Kritchevsky, 1964). This short duration of the experiments appears to be the main advantage of this model, although even then with much lower amounts of dietary cholesterol (e.g. 0,25 g/day) almost the same degree of atheroma formation may be induced with cholesterol values in the serum as high as 500 mg/100 ml. When the vascular lesions, to be induced, are only instrumental and not by themselves object of research, the matter of validity is not very relevant (Cranenburgh & Emde Boas, 1973). If, however, more is to be known about the pathogenesis, other models, which afford a longer experimental duration, are required.

4.1.2 Short-term cholesterol loading

If rabbits are exposed to lipemia for as long as is necessary for the production of foam cell lesions, and cholesterol feeding is then discontinued, the experimental lesions gradually turn into structures that are very similar to the advanced human plaques (Constantinides et al., 1960). Under such conditions in most of the lesions fibrous capsule and gruel develop and calcification occurs. The whole process takes at least 6 months and is intensified by additional time. Albrecht & Schuler (1965) demonstrated that after completion of 3% cholesterol feeding for 21 days a tremendous increase of aorta cholesterol occurred whereas serum cholesterol decreased rapidly during the next 80 – 120 days. The aortic cholesterol values were almost the same after continuous cholesterol feeding as after completion of cholesterol feeding at 21 days.

4.1.3 Intermittent cholesterol feeding

A modification of this 'loading' method was found as an even more effective way of producing such advanced atherosclerotic lesions. This method is based on exposure of rabbits to prolonged intermittent lipemia, i.e. to alternate periods of cholesterol feeding with periods on a normal diet (Constantinides et al., 1960; Constantinides, 1965) for 1 or 2 years. The main advantage of this method is that it permits the animals to live long enough with lipemia so as to develop the destructive elements of advanced atherosclerosis. Continuous feeding of cholesterol would lead to an accumulation of such tremendous quantities of lipid in their liver, spleen, kidneys, lung, etc. that they would die of cholesterol poisoning before having a chance to develop advanced plaques.

By intermittent cholesterol feeding the animals are able to recover and to dispose of a lot of the lipid excess during the cholesterol-free intervals. However, never sufficient time is allowed for their blood lipids to become normal again. During the normal diet-periods the intensity of the lipid attack on the arterial wall declines, which allows the characteristic processes of fibroplasia and regeneration to occur (Constantinides, 1965). The resulting changes in the atherosclerotic process by the healing periods make the lesions resemble that in man much more closely than those reported by the first method (Wissler & Vesselinovitch, 1968).

The lesions include a central area of necrosis filled with fatty gruel, including cholesterol crystals, a fibrous or fibromuscular 'capsule' that surrounds the necrotic atheromatous material, significant medial extension of the process, capillarization of the more advanced atheromatous plaques, haemorrhage in some of the severe plaques, calcification, etc.

Large thromboses in the aorta of rabbits with advanced atherosclerosis, by inducing hypercoagulability and acute hypertension, were produced by Constantinides et al. (1961, 1962, 1963). Microscopic thromboses in the coronaries were seen, but only if the animals were suffering from advanced atherosclerosis. In Buchwald's experiments (1965) myocardial infarction resulting from gross coronary atheroma was provoked in

40% of rabbits fed solely on a 2%-cholesterol diet for four months. The later stages of cholesterol-induced atherosclerosis in the rabbit, therefore, closely resemble the human disease. The early stage of the experimental condition should be regarded as a convenient exaggeration of the fatty streak lesions that are seen in the aortas of children and young adults (Adams, 1967, p. 61).

It is often argued that the high-cholesterol diet fed to rabbits is quite unphysiological and in no way represents the cholesterol intake in human diets. This argument was met by Beckel (1964) who showed that rabbits develop slight to moderate aortic atheroma when fed a simulated human diet for 7 – 12 months. This diet contained 0.1% cholesterol and the animals' daily consumption of this compound was calculated at about 100 mg, resulting in serum cholesterol values between 50 to 324 mg per 100 ml.

The same kind of experiment was carried out with rhesus monkeys (*Macaca mulatta*) (Wissler et al., 1965). It was demonstrated that atherosclerosis will also develop in this species with rather low levels of hypercholesterolemia produced by a table-prepared, average American diet for 107 weeks. In these studies, there was relative protection against hyperlipemia and atherosclerosis if a table-prepared 'prudent' diet, recommended by the American Heart Association, was substituted for the average American Diet. The low-density lipoproteins from the monkeys fed on the usual American diet appear to produce more sustained damage to the multipotential, medial, mesenchymal cell than do the same lipoprotein fractions from the monkeys fed on a prudent diet (Wissler et al., 1964). High serum cholesterol values have been likewise found in pet dogs (225 mg per 100 ml), consuming the habitual diet of their masters, compared with the low values (133 mg per 100 ml) observed in dogs consuming commercial laboratory biscuits (Schiller et al., 1964; Malmros & Sternby, 1968). It has been argued that the serum lipids of these dogs may very well reflect those of their masters or possibly an inverse relationship may exist between the owner, who does not eat fatty meat, and his dog which gets it (Adams, 1967, p. 62).

4.1.4 Cholesterol dose

High amounts of dietary cholesterol are obviously not needed for rabbits to induce a moderate hypercholesterolemia and extensive atheroma formation. This was clearly shown by Gottenbos & Thomasson (1961) who fed rabbits on low-fat diets containing 0.04, 0.08 and 0.16% cholesterol during a 52-week experiment. With the 0.04% cholesterol diet no increase in serum cholesterol was observed, but with 0.08% the values averaged at about 300 mg per 100 ml and with 0.16% at about 800 mg per 100 ml. The atheroma score, using an index from 0 – 4, was for the 0.04 diet 0.49, for the 0.08 diet 1.82 and for the 0.16 diet 3.27, closely reflecting the serum cholesterol values. The kind of fat in the diet profoundly influences the effects of cholesterol feeding in rabbits. Kritchevsky (1964) summarized a lot of these experiments and concluded two things: first, that in general the saturated fat is the atherogenic one, and second that among the saturated fats those containing shorter-chain fatty acids are more atherogenic.

This second conclusion may, however, be erroneous as the most frequently studied, in the longest experiments, were exactly those fats containing shorter-chain fatty acids, i.e. coconut oil and butter fat.

4.1.5 Fat and cholesterol

In the experiments of Anitschkow (1913) in which the animals only received vegetable oils, as sunflower-seed, olive, linseed, arachis, and cottonseed oil, without cholesterol, the animals never developed atherosclerosis. After Rittenberg & Schönheimer (1937) and Bloch & Rittenberg (1942) had found evidence for the endogenous synthesis of cholesterol from small units, finding that acetate was the precursor, and as fatty acids are broken down to acetate units, interest began to focus on the total fat content of the diet.

Low-fat diets were shown by Kempner (1949) and Keys (1953) to have the ability to depress the serum cholesterol content in patients with hypercholesterolemia. Attention to the sharp drop in death-rate from circulatory diseases during World War II was drawn by Malmros (1950), who showed that this drop was accompanied by a decrease in the consumption of milk, butter, cheese and eggs. A link between diet, dietary fat and serum cholesterol was proposed by de Langen in 1916. The possibility that the extent of unsaturation of dietary fat might influence the progression of atherosclerosis was mentioned by Snapper (1941) in his book 'Chinese lessons to Western Medicine'. He suggested that the low incidence of atherosclerosis in China might have something to do with the abundant amounts of unsaturated fat in the Chinese diet.

This thread was taken up by Groen et al. (1952) and Kinsell et al. (1952) who simultaneously demonstrated that vegetable fats, they had the luck not to have used coconut oil, significantly lowered serum cholesterol values while animal fats increased their level, both in patients and in ambulant human volunteers. These observations have been confirmed, extended and systematically elaborated by many investigators using human patients or volunteers, e.g. Kinsell et al. (1953, 1954, 1955), Ahrens et al. (1954, 1955, 1957), Beveridge et al. (1955, 1956, 1957), Malmros & Wigand (1955, 1957), Keys et al. (1957a, b, c, 1965a, b), Hegsted et al. (1965, 1968), Thomasson et al. (1967), Grande et al. (1972), Vergroesen et al. (1970) and many others. A more complete list of references can be found in the reviews of Van Schaik (1973), Reiser (1973) and Keys et al. (1974).

The investigations showed that the consumption of oils with a high content of polyunsaturated fatty acids tends to decrease serum lipids while fats with predominantly saturated fatty acids with a chain length of 12 to 18 carbon atoms tend to increase the same lipids.

4.2 The ideal animal model

Though information has been gained by looking at serum lipids on different dietary fats in man, the need for satisfactory animal models arose in part from the defects of man as a model. Scott et al. (1972) formulated the properties which the ideal animal model should possess as follows. It should be possible to induce atherosclerosis in the animal rapidly, without resource to either expensive or extremely unphysiological means; the animal itself should preferably be large enough so that there will be enough tissue for multiple studies; the animal preferably should be in plentiful supply, inexpensive and easy to house and feed; the lesions produced experimentally should closely resemble those seen in the human. An animal model satisfying all of these requirements has yet to be developed. The rabbit, however, fulfilled most of these criteria.

In nutrition experiments, the need for exogenous cholesterol, applied in huge amounts, producing arterial lesions within three months of time, was a serious drawback. This was overcome by Steiner & Dayton (1956) who fed their rabbits on a diet consisting of 50 – 75 per cent of ground peanuts, without cholesterol and produced an increase in serum cholesterol to several times the original value in rabbits. After 10 – 12 months 2 of 33 animals showed small areas of aortic atherosclerosis.

4.2.1 Cholesterol-free and semi-synthetic diets

Wigand (1959) and Lambert et al. (1958) described a method to produce hypercholesterolemia and arterial lesions in rabbits without exogenous cholesterol using purified or semi-synthetic diets. Lambert et al. (1958) fed their rabbits either on hydrogenated coconut oil or on safflower oil at 40% of the calories. Casein (25% by weight) was used as the dietary protein. All animals were pair-fed, that is daily food ingestion was limited to an amount which all rabbits would eat. After 100 days on experimental diets, plasma cholesterol level was elevated 12 times over normal by coconut oil and only 1.5 times by the safflower oil diet. All animals on coconut oil had clear-cut aortic atheromatous lesions, whereas only 4 of 10 on safflower oil showed any aortic involvement. All animals on safflower oil survived the experiment while 3 out of 10 on coconut oil died. Moreover, rabbits on the coconut oil diet evidenced considerable loss of hair and a general 'ruffled' appearance, while those on the safflower oil were robust and apparently healthy.

The simultaneously, but independently reported data of Wigand (1959) are in agreement with the former. He used a diet, consisting of 26% (of calories) fat and 34% (of calories) casein. Significant effects of different fats could be demonstrated. Feeding butter fat or hydrogenated coconut oil for about 4 months resulted in a distinct hypercholesterolemia with subsequent gross changes in the aorta, resembling those seen in cholesterol-induced atherosclerosis. Feeding rapeseed oil or corn oil produced only slight changes in the serum cholesterol and no aortic lipid deposits. A great variability was found in the onset and the degree of hypercholesterolemia, suggesting

a kind of resistance in the animals. The feeding of oil was accompanied by weight gain while several animals on the coconut oil showed moderate weight loss. Supplements of linoleic acid improved weight gain. While serum triglycerides also increased on the coconut oil diet, they did not on the butter diet and are therefore not considered essential for the development of experimental lesions. Most of the animals on coconut oil and butter fat lost hair during the four months period but when kept for a year the hair regrew spontaneously without any change in the diet. No definite correlation was found between the fur changes and loss of body weight. The nigh fat content of the diets caused no troubles as to digestion and absorption, the absorption percentages ranging from 94 to 100%. A description of the lesions of these animals was given by Stormby & Wigand (1963) as severly sclerotic, comparable to those seen in man.

4.2.2 Mechanism of action of semi-synthetic diets

This model has been used by several investigators during the last decade. It has, however, several limitations. Wilson et al. (1973) pointed to the disappointing results they got with these diets because of the rather poor weight gain, the degree of fatty liver produced, and the high mortality. These difficulties have also existed in studies of others (Funch et al., 1962; Kritchevsky & Tepper, 1964, 1968; Vles & Kloeze, 1967; Vles et al., 1964).

Because of the divergent results obtained when fats are added to a common chow diet for laboratory rabbits compared with a semi-synthetic diet several groups of investigators have tried to find out which component of the chow diet was protective (Kritchevsky & Tepper, 1968; Howard et al., 1965) or which factor in the semi-synthetic diet was lacking or deleterious (Wilson et al., 1973; Carroll, 1971). Howard et al. (1965) concluded that an extracted soya bean meal contained a factor which was protective in rabbits for the effects of saturated fats. They reasoned that such a factor, which was never exactly defined, might be similar to the protective factor in chow diets or the rabbit's natural diet.

Kritchevsky & Tepper (1968), starting from the chow diet, extracted its fat and added this to a semi-synthetic diet with 12% (W/W) hydrogenated coconut oil. They observed no protection by the fat, extracted from the chow. The fat-extracted chow with 14% hydrogenated coconut oil added, caused only slightly higher serum cholesterol values than the complete chow, supplemented with 14% of the oil. No differences were observed in degree of atheroma formation. These data suggest that the complete chow is required to overcome the effects of coconut oil on serum lipids, but that the fat-extracted chow residue causes the main part of this action, at least over a six month feeding period. It was suggested that the carbohydrate source might be of importance. However, starch was found in subsequent experiments to be more hypercholesteremic and atherogenic in rabbits on a semi-synthetic diet than glucose (Kritchevsky et al., 1968; Kritchevsky et al., 1973). Starch was the main carbohydrate of chow and glucose the carbohydrate in Kritchevsky's experiments.

4.2.3 The role of protein

That protein might be the determinant factor in this kind of diet was indicated by the work of Carroll (1967, 1971). He observed a moderate increase of plasma cholesterol levels when casein was added to commercial diets, except when poly-unsaturated oils were present. This was not a dilution effect as added dextrose had little or no effect. Also the experiments of Howard et al. (1965) and Kritchevsky & Tepper (1968) do not exclude the possibility that the casein as dietary protein is the mediating factor for the effects of different fats. In rats, thiouracyl was always required as a hypercholesterolemic agent, but Renaud & Allard (1964) demonstrated that this substance could be omitted from the diet providing the protein, supplied as casein, content of the diet was reduced from 22 to 10%. It was shown that the precipitating factor for both hypercholesterolemia and trombus formation was a methionine deficiency (Renaud, 1966). The effects of dietary protein level and quality will be discussed later 4.7).

In studying effects of different dietary fats on blood lipids and atherosclerosis, using rabbits on semi-synthetic diets as a model, the modifying effects of proteins other than casein should be studied likewise. This may not only lead to an improved model but also to a better understanding of the role played by all dietary components in their mutual relationships.

4.3 Nutritional requirements of rabbits: fat, fibre, vitamins, minerals, protein and amino acids

Though the rabbit is one of the most frequently used experimental animals in this field of research, its nutritional requirements are still poorly understood. The National Academy of Sciences, National Research Council (NAS/NRC) (1966) stated not very long ago, e.g., that the rabbit is relatively insensitive to the quality of the dietary protein. The literature concerning the protein requirements tends however, to the opposite view (e.g. Cheeke & Amberg, 1972), indicating that the rabbit has very particular protein and amino acid requirements.

A general objection against the use of the rabbit as experimental animal in atherosclerosis research is its herbivorous nature. This would imply that dietary *fat* is not very well tolerated and that large amounts of dietary fibre are needed and also used by the rabbit. The question of the optimal amount of fat to be incorporated in purified diets was investigated by Thacker (1956). It was in fact shown that 5% fat (by weight) resulted in the lowest weight gains, while higher amounts from 10 to 25% (by weight) were equal in this respect. The apparent digestibility of the fat, a hydrogenated vegetable oil, was between 97 and 99 per cent for all levels. Wigand (1959) determined the absorbability of the several fats in his experiment, incorporated at a level of 8% (by weight), as between 98.0 and 99.9% with only one exception, rapeseed oil with an absorption percentage of 94.3.

In the experiments of Carroll (1971) different fats were added to a commercial diet

at the 15% (by weight) level. This resulted in only a slightly decreased weight gain as compared with the controls on the commercial diet alone containing only 3% fat. If one takes into account that by this procedure other nutrients, particularly protein, must be diluted, these results as well as the former ones cannot be taken as evidence for adverse effects of fats per se in rabbit diets.

The optimal amount of *fibre* in purified diets was studied by Gaman et al. (1970) at the 7, 15 and 30% levels. They found no differences in performance or weight gain of the rabbits. On practical grounds they suggest 12 – 16% fibre. Contrary to common belief, the rabbit digests fibre extremely poorly. For example, Slade & Hintz (1960) found the digestibility of alfalfa fibre to be 16.2% in the rabbit, compared to 34.7 for the horse, 38.1 for the pony, and 38.2 for the guinea pig. The digestibility of alfalfa fibre in swine diets has been estimated at about 35 – 40% (Danielson et al., 1969). Probably the source of fibre and the composition of the diet influence this digestive process, as Hoover & Heitman (1972) observed a digestibility rate for a mixed fibre in rabbits of about 34%. This value was the same whether low or high fibre diets, 14.7 or 29.4% respectively, were fed. They estimated that caecal fermentation in rabbits produced a quantity of volatile fatty acisd equivalent to 10 to 12% of the daily basal caloric requirement.

The rabbit does not appear to require *Vitamin C* (Harris et al., 1956). The content of blood and organs after 25 weeks on a diet free from ascorbic acid was of the same order as in rats or chicks while the excretion in the urine continued. The rabbit requires *niacin* (Wooley & Sebrell, 1944) on a purified diet, *choline* (Hove et al., 1954), *pyridoxin* (Hove & Herndon, 1957a) and probably all *B vitamins* (Hogan & Hamilton, 1942) because supplementation of casein diets with yeast or liver extract promoted growth. Kunkel et al. (1948) also noted the beneficial effect of liver extract for rabbit growth and maintenance of the fur. *Vitamin A* (Payne et al., 1972) and *E* (Jenkins et al., 1970) requirements were confirmed and estimated at 600 and 25 – 50 iu per kg diet respectively.

The rabbit has a high *potassium* requirement (Hove & Herndon, 1955) and perhaps a high *sodium* and *calcium* requirement (Wooley & Mickelsen, 1954). Gaman et al. (1970) produced evidence that rabbits require a large amount of *magnesium* to cease pulling and eating their fur. The peculiar mineral metabolism of rabbits was studied by Cheeke & Amberg (1973). Rabbits excrete large quantities of calcium in the urine, as compared with rats. Amounts up to 60% of those ingested are found in the urine. The urine may contain such large amounts of white precipitate that it looks cloudy or creamy (Flatt & Carpenter, 1967). Besides this peculiarity of the rabbit's calcium metabolism, the rabbit is exceptional in that the plasma calcium level reflects the dietary level (Chapin & Smith, 1967). Also for magnesium the urine is the major route of excretion, resembling calcium (Cheeke & Amberg, 1973), while for phosphorus the major excretory route was the faeces. Though a high dietary fat level (20%) reduced calcium availability somewhat, still more than 50% of the ingested calcium (4%) was excreted in the urine. Of interest was the observation that high calcium ingestion resulted in marked negative magnesium balance.

The literature on *protein* and *amino acid* needs is still more fragmentary and conflicting. Hogan & Hamilton (1942) and Kunkel et al. (1948) improved their casein diets for growth rate and appearance of fur by adding yeast or liver extract. Thacker & Ellis (1948) observed a low rate of growth on a diet of whole cow's milk supplemented with vitamins and minerals. The high lactose content of milk may, however, cause extreme variation in anorexia and occasional diarrhea (Wells & Anderson, 1959). The poor growth of rabbits on casein was extensively studied by Wooley (1954) and Herndon (1956).

Wooley (1954) reported improved growth when 40% kale meal or alfalfa meal was added to the otherwise purified diet, or when the percentage of casein was increased from 20 to 30%. In this last case, however, the mineral mix was different and in a subsequent experiment he demonstrated that the potassium content of the diet is very important. When casein was increased to 30% in the low-potassium diet (0.6%), 8 of 11 rabbits died between the second and fourth week of the test. After supplementation with potassium all animals lived and almost doubled their weight gain. From these experiments it is concluded that if casein is the sole protein source the requirement is 25 - 30%. If the protein is partly or completely from vegetables, as in the kale and alfalfa experiments and in commercial food, equally good growth will be attained with 20% protein. The 30% casein level is probably harmful for the rabbit unless potassium is increased. On a 20%-casein diet 0.8% potassium was found as minimum. The potassium requirement of 0.8%, is five times that required by the rat.

In later experiments, Wooley & Mickelsen (1954) confirmed these earlier findings and demonstrated that the protective effect of high potassium intake was also exerted by sodium and calcium, resulting in increased growth and decreased mortality, especially on diets containing 30% casein. A similar high requirement for potassium was estimated by Hove & Herndon (1955).

Herndon (1956) and Hove & Herndon (1957b) reported the results of a large series of experiments which were undertaken to discover why rabbits grow exceptionally well on soya bean meal but do very poorly on purified casein diets. Increasing the casein in the diet stepwise from 10 till 50% resulted in increased weight gain, but neither was the growth reached with commercial diets, containing only 18% protein, nor was the effect maintained after 6 - 7 weeks on this diet. About one-third of the animals fed on the casein diets showed marked loss of hair, regardless of the casein level. Soya bean meal at 40% of the diet, containing 50% protein, produced almost the same growth as the commercial diet. The same growth was also reached by supplementing 20% casein with 15% soya bean meal. The growth promoting factor of soya bean meal must be present in the water-insoluble residue of the meal. Amino acid supplementation to 20% casein, with arginine, glycine, methionine and tryptophan produced erratic but positive results, reaching the weight gain on 50% casein but remaining for below soya bean or yeast supplemented groups.

The digestibility of the casein appeared not to be disturbed in vitro (Herndon, 1956) as was already shown by Thacker in vivo (1956), but an enzymatic digest of casein produced slightly better growth than casein itself. Though the digestibility

studies indicate that rabbits digest casein efficiently, they also indicate (Herndon, 1956) that rates of consumption and fecal excretion of rabbits fed on casein diets were low. Eighty percent more nitrogen matter was present in the caeca of rabbits fed 28 per cent casein diets than in the caeca of rabbits fed the soya bean meal diets or the casein hydrolysate diets. The dried caeca of casein rabbits were heavier than of the 'soya rabbits'. This may indicate a type of intestinal stasis with slower passage of ingesta through the tract. From these data it is tempting to speculate that casein has specific characteristics and does not lend itself very well for determination of protein requirements in rabbits.

After surgical removal of the ceacum (Herndon & Hove, 1955) growth of 8-week-old animals was only slightly below that of normal animals on the same 25% casein diet. Digestibility of fat and protein were somewhat decreased but of cellulose was normal. The losses of sodium and potassium were five times normal. The two types of faeces, commonly occurring in rabbits, remained. One type hard, dry and white. The other type dark irregularly shaped and moist. This last type did not show the high protein content the mucous covering or the grape-like clustering of true soft faeces. The two differed only in the presence of bile in the dark. The animals did not eat their faeces. This experiment once again points to the relatively unimportant role of the caecum of rabbits.

As was already observed by Wooley (1954) an amount of about 20% mixed vegetable proteins may be sufficient for maximal growth. This amount was further decreased in experiments of Merkusin (1966) who observed no growth improvement between 13.5 and 16.8% protein. For rabbits of all ages and at all stages, including pregnancy, Gaman et al. (1970) developed a semi-synthetic diet containing 20% soya bean protein, supplemented with 0.2% DL-methionine. Diets based on natural food stuffs and supplemented with fish meal, cottonseed meal and soya bean meal, such that these provided about half of the 15% crude protein of the diet, were compared by Cheeke & Amberg (1972). Only cottonseed meal was inferior but was restored by supplementing with methionine and lysine.

Spreadbury (1974) estimated the protein need supplied by a natural mixed diet at 14 - 15% crude mixed cereal/fish meal protein. The protein quality was investigated by him by supplementing half the crude protein in an oat-based diet by a variety of protein concentrates. Fish meal, soya bean meal or casein gave good growth approaching the mximal weight gain, but groundnut meal, gelatin or maize gluten produced significantly lower weight gains. These results indicate that on a natural diet or when casein is not the sole protein source, the amount and quality of the protein are not very critical. For obvious reasons, however, it is more attractive to use in dietary experiments a well-defined protein like casein than a crude product like soya bean meal or a protein isolate. Therefore attempts have continued to define the amino acid requirements of the rabbit to try to supplement the limiting ones to casein.

After Hove & Herndon (1957) had found variable results with the supplementation of casein with arginine, glycine, methionine or tryptophan, the next investigation was reported by McWard et al. (1967). They confirmed the better growth on a higher

casein level, 3.75% nitrogen, than on a lower level, 2.75% nitrogen. The arginine level, required to produce maximal growth, was estimated as 1.23% of the diet or 5.14% of the protein for the high casein level and 0.98% of the diet or 5.59% of the protein for the diet containing 2.75% nitrogen. Gaman & Fisher (1970) confirmed the essentiality of arginine and also showed methionine and lysine to be required for growth.

By omission of single amino acids from a mixture of amino acids patterned after the composition of methionine-supplemented soya bean protein, Adamson & Fisher (1971) were able to ascertain the essentiality of histidine, arginine, glycine, isoleucine, leucine, lysine, threonine, tryptophan, valine, phenylalanine and methionine. Arginine and isoleucine omission resulted in neither weight loss nor weight gain, while glycine appeared only useful for rapid growth. Of interest was their observation that the weight gain on the amino acid mixture was only moderately decreased as compared with isolated soya protein but that an amino acid mixture resembling whole-egg protein produced no gain at all.

Approximate values for the requirements of lysine, methionine and arginine were established by Cheeke (1971) in diets composed of natural feedstuffs. Levels of 0.88% arginine, 0.93% lysine and 0.45% methionine are suggested. A significant reduction in growth was observed at a lysine level calculated to be 1.63% of the diet. Absence of growth on diets containing zein or gelatin as protein indicates that the assumption of the NAS/NRC (1966), that bacterial synthesis of amino acids in the caecum and colon renders the rabbit insensitive to protein quality is no longer tenable.

The high arginine requirement and the sensitivity for adverse effects of moderately increased lysine amounts, compared with rats (Acheampong-Mensah & Hill, 1970), show that the rabbit resembles the chicken in this respect (Jones, 1964). Uric acid represents a negligible fraction of the total nitrogen excreted in the urine of rabbits (Cheeke & Amberg, 1972), urea accounting for about 60% of the total nitrogen. Thus it is apparent that the high dietary arginine requirement is not due to a lack of enzyme activity in the urea cycle. This was also confirmed by feeding benzoic acid which had no detrimental effects on growth, in contrast to the situation in chickens (Scott et al. 1969). Benzoic acid is normally detoxified by reacting with glycine to produce hippuric acid, excreted in the urine. In chickens benzoic acid is detoxified by conjugating with ornithine, which arises from degradation of arginine and thus precipitates an arginine deficiency.

Major progress has been made in the area of quantitative amino acid requirements while the experiments reported in this thesis were almost completed. Adamson & Fisher (1973) supplied the protein needs as amino acids simulating the composition of methionine-supplemented isolated soya protein. The non-essential amino acids were replaced by glutamic acid and glycine in a 50:50 ratio. The rabbits were held as usual in nutrition experiments, i.e. individually housed, in metal cages, in a temperature-controlled laboratory, on wire screen bottoms which allowed faeces and urine to pass. The rabbits had little contact with their voided faeces although no special attempt was made to prevent coprophagy. At least four levels of each amino acid

under investigation were tested in alphabetical order. Maximal weight gains were obtained at the following levels: arginine, 1%; histidine, 0.45%; isoleucine, 0.70%; leucine, 0.90%; lysine, 0.70%; phenylalanine, 0.60%, including tyrosine need; methionine, 0.60%, including cystine need; threonine, 0.50%; tryptophan, 0.15%; valine, 0.70%.

The requirements are estimated for rapidly growing 6 – 7 week-old rabbits, based on pure amino acids, over a test period of 8 days with great interindividual variability, and therefore need confirmation under differing conditions. It is evident that the rabbit is rather peculiar in its amino acid requirements. Also the growth-depressing effect of several amino acids above their optimal level, as was shown for lysine, at levels of 0.9 and 1.0%, for isoleucine at 0.8%, for phenylalanine at 0.9%, and for threonine at 0.7 and 0.8%, support the hypothesis that the rabbit is very sensitive for an amino acid imbalance. In a diet of 20% casein these are the very amino acids which are present far in excess of the requirements, lysine 1.7, isoleucine 1.2, phenylalanine and tyrosine 2.1, threonine 0.9%, while the sulphur-containing amino acids are just sufficient at 0.6% and arginine is slightly below this requirement at 0.8% (personal calculation).

The concept of amino acid imbalance might explain many of the problems encountered in using casein as the sole dietary protein (Harper, 1959; Nasset, 1972). Anorexia is very easily induced in experimental animals fed on amino acid imbalanced diets and demonstrable in about two days (Kumta & Harper, 1960). It is suggested by Fisher et al. (1960) that the homeostatic mechanism against the build-up of free amino acids and nitrogenous and other degradation products resulting from the catabolism of the excess amino acids involves the regulation of food intake. The imbalance manifests itself in a reduced food consumption and therefore a reduced consumption of the limiting amino acid. In view of the complex effects undernutrition may have on cholesterol metabolism and atherogenesis in rabbits (Kritchevsky, 1964), it is very important to provide the animals with a nutritionally balanced and adequate diet.

4.4 Diet, blood lipids and atherosclerosis

4.4.1 Introduction

The major role played by blood lipids and lipoproteins in atherogenesis and CHD, either directly or indirectly, has been established (see sections 2.2, 2.2.2.3, 3.3, 4.1). Much attention is given to the effects of different dietary components on blood lipids or atherogenesis either in man or animals. For details and many references regarding studies about the relationship between dietary fat and blood lipids in man the reader is referred to the extensive literature, e.g.: Beveridge et al., 1956; Hegsted et al., 1965, 1968; Keys et al., 1965a, b, 1974; Fidanza et al., 1970; Inter-Society Commission for Heart Disease Resources, 1970; Jones, 1970; Brown, 1971; Fidanza, 1972; Grande et al., 1972; Shaper, 1972; Vergroesen, 1972, 1975; Reiser, 1973; Van Schaik, 1973;

and many others.

Even more investigations have been reported about the effects of dietary fats on blood lipids and atherosclerosis in many animal species, e.g. Malmros & Wigand, 1960; Kritchevsky, 1964; Büller & Vles, 1966; Adams, 1967; Frahm & Lembke, 1967; Wissler & Vesselinovitch, 1968; Clarkson, 1972; Scott et al., 1972; Jacobson, 1974. It has been a long way from the first observations by Groen et al. (1952) and Kinsell et al. (1952), who reported different effects of different fats on blood lipids till the sophisticated knowledge which is available now.

Most animal studies provide evidence for a simple, straight relationship between the level of blood lipids, mainly cholesterol or beta-lipoproteins, and the degree of atherosclerosis. A major point of interest has been the way dietary fats or fatty acids affect those blood lipids. The cholesterol-lowering effect of oils containing predominantly polyunsaturated fatty acids of which linoleic acid is the main representative, has attracted most attention. This has led to the suggestion that, depending on the amount of dietary cholesterol (200 – 550 mg), 15 – 23 per cent of calories should be supplied by poly-unsaturated fatty acids to achieve a maximal reduction in serum cholesterol of 18 to 22 per cent (Brown, 1971). McGandy et al. (1972) did not reach this maximal reduction with 13 per cent of calories as linoleic acid while Vergroesen (1972) reported that on 'normal' diets, without restrictions of cholesterol-containing foods, supplying 20 – 25 per cent of calories as linoleic acid, cholesterol was reduced with 20 – 25%.

Interest in linoleic acid is not only justified with regard to this cholesterol-lowering effect. Many other aspects of this lipid are not less interesting. Linoleic acid may act on processes in relation to atherosclerosis through: lowering of blood lipids (e.g. Brown, 1971; Vergroesen, 1972); decreasing thrombosis tendency (e.g. Hornstra, 1975); providing substrate for prostaglandin synthesis (Thomasson, 1969); changing lipids into less sclerogenic compounds (Adams, 1967; Gottenbos, 1975).

That diet as a whole, and not only linoleic acid, may have profound influence on blood lipids was recently shown by a very practical experiment of Anderson et al. (1973). They compared the effect of four diets on blood lipids in man. The diets were either a rich diet, with 42 per cent of calories as fat and rich in eggs and butterfat, a standard American diet, with 40 per cent of calories as fat and 570 mg cholesterol and two cholesterol-lowering diets, L-1 and L-2, intended to decrease cholesterol moderately or strictly. The L-2 diet was designed with the use of all kinds of cholesterol-lowering ingredients known, as fruits and cereals, mature leguminous seeds, elimination of butterfat, one egg a week, reduction of fat calories till 27 per cent and increase of poly-unsaturated fats till 14 per cent of calories. Compared with the standard diet, serum cholesterol increased on the rich diet by 9% and decreased on the L-1 diet by 17%. These changes were nearly equal to those which could be expected from the changes in dietary fat and cholesterol and could have been predicted with the predictive formula of Keys (1965b) based on middle-aged man. The decrease on the L-2 diet of 29 per cent was much greater than could be predicted. It is likely that the larger decrease in cholesterol was due to other changes in the diet, such as the increased

amount of fruits, vegetables (Keys et al., 1961) and dry leguminous seeds (Grande, 1967).

A similar phenomenon is also observed when several populations with widely divergent saturated fat intakes are compared (Keys, 1970). Here the differences between populations in serum cholesterol levels are almost twice the expected differences, based on the predictive formula. Other food components may play a role in enhancing the difference or a lifelong consumption of a diet affects serum cholesterol possibly more than in a short-term experiment (Fidanza, 1969).

4.4.2 The role of sugar

The importance of the role of sugar in atherosclerosis was put forward by Yudkin & Roddy (1964). Sucrose may act either through effects on blood lipids, on platelet adhesiveness or hormone balance (Yudkin, 1972). Arguments against this hypothesis are that epidemiological investigations do not support the hypothesis (Stamler et al., 1972; Paul et al., 1968; Elwood et al., 1970), that effects on blood lipids, particularly triglycerides, are minimal (Anderson, 1967; Grande, 1967) or may be related to caloric imbalance (Dunnigan et al., 1970), and last that in nonhuman primates atherosclerosis was variably induced by either sucrose or dextrin (Lang & Barthel, 1972) or not induced at all (Armstrong et al., 1970) by sucrose.

Of interest is the observation by Mcdonald (1967) of an interrelationship between the influences of sucrose and saturated fat in normal people. This was also confirmed by Nestel et al. (1970) in normal people and by Antar et al. (1970) in hyperlipoproteinemic subjects. If simultaneously present in the diet the triglyceride-lowering effect of polyunsaturated fat is greater than the increasing effect of fructose (Macdonald, 1972). Therefore the type of fat is of greater significance than the type of carbohydrate in terms of blood lipids. As it is outside the scope of this thesis to discuss these relationships in detail, the reader is referred to some authoritative reviews (Bender & Damji, 1972; Albrink, 1973; Macdonald, 1973; Yudkin, 1972; Dalderup & Van Haard, 1971b).

4.4.3 The role of milk fat

Milk fat has been used in many experiments since the effects of dietary fats on blood lipids and atherosclerosis were first studied. Though this fat constitutes an important part of the diet in populations where cardiovascular diseases are endemic, detailed knowledge about the effects of its several constituents is grossly lacking. Milk fat, from cow's milk, is characterized by the presence of short and medium chain-length fatty acids, a low content of linoleic acid and a fair amount of cholesterol. A discussion on the hyperlipidemic role of milk fat should therefore pay attention to these three points. Lastly the scanty literature on dietary protein, blood lipids and atherosclerosis will be reviewed, as well as some phenomena related to milk consumption.

4.5 Milk fat, cholesterol, blood lipids and atherosclerosis

In the numerous studies reported in the literature, comparing effects of saturated and unsaturated fats, mainly coconut oil or milk fat are used as representatives of the saturated fat group. Therefore a wealth of information is available relating the effects of milk fat to other fats in many species. However, studies to define the components in milk fat which are principally responsible for its properties are scarce.

4.5.1 Findings with various animals

In *rats* the high serum cholesterol, induced by 20% coconut oil could be decreased by sunflower-seed oil or fish oil, was not influenced by peanut oil but was enhanced by butter or tallow (Wilkens & Witt, 1962). The effects on serum cholesterol were proportional to the amounts of fat in the diet. In a comparison between 40% butter, corn oil, butter oil, or lard the lowest serum cholesterol values were observed with corn oil, the highest with butter or butter oil (Suzuki, 1959). Coleman & Beveridge (1960) found no effects of dietary fats in rats without bleeding stress but observed a similar cholesterol increase for corn oil and butter with severe bleeding stress. Amounts of cholesterol, equal to that in butter or fractions of the butter were likewise without effect. Butter and several margarines, composed largely of hydrogenated fish oil, coconut oil and rapeseed oil were compared at three weight levels, 12, 24 and 48% in rats by Funch et al. (1960a) in purified diets. At the high intake level butter induced the highest serum cholesterol values whereas at lower levels the differences became smaller, but still with the highest values for butter.

In *chickens*, blood lipids are strongly related to dietary protein, but independent of dietary protein blood cholesterol values were always higher on butter or lard than on unsaturated fats as cottonseed oil, corn oil or linoleic acid at the 10% level (Stamler et al., 1959). Brechter (1960) confirmed the hypercholesterolemic effect of butter and coconut oil compared with corn oil in cockerels.

Essentially the same trend is observed in experiments with *dogs* fed on diets supplemented with cholesterol and thiouracil. Sunflower-seed oil induced much lower values of serum cholesterol than butter (Dolidze, 1962). After thyroidectomy a mixture of lard and butter provoked a hypercholesterolemia, while fish-liver oil reduced serum cholesterol (Ancona & Coloza, 1963) compared with a standard diet. Butter or butter with cholesterol induced only a moderate hypercholesterolemia, while the supplementation with thiouracil induced very high cholesterol values and severe atherosclerosis. With corn oil these changes were less extensive or absent (Guidry & Wu, 1967).

In *rhesus monkeys* fed on butter-enriched diets higher blood cholesterol levels and more extensive atherosclerotic lesions were observed by Pickering et al. (1961), Wissler et al. (1965), and Greenberg & Wheeler (1972), compared with a mixture of vegetable oils, a 'prudent' diet or several infant formulas, including human milk. Though butter caused higher serum cholesterol levels in rhesus monkeys fed on

cholesterol-containing diets than peanut oil, intimal proliferation and fibrosis were more severe on peanut oil (Vesselinovitsch et al., 1974).

The addition of butter to the diet of *swine* caused a significant elevation in serum cholesterol levels. This did not occur in animals fed on isocaloric amounts of corn oil (Bragdon et al., 1957). After nine weeks arterial lesions were present in both groups with the same incidence. Rowsell et al. (1958) studied blood lipids, atherosclerosis and coagulation indices in swine fed either a low-fat diet or isocaloric diets rich in butter or margarine (not specified). Some atherosclerosis was found on the low-fat and the margarine-supplemented diet, while butter caused a considerable increase in the amount of atherosclerosis. Blood lipids did not increase on either margarine or butter. The coagulation indices were most active in the butter-fed swine and appeared to parallel the degree of atherosclerosis.

In a later experiment the same group (Rowsell et al., 1960) compared the effects of butter and egg yolk on the development of atherosclerosis and related factors over a period of one year. The swine fed on egg yolk had approximately six times as much aortic atherosclerosis as the controls that eat the commercial pig ration, and the butter-fed group three times as much. This holds not only for the aorta but for the coronary arteries as well. The blood lipid levels were only slightly elevated with butter but significantly with egg yolk. Both groups showed a positive relationship between changes in the activity of clotting and extent of aortic atherosclerosis.

4.5.2 Findings with rabbits

Increased serum cholesterol levels were induced in rabbits after incorporation of butter or butterfat in their diets (Wigand, 1959; Gresham & Howard, 1962; Kobernick et al., 1964; Funch et al., 1960b, 1962; Krogh et al., 1961; Wilson et al., 1973; Carroll, 1971; Moore & Williams, 1963, 1964a, b, c; Moore, 1969; Moore & Noble, 1969; Malmros et al., 1964). They mainly used semi-synthetic diets with minor modifications and without exogenous cholesterol.

In the study of Wigand (1959) 26% of calories were fed as fats. Butterfat was compared with hydrogenated coconut oil, corn oil, rapeseed oil and glyceryl trilaurate. While the serum cholesterol values rose only slightly on the corn and rapeseed oil diet, the levels on butterfat increased from 29 to 358 mg per 100 ml on the average. A hypercholesterolemia still more marked was noted in the coconut oil group. The values with trilaurate feeding were comparable to those on coconut oil. The addition of linoleic acid or corn oil (13% of the calories) to the glyceryl trilaurate ration induced only half the increase in cholesterol. The serum triglyceride values were highest on coconut oil, intermediate on butterfat and lowest on corn oil and rapeseed oil. Trilaurate again was comparable with coconut oil, while supplementation with linoleic acid induced smaller increases. The increase in serum lipids on coconut oil, butterfat and trilaurate was entirely reflected in increased β-lipoproteins. A close correlation was reported between the severity of aortic atherosclerosis and the concentration of free ($r = 0.77$), esterified ($r = 0.82$) and total ($r = 0.82$) cholesterol in

the serum after an experimental period of 4 months.

In subsequent experiments (Malmros et al., 1964) it was shown that neither groundnut, corn, rapeseed, poppy-seed, or cottonseed oil induced increased cholesterol levels in rabbits while hydrogenated oils enhanced the serum cholesterol levels.

The effects of pure fatty acids, fed as triglycerides, were studied by the same group. Neither butyric acid, palmitic acid, stearic acid, or behenic acid produced an elevation of serum cholesterol. Absorbability problems must have interfered with the results for the longer chain acids. Caprylic acid was slightly hypercholesterolemic, but capric, myristic and particularly lauric acid induced marked increases in serum cholesterol and atherosclerosis. As serum cholesterol and atherosclerosis also increased on a fat-free diet, devoid of saturated and unsaturated fatty acids, it was concluded that atherosclerosis is a deficiency disease, i.e. a relative deficiency of linoleic acid. Malmros et al. (1964) could, however, find no biochemical or biological signs of such a deficiency in their animals. The possibility of a cholesterol-increasing potency of their basal semi-synthetic diet, otherwise than by the absence of linoleic acid, was not consedered.

Moore & Williams (1964a) also observed that a low-fat diet, supplying exactly the same quantity of linoleic acid as a 35% (of calories) butter-containing diet induced the same degree of atherosclerosis but definitely lower cholesterol values in serum. Butter and an equal amount of butterfat, on a fat-base, induced similar changes. Rabbits on corn oil had no atherosclerosis and the lowest cholesterol values. Though the mean cholesterol values for butterfat-fed and coconut oil-fed animals over the entire period were about equal, the graphs showing the course of serum cholesterol over the entire period for these two fats were quite different. Butterfat induced a rapid rise in serum cholesterol and maintained a constant level afterwards while coconut oil remained for a long period (29 weeks) rather low and than started to rise, which continued till the end of the experiment at 37 weeks. Both fats induced the same degree of atherosclerosis. The incidence of coronary atheroma was similar to the incidence of aortic atheroma in the various groups.

Substitution of corn oil for half of the butterfat induced lower serum cholesterol values than the butterfat alone, almost equal to those on a low fat diet, but with definitely less atherosclerosis, almost equalling the rabbits given the corn oil diet (Moore & Williams, 1964a). Substitution of 10% corn oil for the corresponding amount of starch led to similar findings. From these observations, Moore & Williams (1964a) concluded that saturated fats per se do not exert a positive atherogenic effect but that the atherogenic properties of the high-starch and high-butterfat diets may be ascribed simply to their lack of polyunsaturated fatty acids. Their conclusions are, however, based on experiments, resulting in very small differences in serum cholesterol at relatively low absolute levels and with an extreme variability. No direct relationship was found (Moore & Williams, 1964b) between the degree of aortic atherosclerosis and total, free or esterified cholesterol, triglycerides or the ratio phospholipid : esterified cholesterol. A very close relationship was reported between degree of atherosclerosis and the ratio phospholipid: free cholesterol. A ratio of

more than 2.0 (weight) or 1.0 (molar) would predict the almost complete absence of any atheromatosis.

In a 42-weeks experiment Funch et al. (1960) fed rabbits on purified diets with 20% (by weight) fat, either butter, peanut oil or three blends of margarine, containing roughly 10, 7 or 22% polyunsaturated fatty acids. The butterfat diet induced the highest serum cholesterol levels, followed by the low-unsaturated fat margarine. The α-lipoproteins showed no appreciable changes but a close correlation was found between cholesterol and β-lipoproteins. The high serum cholesterol values of rabbits fed on butter were associated with marked atheromatous lesions in the aorta and pulmonary arteries. From the serum fatty acids 14% were dienoic with the butterfat diet (Krogh et al., 1961) and from the aorta lipids only 2.5%. The content of cholesterol in aortic tissue rose with the values for atheroma grading. Though the exact degree of atherosclerosis associated with a certain cholesterol value in the serum could not be predicted in individual rabbits, it was nevertheless concluded that the serum cholesterol level gave a good index of whether or not conditions were favourable for the development of lesions.

4.5.3 Hypercholesterolemic factors in milk fat: cholesterol and linoleic acid

The factors in butterfat that might be responsible for the observed hypercholesterolemic and atherogenic effects in rabbits were studied by Funch et al. (1962). They supposed that these might be related to the cholesterol content, the low content of unsaturated fatty acids, the high content of short-chain fatty acids or a combination of these factors in butterfat. The effect of butter cholesterol was studied by adding cholesterol to cocoa butter in an amount equal to that in butterfat. The problem of the low content of linoleic acid was studied by substituting 20% of the butterfat with corn oil. All these fats were compared with hydrogenated arachis oil and fractionated cocoabutter. They were fed at 20% by weight during 38 weeks. The results of the butterfat group and the cholesterol supplemented cocoa butter group were similar with respect to serum cholesterol and aortic atherosclerosis. As the cholesterol supplement to the cocoa butter increased both characteristics compared to the plain cocoa butter, Funch et al. (1962) concluded that short-chain fatty acids apparently have no influence on these processes while the effectiveness of butterfat in increasing serum cholesterol levels and incidence of atherosclerosis in rabbits must be in part ascribed to its cholesterol content.

The same conclusion was reached by Beveridge et al. (1960) for human beings. They observed an increase in serum cholesterol after the ingestion of small amounts of cholesterol added to a butterfat fraction from which the natural cholesterol had been removed.

The minimal amount of dietary cholesterol, able to raise blood lipids in rabbits on natural diets, was established by Kobernick et al. (1964) at 0.25%. The cholesterol raising effect of a 10%-butterfat diet, containing 0.029% cholesterol, could therefore not be ascribed to the cholesterol content of the butterfat. Gottenbos & Thomasson

(1961) observed that addition of 0.04% cholesterol to a basic low-fat diet did not cause either hypercholesterolemia or atheromatosis. The addition of 0.08% cholesterol however induced strongly increased cholesterol levels and atheromatosis, approaching the values induced by 40% (cal/cal..) of coconut oil. The amount of cholesterol added by Funch et al. (1962) was 0.28 g/100 g fat which yields a diet with 0.056% cholesterol. This amount may be sufficient to raise serum cholesterol in rabbits considerably, particularly when applied in high-fat purified diets.

The substitution of corn oil, as a source of linoleic acid, for a part of the butterfat resulted in almost the same serum cholesterol values as obtained with pure butterfat but markedly reduced the incidence of atherosclerosis (Funch et al., 1962). This result is essentially similar to the observation of Moore & Williams (1964a, b) that corn oil reduces atherosclerosis more than could be expected from lowering the cholesterol content. Therefore the other mechanisms of action of linoleic acid might also play a role (see 4.4.1).

The relationship between corn oil and butterfat was further studied by Moore (1969) with rabbits given a series of diets containing different proportions of these two fats, totalling 20% fat by weight in the diet. The concentration of serum cholesterol increased progressively as the butterfat content of the diet was increased and the corn oil content was decreased. Little aortic atheroma was observed in the rabbits given the diets containing 0, 10 and 12% butterfat or 20, 10 and 8% corn oil. As the butterfat content of the diet was increased above 12%, pronounced increases occurred in the extent of atheromatous degeneration of the aorta. The concentration of linoleic acid in the serum cholesteryl esters was inversely related to the concentration of cholesterol in the serum.

In view of the suspected effect of the cholesterol inherent in butterfat it would be interesting to know whether the same result would have been obtained if all the diets had contained the same quantity of cholesterol. An indication for this may be obtained from the experiment by Wilson et al. (1973). They compared the effects on serum lipids of rabbits of two fats, butter and corn oil, at two caloric levels, 24.5% and 44.3%. They carefully matched all groups with regard to dietary cholesterol. Higher total lipid, total cholesterol and phospholipid levels in serum of rabbits fed on butter compared with those fed on corn oil, were observed. The two caloric levels of each fat however produced identical values. The same applies to the results of incidence and severity of aortic atherosclerosis. Individual aortic scores correlated well with the individual mean serum cholesterol concentrations.

4.5.4 Interrelationship of fat and vehicle

The aforementioned failure of fats added to commercial diets, to influence serum lipids and degree of aortic atherosclerosis in rabbits (Kritchevsky & Tepper, 1964) was further investigated by Carroll (1971). He confirmed the lack of response of rabbits fed commercial diets containing 15% by weight of medium-chain triglycerides (MCT), coconut oil, butter, tallow or several vegetable oils. The plasma cholesterol

levels were high in rabbits fed on the semi-synthetic diets containing either no fat or the saturated fats. Acetate incorporation into cholesterol by liver slices was generally lower on semi-synthetic than on commercial diets. Added fats had no consistent effects.

In order to find out which factor in the semi-synthetic diet was responsible for the cholesterol promoting effect a mixture of commercial diet with casein (3:1) was given together with several fats or without any fat (Carroll, 1971). Casein supplementation caused a significant increase in the level of plasma cholesterol when it was given to the animals with the low fat diet or the diets containing coconut oil or butter whereas it was without effect when given together with olive oil, corn oil or soya bean oil. As the supplementation with equal amounts of dextrose instead of casein had no influence, the effects of casein cannot be ascribed to dilution. The acetate incorporation into liver cholesterol was again lower on the casein supplemented diets. Increased biosynthesis of cholesterol is therefore unlikely as an explanation for the mechanism of action of casein.

Failure of increased synthesis of cholesterol from mevalonic acid by liver slices from rabbits fed on semi-synthetic, hydrogenated coconut oil diets was already reported by Howard & Gresham (1968) while liver cholesterol had increased twofold. Carroll (1967) observed similar differences in acetate incorporation in rats. Howard & Gresham (1968) concluded from sterol balance experiments in rabbits that the hypercholesterolemia in rabbits fed on semi-synthetic diets was caused by a defect in the excretion of cholesterol and its metabolites. The inclusion of soya bean meal in the diet, which prevents hypercholesterolemia (Howard et al., 1965), enhances the excretion of these compounds several fold. They did not take into account the lower protein content of the soya bean meal compared with casein. They speculated on the presence of some cholesterol sequestering substance in soya bean meal which the rabbit may need for the excretion of cholesterol. Similar substances have been suggested to be present in alfalfa (Cookson et al., 1967), leguminous seeds (Grande et al., 1965), husk and bran of paddy (Vijayagopal & Kurup, 1973), spinach and wakame (Iritani & Nogi, 1972).

4.5.5 Findings with man

After the original observations of Groen et al. (1952) in man that a rich diet, containing mostly animal fats, increased cholesterol in serum compared with a moderate or vegetable diet numerous experiments in man have confirmed the cholesterol elevating effect of several animal fats, particularly milk fat. It would take too much space to discuss all these experiments in detail but the series of investigations carried out by Beveridge and his group with students are very illustrative (Beveridge et al., 1955, 1956, 1957, 1960).

4.5.5.1 Kind and amount of fat

Two levels (28.4 and 58.5% by calories) of a vegetable oil mixture (corn oil and margarine) in liquid formula diets induced cholesterol reductions of 33 and 47 mg per 100 ml, respectively (Beveridge et al., 1955). Supplementation of the diets with a mixture of vegetable oils with about 160 – 200 mg cholesterol per 1000 calories, this amount being equal to that supplied by butter, did not affect serum cholesterol levels. When 58.5% of the calories were supplied as butter, serum cholesterol rose and was significantly higher than on the cholesterol-supplemented vegetable oil diet. These results suggest that increasing the fat content of the diet with unsaturated fat decreases cholesterol and other serum lipid levels, while the addition of cholesterol to such diets has no influence (see Brown, 1971).

In subsequent experiments it was shown (Beveridge et al., 1956) that changing the experimental subjects from a diet, supplying 58.5% of the calories like corn oil, to a diet containing several animal fats, always resulted in higher serum cholesterol values. The fats tested were butter, chicken fat, lard, and beef-dripping, the butter-fed group showing the highest increase of about 29%. Because it was impossible to decide whether the elimination of corn oil or the addition of the butter caused the increase in serum cholesterol concentration, an experiment was performed (Beveridge et al., 1956) in which all subjects were first placed on a fat-free diet and afterwards partly transferred to diets in which butterfat or corn oil, providing 20 and 60% of total calories, were substituted for a part of the dextri-maltose moiety. On the fat-free diet serum cholesterol decreased 22% compared with the habitual free diet. Whereas those continuing on the fat-free diet after the first 12 days showed no further changes, those who were replaced to the 20 and 60% corn oil diets manifested further decreases of 7 and 15%, respectively. The subjects provided with diets containing 20 and 60% of calories as butterfat showed increases in plasma cholesterol of 7% and 22% respectively. From these experiments it may be concluded that corn oil contains substances capable of depressing serum cholesterol while butter has cholesterol-enhancing properties.

A possible interaction between two kinds of fat was tested (Beveridge et al., 1956) by supplying various mixtures of butterfat and corn oil at 60% of calories. Compared with a 60% butterfat diet, the groups consuming diets which provided the following percentages of calories from butterfat and corn oil: 45/15; 30/30; 15/45; and 0/60 showed the following percentage decreases in plasma cholesterol: 3.9; 15.9; 27.1 and 32.0, respectively. The greatest decrease was therefore observed with 45 per cent and 60 per cent corn oil. A small amount of corn oil is almost without effect. These results agree very close with those obtained by Vergroesen (1972) which suggest no effect of accompanying saturated fatty acids above a certain level of linoleic acid.

Whether the use of butter or a poly-unsaturated margarine under household conditions would influence serum cholesterol was studied by Dalderup et al. (1969) in a crossing-over procedure. Each dietary treatment lasted three months. The people were asked not to change their habitual diet. Compared with their free diet, butter

induced an increase of serum cholesterol of 4 mg per 100 ml while the poly-unsaturated margarine induced a decrease of 12 mg per 100 ml. Taking into account that only part of the total fat could be changed, i.e. only the visible fat which provided 24% of calories, and that dietary cholesterol was about the average for the Netherlands, i.e. 500 mg per day, makes it unlikely that a greater difference could be expected. In fact from applying the first equation of Keys an increase of 2 mg on the butter diet and a decrease of about 11 mg on the special margarine could be expected.

The nature of the substances in fats responsible for the effects of different fats were studied by Beveridge et al. (1957). The addition of 10 or 200 mg of α-tocopherol per 950 calories to a diet, providing 60% of calories as butter, resulted in no change of serum cholesterol. Supplementation with 7 g of β-sitosterol per 950 kcalories to the butter diet resulted in a decrease of serum cholesterol till values similar to those obtained with corn oil. Several cuts of corn oil obtained by molecular distillation all brought about significant decreases in plasma cholesterol. The fraction containing most of the unsaponifiables produced the greatest decrease but not significantly different from the others. It may thus be concluded that sitosterol has a cholesterol-lowering ability but the amount present in corn oil is not sufficient to account for the effects of corn oil.

4.5.5.2 Dietary cholesterol

By working along the same line with butterfat fractions, it was shown (Beveridge et al., 1957) that all fractions induced significant increases in plasma cholesterol, the greatest change being noted with the most volatile fraction. This fraction contains most of the unsaponifiable material, particularly cholesterol, and the fatty acids of relatively short-chain length. That the cholesterol present in butter accounts, at least in part, for its hypercholesterolemic activity was demonstrated by Beveridge et al. (1959) more directly. They established a dose-response effect in men fed on liquid formula diets (Beveridge et al., 1960). Various supplements of cholesterol were dissolved in 30% stripped butterfat as to provide 25 – 1600 mg cholesterol per 950 k calories diet. Between intakes of about 13 mg cholesterol per day to 634 mg per day the serum cholesterol increased sharply but there was no further significant increase obtained by daily intakes of 1300 to 4500 mg. Compared with a fat-free period the stripped butterfat caused also an increase in serum cholesterol of about 15%. At the level of cholesterol present in butterfat, only a moderate increase of serum cholesterol can be expected of about 10 – 15 mg per 100 ml.

This agrees very closely with the change observed by Mattson et al. (1972) with liquid formula diets in which the fat contained predominantly saturated and mono-unsaturated fatty acids. From their regression equation one may calculate that a diet containing 100 mg cholesterol per 1000 kcalories induces an increase in serum cholesterol of about 13 mg per 100 ml compared with a cholesterol-free diet. From the Keys' equation (Keys et al., 1965a), an increase may be calculated of 15 mg per 100 ml and with that from Hegsted et al. (1965) of 19 mg. All these calculations are based on

an intake of about 100 g butterfat in a 30% (cal/cal) fat diet containing 100 mg cholesterol per 1000 kcalories. The observed increase on a 60% (cal/cal) butterfat diet of about 40 mg serum cholesterol (Beveridge et al., 1956) compared with a fat-free diet can therefore easily for more than 50 per cent be attributed to dietary cholesterol. For the increase on a 20% (cal/cal) butterfat diet of slightly more than 10 mg holds the same.

Iongh et al. (1965) investigated the influence of some dietary fats on serum lipids in healthy man consuming a normal diet containing 33% of the calories from the fat under investigation. They observed a difference in serum cholesterol between sunflower-seed oil and butter of 39 mg/100 ml. Assuming a dietary intake of about 100 g fat and 2400 kcalories one may calculate a cholesterol intake of about 280 mg per day or 116 mg per 1000 kcalories from the butterfat. As the diet was otherwise poor in cholesterol it may be assumed that total cholesterol intake was well below the maximal effective intake of 600 mg per day (Beveridge et al., 1960). From the observed difference of 39 mg/100 ml at least 16 mg (Keys et al., 1965), 19 mg (Hegsted et al., 1965) or 14 mg (Mattson et al., 1972) i.e. about 40% of the cholesterol-elevating effect of butterfat may be ascribed to its cholesterol.

In an earlier study Malmros & Wigand (1957) compared the effects of 40% of the calories supplied as corn oil or as milk fat, added to a fat-poor diet composed of natural foodstuffs as bread, cereals, vegetables, potatoes, rice, fruit and sugar. They observed also a difference in serum cholesterol of about 35 - 40 mg/100 ml. Their statement that replacement of corn oil by milk fat increased serum cholesterol, but did not quite reach its original level is somewhat euphemistic as the original level on a free diet was 215 mg per 100 ml, the level on corn oil was about 140 mg and on milk fat 180 mg per 100 ml.

4.5.5.3 Interrelationship of cholesterol and vehicle

The evidence (Malmros & Wigand, 1957) that dietary cholesterol as in milk fat had no influence, was shown by the addition of 0.5 g cholesterol to the corn oil diet. This is, however, only support for the calculations by Brown (1971) that a diet containing 40% of the calories as corn oil, thus supplying about 24% of the calories as linoleic acid, can induce a maximal cholesterol-decrease of 22% even in the presence of 550 mg dietary cholesterol. These are exactly the experimental conditions as indicated by Malmros & Wigand (1957). The absence of a serum cholesterol elevating effect of cholesterol added to corn oil cannot be taken as evidence for the influence of cholesterol in milk fat.

The importance of the interplay between dietary cholesterol and poly-unsaturated fatty acids, as outlined by Brown (1971) can be illustrated with the results of a study by Connor et al. (1964) with human subjects. They fed mixed natural foods with 40% of the calories of two different fats at two cholesterol levels, zero and 725 mg per day. The fat supplied either 4 or 12.5% of the calories as polyunsaturated fatty acids (pufa). They observed on the four diets, high cholesterol-low pufa, low cholesterol-

low pufa, low cholesterol-high pufa, high cholesterol-high pufa, serum cholesterol levels of 213, 175, 174 and 202 mg per 100 ml respectively. They concluded therefore that the influence of dietary cholesterol was much more important than of the kind of fat. According to Brown (1971) however, a decrease of less than 2% of serum cholesterol may be expected if the diet contains 700 – 800 mg cholesterol and if 14 per cent of the calories are to be supplied by poly-unsaturated fatty acids. The decrease observed by Connor et al. (1964) from 213 to 202 is even more than could be expected. Evidently a higher pufa content of the diet or a lower cholesterol content would have been required to detect this interrelationship more clearly.

Another interesting factor might be the interrelationship between dietary cholesterol and the content of trans-isomeric fatty acids. Anderson et al. (1961) described significant rises in serum cholesterol caused by elaidic acid, incorporated in an average American diet, which is rich in cholesterol. This conclusion could not be confirmed from the experiments by McOsker et al. (1962) and Iongh et al. (1965). They used diets virtually free from cholesterol. Vergroesen (1972) concluded from these and his own experiments that trans-isomeric fatty acids may have a cholesterol-elevating effect in cholesterol-containing diets, while in cholesterol-free diets they behave as their cis-isomers.

Only one study in the literature combined these two possibilities in one design (Erickson et al., 1964). From this it was concluded that a partially hydrogenated fat, containing 10% trans-isomers induced the same cholesterol levels as a blend of natural vegetable fats, both on a diet without cholesterol as on a diet containing 742 mg of cholesterol per day. The difference between the two dietary-cholesterol groups was 24 and 27 mg per 100 ml. This study should be considered with some reserve however as the degree of unsaturation of the fat did not influence serum cholesterol either.

It may be of interest to mention here the results reported by McMillan et al. (1963) and Weigensberg & McMillan (1964) for rabbits. They fed rabbits on normal commercial diets supplemented with 1 g cholesterol per day and either 6 g natural olive oil or 6 g elaidinized olive oil. The elaidinized oil contained 68% trans-isomer. In the rabbits fed on elaidinized oil higher serum cholesterol and triglycerides were observed and slightly more visible aortic atherosclerosis than in those fed on natural oil (Mc Millan et al., 1963). In the cholesteryl esters from serum about 15% contained elaidic acid, the same figure was found in spleen and aorta, suggesting no preferential uptake or deposition of the aleidate ester in these tissues (Weigensberg & McMillan, 1964). The phospholipids contained only 2 – 3% of elaidic acid.

4.5.5.4 Conclusion

From all the above mentioned studies it is evident that milk fat has a cholesterol elevating effect, compared with unsaturated vegetable oils or low-fat diets. The influence on serum cholesterol may be partly caused by its cholesterol content, partly by its low content of polyunsaturated fatty acids and partly by its saturated fatty acids. All these data have been derived from controlled experimental studies.

4.6 Components of milk fat, blood lipids and atherosclerosis

In Section 4.5 the influence of butterfat on serum cholesterol and experimental atherosclerosis in several animal species was discussed. It was moreover demonstrated that at least part of its effect could be attributed to the cholesterol present in it. However, milk fat may be consumed in different forms, as part of milk, as cream, as butter or as butter oil. These points as well as the effects of some specific fatty acids from milk fat will be discussed in this section.

4.6.1 Whole milk versus milk fat

First it was shown by Bazzano & Olson (1968) that a liquid milk diet, supplemented with sucrose to reach the desired caloric level, is no more hypercholesterolemic in man than a control diet, simulating the usual American diet. As the control diet was composed of natural foods and as the milk diet may be regarded as a liquid formula diet a decrease could have been expected on this formula diet (Beveridge et al., 1956; Hashim et al., 1960; Mattson et al., 1972). The absence of such a cholesterol-lowering effect in the presence of milk should be considered as a cholesterol-elevating effect. From the evidence put forward by Wissler et al. (1965) that a table-prepared average American diet induces moderate hypercholesterolemia and atherosclerosis in the rhesus monkey it may be inferred that also a milk diet is hypercholesterolemic and atherogenic.

No differences in serum cholesterol between whole milk and cream or butter, incorporated in liquid formula diets were observed by Goldsmith et al. (1968) in man. The protein source was either casein or dried skim milk. When cottonseed oil was used as fat source, serum cholesterol was always lower. If a liquid formula diet, containing 42% of calories as butter and 12% protein was given to human volunteers no influence of different carbohydrates, be it sucrose, lactose or starch, could be detected on serum lipids. The protein in this experiment was a combination of the proteins of milk in approximately the same ratio as that found in milk. The same investigators (Goldsmith et al., 1968) also reported about a comparison, using a formula diet, of butter, butter oil and butter oil plus the phospholipids removed from the butter. No changes were observed in serum cholesterol concentration during this experiment. Similar findings for serum lipids and atherosclerosis were reported by Moore & Williams (1964a).

The information on the physiological effects of the different physical forms in which milk fat is ingested is still scanty. It might nevertheless be concluded that the fat has about the same influence on blood lipids, irrespective of the form in which it is consumed. This suggests that the main hypercholesterolemic effect of milk fat is caused by its cholesterol and by its triglyceride component or the fatty acids composing these triglycerides.

4.6.2 The role of different milk fat fractions

The influence of different triglycerides was studied by feeding butterfat fractions obtained after molecular distillation or by fractional crystallization without solvent or from acetone. Fractions obtained by the first procedure were used by Beveridge et al. (1957). The greatest increase of serum cholesterol was observed with the most volatile fraction, containing short-chain fatty acids and most of the cholesterol.

Fractions obtained by crystallization in the absence of solvent were prepared by Richardson (1968) at intervals of 5°C from 30°C to 15°C. The first fraction, solid at 30°C, had a yield of 40%, the other fractions had comparable yields of about 15%. The concentration of cholesterol, both free and esterified, tended to be similar in all fractions, about 240 – 270 mg per 100 g fat, with a somewhat higher content of 340 mg in the fraction liquid at 15°C. The fatty acid composition of these fractions showed a definite though small trend. The saturates 4:0 through 12:0 and all unsaturates tended to increase from Fraction 1 (solid at 30°C) to Fraction 5 (liquid at 15°C), while 14:0 through 18:0 saturates tended to decrease. These results in general were also observed by Schulz & Timmer (1966). That differences were small may be seen from the content of butyric acid, which increased from 3.24% (by weight) to 5.34% while oleic acid increased from 22.4% to 29.15%. The sum of short-chain fatty acids, from 4:0 till 10:0 increased from 9.10% to 13.00%. Interesterification of these fractions hardened all fractions except the highest melting fraction and caused a significant loss of butyrate. By interesterification the content of high-melting glycerides of each fraction was increased. It was concluded (Richardson, 1968) that small differences in fatty acid composition and unsaturation contrast with the gross differences in physical properties of the fractions. Differences in triglyceride structure may account for differences in physical properties.

Fractions from this kind were used by Campbell et al. (1968) in liquid formula diets supplying 40% of calories. They compared the parent cream, the fraction solid at 30°C and the fraction liquid at 30°C in successive periods of three weeks. The liquid fractions caused a modest but significant rise in serum cholesterol while the solid fractions caused the opposite change. There were hardly any differences in fatty acid composition of these fats. The observed difference may be related to the small difference in sterol content of the two fractions.

Another method to prepare fat fractions is by fractional crystallization from a solvent, e.g. acetone (Chen & De Man, 1966) at different temperatures. By this procedure eight fractions were obtained with a much more variable composition than with the former procedure (Man, 1968). From the first fraction till the residue the content of trisaturated glycerides decreased from 67% to 27%, total saturated fatty acids from 82 to 46%. The total unsaturated fatty acids increased from 18 to 54, the short-chain fatty acids from 0.9 to 16.8%. The distribution of cholesterol over the fractions was also quite dissimilar. The fractions obtained at 15 and 5°C contained only traces of cholesterol, those crystallizing from −5°C till −45°C contained increasing amounts from 60 to 160 mg per 100 g fat. The residue was very rich in

cholesterol: 1020 mg per 100 g whereas the original fat contained 240 mg.

The −15°C fraction was used by Campbell et al. (1968) in formula diets and compared with the parent milk fat. This fraction contained 9% short-chain and medium-chain fatty acids from 4:0 till 10:0, 38% mono-unsaturated fatty acids, 6% poly-unsaturated fatty acids and 47% saturated fatty acids. The parent fat contained 6, 37, 4 and 53% of these fatty acids, respectively. The cholesterol content of the fraction was calculated as 93 mg per 100 g (Man, 1968). The serum lipids did not exhibit a striking change, with the exception of a transient rise in triglycerides after switching from the parent fat to the −15°C fraction (Campbell et al., 1968).

In another experiment the combined −45°C and residue fractions were compared with the parent butterfat. This fraction contained appreciable quantities of short-chain fatty acids, 12.7%, of oleic acid, 43%, and of linoleic acid, 5.5%, compared with the original fat, 7.8%, 30.5% and 3.2%. The cholesterol content of this fraction was not mentioned but may be calculated from the figures of Man (1968) as about 500 mg per 100 g fat. The decrease observed with the low-melting fraction was 10 - 12 mg per 100 ml compared with the original fat (Campbell et al., 1968), which is not very striking. The advantage of the fatty acid composition may very well be counteracted by the higher cholesterol content of the low-melting fraction.

Application of the predictive formulas of Hegsted et al. (1965) or Keys et al. (1965b), without considering the change in dietary cholesterol would yield an expected decrease of serum cholesterol of 24 mg or 18 mg, respectively when the diet was changed from the butterfat to the butterfat fraction. Inclusion of dietary cholesterol and supposing that the calculation of the cholesterol content was valid would result in an expected decrease of 6 mg or 3 mg respectively. Maybe the cholesterol content is somewhat overestimated.

However it may be concluded that even the most extreme butterfat fraction prepared up till now has only a very limited cholesterol-lowering capacity. The influence on serum cholesterol of this fraction appears to fit in the formulas derived from a wide range of dietary fats. The influence on serum cholesterol of milk fat and its fractions is therefore attributable to its fatty acid composition and its cholesterol content.

4.6.3 Specific fatty acids

The fatty acid composition of milk fat is more complicated than of most other fats (Mulder & Walstra, 1974; Renner, 1974). Up till now at least 142 different fatty acids have been demonstrated, many however occur only in trace amounts. There are only 15 fatty acids which contribute more than 1% to milk fat. These comprise all the saturated fatty acids with a chain length from 4 to 18, the mono-unsaturated fatty acids myristoleic, palmitoleic and oleic acid and the poly-unsaturated fatty acids, linoleic and linolenic acid. The presence of the fatty acids with chain length of less than 12 carbon atoms is rather characteristic for milk fat. Of other natural fats only coconut oil and palm kernel oil contain appreciable quantities of these fatty acids.

The fatty acid composition of cow's milk is rather variable, reflecting many environ-

mental conditions, especially nutrition. The dienoic acid content was given by Mulder & Walstra (1974) as 2.5 mol %(mol/mol × 100) with a range from 1 to 4.3%. From a nutritional point of view this relatively wide range is, however, of limited significance as it is for the other fatty acids.

Since the first observations of a cholesterol-elevating effect of particularly coconut oil and milk fat (e.g. Ahrens et al., 1957) it has been suggested that the short-chain and medium-chain fatty acids might be in part responsible for this effect. An opposite view has, however, also been expressed, namely that the short-chain fatty acids of milk fat are the protective factor against heart disease (Malhotra, 1967a, b; 1968).

An important point in this debate is how fatty acids are classified according to chain length. This may be on physical, chemical or physiological grounds, depending on the goal of such a classification. But even from a physiological or nutritional point of view there is hardly any agreement. Sharp dividing lines cannot be drawn. Bach & Métais (1970) classified the fatty acids into three groups: long-chain fatty acids with more than 14 carbon atoms; medium-chain fatty acids from 9 till 15 carbon atoms, and short-chain fatty acids with less than 9 carbon atoms. They argued that up till octanoic acid the fatty acids are liquid at room temperature. However the synthetic fat which is called MCT consists for about 70% of octanoic acid (Babayan, 1968), in the terminology of Bach & Métais (1970) a short-chain fatty acid. It is because of this confusion that it appears better not to use these terms but to mention the fatty acids with their own names and symbols.

From a nutritional point of view the chain length of fatty acids at the lower end of the scale is interesting mainly for two reasons: the rate and ease of digestion and absorption and the way they are transported and metabolized. During recent years several extensive reviews have been written on these aspects and it would be superfluous to discuss the particularities of digestion and absorption here at length (Hashim, 1967; Harkins & Sarrett, 1968a; Holt, 1967; Greenberger & Skillman, 1969; Skála & Horácková, 1969; Hermus, 1970; Senior, 1968; Bach & Métais, 1970).

From this literature the properties of fatty acids with shorter chain length compared with those with a longer chain length can be summarized as follows: they are more readily and completely hydrolysed in the intestinal lumen by pancreatic enzymes; the distribution over the glycerol molecule is not important; the presence of bile in the intestinal lumen is not necessary for normal absorption; as triglyceride they may be absorbed intact by the small intestine and these are completely hydrolysed in mucosal cells; there is a greater capacity to absorb shorter than longer chain fatty acids and triglycerides; the shorter the chain length the less fatty acids are incorporated into triglycerides and chylomicrons in the mucosa and the more they are transported by the portal vein and not by the lymphatics; they disappear much more rapidly from the bloodstream; they are preferentially taken up by the liver; finally they are more rapidly oxidized by many tissues, including liver and skeletal muscle, and are only minimally used for lipid synthesis.

4.6.4 Shorter-chain fatty acids; findings with various animals

The influence of shorter-chain fatty acids on serum cholesterol levels has been described during the last decade in many animal species, including man in normal and pathologic states. Most studies were carried out by feeding all or most of the fat as MCT which contains predominantly octanoate.

The serum cholesterol level in *rats* fed 20 per cent of diet by weight as MCT, coconut oil or corn oil was reported by Kritchevsky & Tepper (1965a). Feeding of MCT induced the lowest serum cholesterol. When the diet was supplemented with 2% cholesterol the MCT-fed rats had serum cholesterol levels half those of animals fed on the other fats. The same trend was observed for the liver cholesterol levels. These results are consistent with those of Kaunitz et al. (1959) and Fisher & Kaunitz (1964) who demonstrated lower liver cholesterol levels in MCT-fed rats than in rats fed long-chain saturated triglycerides or low-fat diets. Harkins & Sarrett (1968b) compared in a 47 weeks experiment the influence of MCT with several other fats in purified diets on serum and liver lipids. Serum cholesterol values were lowest on the MCT diet and highest on the coconut oil. The total liver lipids and liver cholesterol were again lowest with MCT as fat source. Liver cholesterol was highest with sunflower oil and coconut oil.

In a comparison of corn oil, coconut oil and MCT in rats, Leveille et al. (1967a) observed that ingestion of a diet, free from MCT and cholesterol, depressed plasma and liver cholesterol as compared with corn oil or coconut oil. In the liver tissue of these rats the chain-elongation and desaturation mechanisms were enhanced by feeding MCT. They also presented evidence that the enhanced incorporation of acetate into fatty acids by the adipose tissue of rats fed on MCT represents synthesis de novo and not chain lengthening.

Reiser et al. (1965) reported the lowest serum cholesterol levels when rats were fed 30% of their diets as tributyrin, compared with other pure triglycerides. Trimyristin induced the highest serum cholesterol values. Completely different results were obtained by Caster (1969) who found caproic acid as the sole hypercholesterolemic fatty acid in rats.

The findings in *chicken* are somewhat confusing. Originally MCT feeding was found to elevate serum cholesterol in comparison with coconut oil, although MCT lowered liver cholesterol levels (Fisher & Kaunitz, 1964). On the other hand it was shown in young chicks that the substitution of MCT for coconut oil lowered both serum and liver cholesterol levels, but not as much as when corn oil was substituted (Whiteside et al., 1965). If the chick diet contained 2% cholesterol, an increase of plasma and liver cholesterol was observed on a MCT diet. This increase was, however, of lower magnitude than that observed when the cholesterol was added to a diet containing 14% corn oil or 12% coconut oil and 2% corn oil (Leveille et al., 1967b). The MCT diet contained also 2% corn oil. Fisher & Griminger (1970) found no elevation of plasma or liver cholesterol when 2% cholesterol was added to a diet containing 7% MCT. These findings led Kenney & Fisher (1973) to postulate that dietary MCT

might influence cholesterol absorption. They actually demonstrated that on corn oil the absorption of cholesterol was twice as great as on MCT with or without cholic acid.

In *dogs* MCT caused lower serum cholesterol values than coconut oil (Grande, 1962). In *swine* feeding 10% fat as corn oil, lard, tallow, or coconut oil elevated serum cholesterol, compared with a low-fat control diet, whereas MCT did not (Allee et al., 1972). MCT was less inhibitory for fatty acid synthesis by adipose tissue than the other sources of cietary fat.

In a study with *rabbits*, Kritchevsky & Tepper (1965b) compared effects of hypercholesterolemic diets containing 2% cholesterol and MCT, corn oil or coconut oil. MCT-fed animals were found to have the lowest serum cholesterol. Malmros et al. (1964) studied the effect of chain length of fatty acids in rabbits fed on purified diets. The fatty acids were fed as pure triglycerides. Tributyrin induced cholesterol levels below 100 mg per 100 ml. Trioctanoate however caused an increase till 250 mg per 100 ml after 18 weeks and tridecanoate till 450 mg per 100 ml. No linoleic acid was present in the diet. The highest cholesterol values were observed after feeding triglycerides of lauric and myristic acid.

In a recent study, Malmros et al. (1972) observed severe hypercholesterolemia and moderate triglyceridemia in rabbits fed on purified diets and MCT. The results were in the same range as with the pure triglycerides. Lack of a control group makes this study difficult to interpret. It should be remembered that a fat-free semisynthetic diet is also hypercholesterolemic for rabbits (Malmros et al., 1964). Carroll (1971) compared the effects of several fats added to semi-synthetic or commercial diets. The addition of MCT to a semi-synthetic diet induced even higher serum cholesterol levels than the fat-free diet or the semi-synthetic diets supplemented with butterfat, beef-tallow or unsaturated oil. Adding MCT to a commercial diet decreased serum cholesterol levels till the low corn and sunflower oil level. Liver cholesterol was in both cases lowest in the MCT-fed animals, while acetate incorporation into cholesterol was only depressed with MCT added to the commercial diet.

4.6.5 Shorter-chain fatty acids; findings with man

Studies of effects of MCT on serum cholesterol in healthy man are generally in agreement with those done in animals. Beveridge et al. (1959) observed in 8-day studies, involving apparently healthy students, no significant change in serum cholesterol levels when isocaloric amounts of an MCT-formula replaced a fat-free regimen. These levels rose slightly and markedly after replacement with coconut oil or butter, respectively. In another similar study Hashim et al. (1960) observed the lowest serum cholesterol values during corn oil feeding, the highest during butter and intermediate during MCT feeding.

From the results of these and their own studies Keys et al. (1965b) and Hegsted et al. (1965) concluded that the only variables in fats which could be identified to influence the level of serum cholesterol in man were either lauric acid, myristic acid, palmitic

acid, poly-unsaturated fatty acids and cholesterol (Keys et al., 1965b) or myristic acid, palmitic acid, poly-unsaturated fatty acids and cholesterol (Hegsted et al., 1965). To the latter lauric acid and stearic acid were added (McGandy et al., 1970) after testing a wider range of fats. The fatty acids with a chain length shorter than 12 carbon atoms are considered by both groups as without effect.

MCT was fed to a number of subjects with malabsorption (Furman, 1968). This led to diminished faecal fat loss, improved nutrition and increased body weight, and slightly increased serum lipid levels, usually from low to more nearly normal levels. In subjects with hyperchylomicronemia MCT feeding markedly lowers serum triglyceride levels and abolishes lactescence. Carbohydrate-accentuated lipemia may be markedly diminished by high MCT diets as with longer chain unsaturated fats. Though MCT has many features in common with carbohydrates (Kuo & Huang, 1965; Hermus, 1970) they cannot be regarded metabolically as carbohydrates for this reason.

4.7 Dietary protein, blood lipids and atherosclerosis

4.7.1 Findings with various animals

Dietary protein is generally not regarded as an important determinant of blood lipids or atherosclerosis in man (Anderson et al., 1973). In many animal experimental studies, however, and in some experiments with man the quality and quantity of protein or certain amino acids had a profound influence on blood lipids and consequently on atherosclerosis. In view of the remarkable results which may be obtained with rabbits fed on semi-synthetic diets containing casein, it appeared worthwhile to reconsider the protein question with special reference to rabbits (see Sections 4.2 and 4.3). The influence of dietary protein or specific amino acids was studied in pigs, rats, chickens, rabbits and primates.

In a comparison in adult *swine* of two protein levels, 13.7 and 4.9%, provided as soya bean protein, no differences in serum cholesterol levels could be detected (Barnes et al., 1959a). When all protein was removed from the diet and the swine were continued on this regimen for more than 4 weeks some elevation of serum cholesterol was observed. In a similar study in young growing swine (Barnes et al., 1959b), to compare 16% and 9% protein with and without 10% beef tallow, evidence of protein malnutrition was most marked in the low-protein, high-fat group. The protein level was not corrected for the higher caloric density of the diet due to the supplementation with 10% by weight of tallow. The low protein intake resulted in increase in serum cholesterol, the values reaching a peak and then declining slowly towards the levels found in adults. This return was less rapid for the low-protein, high-fat group.

A hypercholesterolemic response to low-protein diets has invariably been found in *mice* (Fillios & Mann, 1954) and *rats* (see Groot, 1958, 1959, 1960; Chen et al., 1972). Feeding graded levels of casein to rats caused a progressive lowering of serum cholesterol concentration for each increase in the casein level from 6–40% of the diet (Nath et al., 1959). A further increase to 70% caused the serum cholesterol again to rise.

This biphasic response was not observed for wheat gluten. The absolute levels induced by this protein were already low. Nath et al. (1959) ascribed the relative absence of hypercholesterolemic properties of wheat gluten to accompanying lipids. Groot (1960) could not confirm this effect of gluten, but he added 5% gluten to a diet containing 15% casein while Nath et al. (1959) gave gluten or extracted gluten as the sole protein. The same effect as with wheat gluten could be obtained with an amino acid mixture made to simulate wheat gluten (Groot, 1960). From this result it was concluded that the cholesterol-lowering effect of wheat gluten added to casein could be ascribed to its amino acid composition.

From a review of the literature (Groot, 1960) it may be concluded that a protein-deficient diet always provokes hypercholesterolemia and atherosclerosis in rats. In almost all experiments casein or soya bean protein was used as dietary protein. Both are limiting in sulphur-containing amino acids. Supplementation of such diets with methionine or cystine always leads to cholesterol lowering (e.g. Okey & Lyman, 1957; Groot, 1960; Fillios & Mann, 1954; Banerjee & Chakrabarti, 1973). It is evident that the amount of biologically available protein may be increased by either increasing the absolute amount of protein or by supplementation of the protein with the limiting amino acid. If therefore a protein is limiting in another amino acid, addition of this amino acid would apparently increase the protein in the diet. This was demonstrated in rats for wheat gluten, deficient in lysine. Supplementation of wheat gluten with 0.7% lysine in diets for weanling rats reduced serum triglycerides and phospholipids (McGregor, 1971). The observed differences were small, probably due to the quite high protein levels in the diet of 20 – 22%.

From his experiments with other proteins as blood meal or sunflower-seed meal, Groot (1960) concluded that the addition of methionine, apart from providing the limiting amino acid, also had a specific cholesterol-lowering effect in rats. This effect may be related to the suggested anti-hypercholesterolemic effect of some non-essential sulphur-containing amino acids tested by Itokawa et al. (1973). They ascribed this effect to the ability to provide endogenous sulphate and taurine. The data of Feland et al. (1973) indicate that the level of sulphate and of sulphate and taurine precursors in the diet may play an important and equal role in the dietary management of hypercholesterolemia in rats.

The protein question may, however, be even more complicated. Banerjee & Chakrabarti (1973) observed differences in the effectiveness of reducing the cholesterol contents of different tissues of rats, fed on different pulse proteins at 12 per cent level. These were supplemented with tryptophan, lysine, methionine and threonine. The amino acid compositions of the pulse proteins were more or less the same. They attributed these differences to the different rate of liberation of amino acids from the proteins. This may be characteristic of the amino acids, their linkage or sequence in the different proteins.

The importance of sufficient sulphur-containing amino acids was demonstrated in sub-human primates by Mann et al. (1953). They were able to induce severe atherosclerosis in the *Cebus albifrons monkey* only when they were fed on a diet deficient in

these amino acids.

The ability of dietary protein to alter the serum cholesterol in the *chick* has been studied by many investigators. Kokatnur et al. (1958) compared three levels of protein, 7.5, 15 and 30%, each at two levels of corn oil, 0.1 and 10%. The low protein intake increased cholesterol levels significantly at both levels of fat intake. The highest cholesterol concentration was induced by the low-protein, high-fat diet. After cessation of cholesterol supplementation normal serum cholesterol values were regained only when the protein level of the diet was high. Johnson et al. (1958) confirmed these findings for both casein and soya bean protein as the dietary source of protein. Supplementation of the 10 and 25% soya bean protein diet with methionine depressed serum cholesterol and increased weight gain. Supplementation of the casein at 10 and 25% of the diet with arginine, methionine or both improved growth and decreased cholesterol levels. The lowest cholesterol concentrations were observed on the 40% casein diet with both arginine and methionine added. The cholesterol-lowering effect of arginine in chicks was confirmed by Kokatnur et al. (1959). The positive effect of arginine supplementation to even a high protein diet (Johnson et al., 1958), containing casein, may be related to the lysine-arginine antagonism in chicks (Jones, 1964; Section 4.3).

The interrelationship between dietary protein level and amount and kind of fat was studied by Leveille & Fisher (1958) and Katz et al. (1958). On the low protein diets, the serum cholesterol rose considerably, whatever the composition or level of fat. Serum cholesterol remained normal on high-protein diets. The addition of 0.3% cholesterol to a 10% protein diet induced similar cholesterol concentrations as 2% cholesterol to a 20% protein diet (Leveille et al., 1961). This similarity in response of chicks to either cholesterol or different protein levels has led to the construction of a nomogram which shows the relationship between serum cholesterol and coronary artery involvement (Kakita et al., 1972).

The mechanism of action of different protein levels was originally ascribed to a greater bile acid excretion (Leveille et al., 1961). This was observed both for cholesterol-free and cholesterol-containing diets. The absolute amount of plasma plus liver cholesterol was similar on a high-protein and a low-protein diet (Kenney & Fisher, 1973). As the high-protein diet induced a greater weight gain, at least part of the protein effect was attributed to body weight differences. The percentage excretion with the faeces of cholesterol and its metabolites was the same at both protein levels but because of differences in voided faeces the absolute excretion of these substances was greater on the high-protein level.

Kenney & Fisher (1973) also compared casein and soya bean protein as nitrogen sources. At the low protein intake the cholesterol pool from liver plus plasma was similar but the steroid excretion was much less with casein. At an 0.5% cholesterol intake the pool size was much greater on the casein diet. At the high protein level without cholesterol the same trend as with the low protein level was observed. Addition of cholesterol induced higher level of serum cholesterol, liver cholesterol and lower steroid excretion on the casein diet. Cholesterol absorption did not appear to

be influenced by the protein level of the diet, with or without supplementation with cholic acid (Kenney & Fisher, 1973).

The influence on cholesterol metabolism of feeding different protein levels to chicks was investigated by Yeh & Leveille (1969, 1972). Both in vivo and in vitro it was shown that acetate incorporation into hepatic cholesterol increased as the dietary protein level increased (Yeh & Leveille, 1969). This was in contrast to earlier results obtained by Nishida et al. (1960). They observed a higher rate of acetate incorporation into liver and serum cholesterol of chicks fed on a low protein diet than of those fed on a high protein diet. The reason for this discrepancy is not clear.

Cholesterol and fatty acid synthesis by several organs was established by Yeh & Leveille (1972) at two dietary protein levels, supplied by methionine-supplemented soya bean meal. It was observed that in the chick total cholesterol synthesis is enhanced and fatty acid synthesis is depressed by increasing the dietary protein level, suggesting a reciprocal relationship between cholesterol and fatty acid synthesis. These findings are thus in accord with Yeh & Leveille (1969) but in contrast to those of Nishida et al. (1960). The contribution in the chick of liver cholesterol synthesis was about 50% while the intestine contributed 10%.

As the plasma cholesterol concentration was lowered and the cholesterol synthesis increased on the high protein diet some other mechanism must be responsible. This may be a higher rate of degradation of cholesterol to bile acids and subsequent excretion or a redistribution of cholesterol to other tissues than plasma and liver. In fact a slightly increased carcass cholesterol level was found. This result is difficult to interpret as body weight differed considerably and the total lipid content of the carcass decreased from 26.5 to 14.5% by increasing dietary protein (Yeh & Leveille, 1972). Also the use of soya bean meal with a protein content of 50% as dietary source of nitrogen may cause specific effects, not related to its protein as has been found in rabbits (Howard et al., 1965). The results are however in agreement with Yeh & Leveille (1969) who used isolated soya protein. Also casein fed at different levels has the same effect on serum cholesterol levels (Johnson et al., 1958; Kenney & Fisher, 1973).

The possibility of increased conversion of cholesterol into bile acids and enhanced excretion of cholesterol and its metabolites was studied recently by Yeh & Leveille (1973). Two levels of soya bean meal, supplying 15 or 30% protein to the diet and supplemented with methionine, were fed to male chicks. The specific activity of plasma cholesterol was determined after intravenous injection of cholesterol-4-^{14}C.

The specific activity of plasma cholesterol against time yielded biphasic curves for both protein levels, suggesting that the two-compartment model of cholesterol turnover is also valid for the chick as it is for man (Goodman & Noble, 1968; Nestel et al., 1969) and rabbits (Iritani & Wells, 1966). The first phase of the curve yielded a half-life of 55 hours for the animals fed on 15% protein and of 41 hours for the 30%-protein group (Yeh & Leveille, 1973). The second phase yielded 7.78 and 4.34 days, respectively. The turnover rates for both phases were more rapid for chicks fed on high-protein diets. As the first phase is a combination of the rapidly and slowly miscible pool, this phase was corrected to represent only the rapidly miscible pool. The then resulting

curve yielded half-lifes of 17 and 16 hours for the low and high protein diets respectively. Differences between the two protein levels are, therefore, mainly due to an alteration in the turnover rate of the slowly equilibrating pool. The more rapid turnover on the high protein diet was accompanied by an increase in faecal steroid excretion. The increase was almost equally distributed between the sterol and bile acid fractions. This confirmed the earlier findings of Leveille et al. (1961) and those of Kenney & Fisher (1973).

The finding of an absence of effect on cholesterol absorption (Kenney & Fisher, 1973), of enhanced cholesterol synthesis (Yeh & Leveille, 1969; 1972) and excretion (Yeh & Leveille, 1973) indicated that the hypocholesterolemic effect of more dietary protein in chicks is mediated through a more rapid turnover of plasma cholesterol.

Lofland and Clarkson (1968) stressed the importance of interrelations between several dietary components, blood lipids and atherosclerosis in *pigeons*. They demonstrated, with very few animals per treatment, that the level of protein in the diet influences the degree of atherosclerosis and the cholesterol content of the aorta more than it did the serum cholesterol level. No clear conclusions can be drawn from their data. In one experiment a high casein level aggravated hypercholesterolemia and atherosclerosis, in another it was relatively protective. Sometimes atherosclerosis parallelled serum cholesterol, sometimes it did not.

Kakita et al. (1972) evaluated more than 2000 coronary arteries from individual cockerels and demonstrated a curvilinear relationship between serum cholesterol and degree of artery involvement ($r = 0.52$), whether hypercholesterolemia was induced by protein level or by dietary cholesterol. The largest effect of serum cholesterol on atherosclerosis occurred in the lower range from 100 – 500 mg per 100 ml. As the pigeons of Lofland & Clarkson (1968) often had cholesterol values above 500 mg, differences become increasingly difficult to detect. One lesson may be learned from their experiments, that results obtained from one variable tested, may heavily depend on the rest of the diet.

4.7.2 Findings with rabbits

The proteins of mechanically defatted beef or casein and skimmed milk were long ago claimed to induce hypercholesterolemia and atherosclerosis in rabbits (Newburgh & Squier, 1920; Newburgh & Clarkson, 1923). Freyberg (1937) observed that rabbits fed on a diet containing cholesterol and a high level of vegetable proteins such as gluten for as long as 11 months failed to develop hypercholesterolemia and atherosclerosis. He suggested that the previously observed effects of animal proteins might be due to some non-protein constituent of the diet. Later Meeker & Kesten (1941) reconciled both results by showing that rabbits on a diet supplemented with 250 mg cholesterol per day, developed hypercholesterolemia and atherosclerosis when casein was fed as protein but were relatively protected when soya bean flour provided the protein.

Weigensberg et al. (1964) suggested the lysine content as the responsible factor for

the differences induced by several proteins. They argued that from epidemiological studies and from evidence available from animal experimental studies it could be derived that lysine-rich products or diets were correlated with the incidence of ischaemic heart disease or atherosclerosis. Rabbits were fed on diets containing 1 g cholesterol per day and providing either an excess (5 g per day), a normal amount (0.55 g per day) or deficient amounts (52 mg or 22 mg per day) of lysine (Weigensberg et al., 1964). The attempt to feed an excess of lysine to the rabbits failed because the animals refused to eat this diet (see Section 4.3). The moderately lysine-deficient diet induced considerably lower serum cholesterol, triglyceride and phospholipid values than the normal or severely deficient diet. Likewise the severity of aortic atherosclerosis was significantly reduced in the animals fed on the moderately deficient diet. The severely deficient animals exhibited the least aortic involvement. Weight and blood lipids nor atherosclerosis were correlated. These results are consistent with the hypothesis but still difficult to interpret. Inclusion of a group ingesting an excess lysine and a group at a subnormal intake avoiding body-weight loss would have made this experiment much more interesting.

Rabbits on a mixed diet with 10% protein mainly from casein, exhibited a definite though small increase of serum cholesterol (Polčak et al., 1965). The addition of meat to the diet in order to increase its protein content to 15%, depressed serum cholesterol. When 1 g cholesterol was added to the diet all serum lipids rose tremendously on the 10%-protein diet. The 15%-protein diet afforded strong protection against this hypercholesterolemia and also against cholesterol accumulation in the liver, kidneys and adrenal glands. The authors attributed most of the favourable effects of meat supplementation to the meat itself. It might be very well possible that in this case the effects are more due to increasing the protein content of the diet from sub-normal to normal, as 10% of even a mixed protein in the diet is probably below the requirements of the rabbit (see Section 4.3).

The protective effect of substituting hexane-extracted soya bean meal for casein in semi-synthetic diets, reported by Howard et al. (1965), has already been mentioned (Section 4.2). The protective effect of soya protein isolate was less or absent. The authors did not take into account the lower protein content of the soya bean meal. Thus not only the quality but also the quantity of protein was changed. It might however be speculated that if the proteins possessed similar effects, the effect of the soya bean meal should have been in the opposite direction due to a reduction in the protein content of the diet. The presence of specific cholesterol-lowering substances in the soya bean meal cannot be excluded (see Section 4.2).

The experiments of Carroll (1967, 1971) point to a clear hypercholesterolemic effect of casein for rabbits. When casein was added to a commercial diet a moderate hypercholesterolemia was induced if the diet was fat-free or contained saturated fats. No such increase was seen when polyunsaturated fat was present. Recently Hamilton & Carroll (1974) reported the results of a study with rabbits fed on semi-synthetic diets, containing no added fat and isonitrogenous amounts of proteins from various sources. Casein, casein hydrolysate, lactalbumin or skim milk powder gave average values of

serum cholesterol of 200 mg per 100 ml or higher. Defatted fish protein concentrate, beef protein and pork protein induced values of 190, 160 and 110 respectively. Vegetable proteins such as wheat gluten, peanut protein or soya bean protein gave values similar to or below those observed on normal diets, i.e. below 100 mg per 100 ml. Addition of butter or corn oil to the soya-protein diet made little difference to the plasma cholesterol level.

These results, together with the data discussed in Sections 4.2 and 4.3 indicate that the rabbit, particularly on a semi-synthetic diet where the protein is provided by one single source, is very sensitive to the protein source. This seems to be related to the particular amino acid requirements of the rabbit which are only beginning to be understood. The adverse effects of proteins high in lysine on food intake, growth, general well-being and blood lipids are worth further studying. Whether the quality of the protein is always mirrored in the serum cholesterol level as was suggested by Gaman & Fisher (1970) deserves further study. It is evident that results obtained with the casein-fed rabbit, should be considered with the foregoing points in mind. It would, however, be worthwhile to try to improve the performance of the rabbits on a casein-based diet without destroying those properties of the experimental model that relate to reactivity to dietary components of the lipid metabolism.

4.7.3 Findings with man

There are very few indications for an effect of dietary protein on blood lipids or atherosclerosis in man. Yudkin (1957), Yerushalmy & Hilleboe (1957) and Annand (1963) have calculated a strong correlation between ingestion of animal protein and the incidence of coronary heart disease in a comparison between populations. This was again confirmed by Connor & Connor (1972), with $r = 0.782$, and Stamler et al. (1972), r being 0.65 – 0.72 for man. No such correlation was found by Keys (1970). Total protein or animal protein is, however, also often strongly correlated with total calories, saturated fat, cholesterol, and sucrose intake (Stamler et al., 1972). Experimental studies are therefore needed to clarify the role of dietary protein relative to dietary fat and cholesterol.

4.7.3.1 Protein quantity

In several animal species it was demonstrated that a low-protein diet, as was found with swine and chickens, or an imbalanced protein, e.g. casein in rabbits, disturbs lipid metabolism and induces hypercholesterolemia. The correlation found between populations would imply that in man a high intake of 'high quality' animal proteins would induce hypercholesterolemia. This reverse reaction of man is also seen in malnourished individuals (Tripathy et al., 1970). The mean serum cholesterol value of protein-depleted adults was found as 113 mg per 100 ml on a diet containing 100 g butterfat. On a repletion diet, providing 50 – 100 g protein from skimmed milk and

chicken at the expense of starch, serum cholesterol rose to about 227 mg per 100 ml. Exogenous cholesterol did not influence serum cholesterol levels on the low-protein diet nor did it influence the increase on the high-protein intake. Feeding of different levels of protein induced a corresponding increment of serum lipid levels with each increment in dietary protein level. The changes were more marked at the levels of 50 g and 100 g protein intake while some individuals exhibited no further increment above 50 g protein intake. The maximum increases in serum cholesterol occurred within 3 weeks after the institution of the high protein diets and preceded any significant weight gain. Weight gain under hypercaloric conditions is known to lead to a moderate elevation in cholesterol levels in man (Anderson et al., 1957).

In children recovering from kwashiorkor, serum cholesterol levels increase by 80 – 100% (Schendel & Hansen, 1958) during the first week of treatment prior to any significant weight gain. Therefore weight gain per se does not play a significant role, during the first weeks of the high protein regimen, on the increase of serum cholesterol.

Malnutrition is usually a combined deficiency of calories, protein, vitamins, and minerals. Tripathy et al. (1970) placed all their subjects on a control diet for 4 – 6 weeks to correct all deficiency states except that of protein to observe the effect of subsequent protein repletion per se. From the higher increment of serum cholesterol compared with serum phospholipids it was hypothesized that the increase in serum cholesterol was due to increased β-lipoprotein secretion from the fatty liver after protein repletion. It is not clear whether this is due to increased synthesis by the liver of protein, phospholipid or cholesterol. From rats it is known that a high protein diet may stimulate hepatic cholesterol synthesis (Cohen & Teitelbaum, 1966).

An increase of the protein in the diet from 8.6 to 17.7% of the calories by skim milk powder and given to human middle-aged volunteers did not influence serum cholesterol (Keys & Anderson, 1957). Albanese et al. (1959) reported that in aged people increases in protein intake by supplementation of the self-selected diet with a milk protein concentrate, providing 0.5 g per kg body weight per day, were associated more frequently with an increased than a decreased blood cholesterol level. Men eating diets with protein contents of 8.5, 15.0 and 23.0% of the calories exhibited no differences in serum cholesterol (Lutz et al., 1959). In one of their series of experiments with students, Beveridge et al. (1963) observed no changes of serum cholesterol when the students were changed from a diet providing 15% of the calories as protein to one providing either 10% or 20 and 25%, respectively. A reduction of the protein content to 5% of the calories increased serum cholesterol, however.

Negative results in comparing effects of low and high protein intakes on serum cholesterol in man were also reported by Connor et al. (1961), Leveille et al. (1962) and Wilcox et al. (1964).

4.7.3.2 Protein quality

Bagchi et al. (1963) indicated that vegetable proteins are as effective as animal proteins in increasing serum cholesterol levels in malnourished children. Another comparison of the effect of equal levels of animal and vegetable proteins was made by Walker et al. (1960). They used two different groups of young women. Compared with the cholesterol level induced by their habitual diet, the vegetable protein diet caused a greater fall than the animal protein diet.

Gluten and a casein-lactalbumin mixture were compared by Campbell et al. (1965) as to their effect on serum lipids. No significant differences could be detected. Recently, Anderson et al. (1971) reported the results of an experiment with students in which the effect of egg white was compared with that of wheat gluten, each providing 8% of calories and half of the dietary protein. They used a switch-over design, each period lasting 28 days. The diets contained 120 g protein. No significant differences were found for serum cholesterol and triglycerides between the two dietary treatments. In an experiment with women Prather (1965) observed an increase in serum cholesterol if the protein content of the diet was increased by 36 g, mainly from beef and milk protein at the expense of carbohydrates.

In other studies often many more components were changed together with the protein component. Hodges et al. (1967) changed male adults from a hospital diet to a vegetable diet, still high in fat. They observed a decrease of serum cholesterol of about 100 mg per 100 ml and attributed most of this effect to the change from mainly animal to vegetable protein. They ascribed only about 30 mg of the change to effects induced by the change in kind of fat. Keys (1967), however, recalculated their data and concluded that about 90 mg of the change of cholesterol could be expected from the changes in dietary fat and cholesterol, thus leaving only a small part for the protein. He had to make some assumptions but the main conclusion remains that it is very dangerous to draw conclusions too rapidly from incidental findings.

A somewhat similar situation exists in relation to the findings of Olson et al. (1958; 1970a). Feeding of 20 g rice or mixed vegetable protein resulted in a sharp decrease of serum cholesterol of about 20%. This was primarily due to a fall in β-lipoproteins with an Sf value between 0 and 12. Their subjects were changed from a diet supplying 100 g protein, mainly of animal origin. Moreover, the subjects were in negative nitrogen balance and their diets were increased with 74 g carbohydrates from cereals, rice and legumes.

4.7.3.3 Specific amino acids

Still more difficult to interpret are the recent findings of Olson et al. (1970a, b) with formula diets containing the eight essential amino acids at levels of three times the tentative daily requirements, plus a source of non-essential nitrogen. Glycine plus ammonium acetate or glutamic acid were used as such. The formula diets were made to be comparable, as to fat content, fatty acid composition and cholesterol content, to

a control diet of natural foodstuffs. Upon changing the control diet to the amino acid formula with glycine no change in serum cholesterol occurred. When the glutamic acid-supplemented formula was given a marked fall in cholesterol and β-lipoproteins, equivalent to 70 and 121 mg respectively, was observed. A full-formula diet containing all the amino acids of the control diet in equal amounts maintained normal lipid values (Olson et al., 1970b). The formula per se thus cannot be argued as responsible for the changes observed.

However, Coles & Macdonald (1972) observed lower cholesterol and higher triglyceride values in serum of young volunteers ingesting sucrose-protein diets, when the protein was replaced by an average amino acid mixture. Calcium caseinate, sodium caseinate, egg albumen, and gelatin all induced the same effects. Graded amounts of glutamate added to a full diet are without cholesterol-lowering effects (Olson et al., 1970b).

The mechanism of action of large amounts of *glutamic acid* (about 100 g) is not clear. A clear-cut amino acid imbalance in the blood of the test persons was not found (Garlich et al., 1970). Such an imbalance may induce fatty liver as was shown in rats (Harper, 1958). A fall in plasma cholesterol may be due to a decreased entry of molecules into the plasma compartment through limitations in the biosynthesis of cholesterol or some other component of the ß-lipoproteins, or to an increased efflux from the plasma pool. This efflux may pass into other parts of the body, a redistribution, or to the liver with subsequent breakdown to bile acids and excretion through the bile in the faeces. An answer to this question can be obtained by labelling the plasma cholesterol pool directly with labelled cholesterol itself or by administration of a labelled precursor of cholesterol, e.g. acetate, and observing the change in slope of the biological decay curve of cholesterol in plasma. Olson et al. (1970b) performed both types of experiments. It was concluded that the fall in cholesterol induced by glutamate is primarily caused by a fall in entry of cholesterol molecules into the plasma pool, followed by a reduction in the departure of cholesterol until a new steady state is reached at a reduced turnover rate. The actual turnover rate of cholesterol changed from 1.7 to 1.1 g per day, when glutamate was added as source of non-essential nitrogen.

From a chemical sterol balance experiment, Olson et al. (1970b) concluded that the glutamate-supplemented diet caused a marked fall of total sterol output. The qualitative distribution of sterols and bile acids varied significantly from experiment to experiment but the net balance decreased by more than 300 mg per day. From the decay curves for labelled cholesterol the size of the rapidly miocible Pool A can be calculated. It was found that this Pool A decreased from 17 g to 11.6 g at the end of the glutamate period. This pool includes plasma, liver and intestine. From the constant slope for the slowly miscible pool B it was concluded that the changes in kinetics were confined to Pool A. All data support the view that a decreased entry of cholesterol into the plasma pool causes the fall in serum cholesterol. The production rate of cholesterol in Pool A decreased from 1.10 g per day to 0.69 g per day after the change

from the glycine amino acid formula to the glutamate formula.

The precise biochemical mechanism is not yet clear. It is, however, of importance that addition of the non-essential amino acids to the diet as glutamate affects lipid metabolism profoundly without affecting several liver function tests.

The finding that the gerbil is an experimental animal in which glutamate has the same effect upon serum lipids as in man (Bazzano, 1969) opened the way for metabolic studies. With this animal model it was found (Bazzano et al., 1972) that glycine or lysine had no cholesterol-lowering effect but glutamic acid, proline or α-ketoglutarate depressed cholesterol with 30 – 60%. With α-ketoglutarate the incorporation in vivo of tritiated water or ^{14}C-labelled acetate into liver cholesterol was depressed by 43 and 66% respectively as compared with glycine. As both proline and glutamic acid can be easily converted to α-ketoglutarate it is suggested that the Krebs-cycle intermediate α-ketoglutarate is the component responsible for the glutamate effect and maybe for the regulation of cholesterol biosynthesis (Bazzano et al., 1972). The practical implications of these findings are not clear.

In the experiment of Anderson et al. (1971) gluten and egg white were compared in man. These two proteins differ in 21 g glutamic acid per day which is much less than the more than 100 g used by Olson et al. (1970a, b) but nevertheless probably close to the maximum difference obtainable by interchanging protein-rich food in usual human diets. Lack of effect in the experiment of Anderson et al. (1971) may also be due to the high protein content of the diet, 120 g or 16% of the calories. It was shown by Olson et al. (1970b) that supplementation with glutamate of an ordinary diet will only lead to cholesterol-lowering when added to a low-protein diet. It is suggested that the ratio of non-essential to essential amino acids in the diet is critical in obtaining a hypocholesterolemic response.

Most of the experiments have clearly shown that amount or kind of proteins in the diet of man have not much influence on serum lipids. Diets very low in protein or otherwise imbalanced may decrease serum lipids but whether this is physiological or pathological remains to be elucidated.

4.8 Miscellaneous aspects of milk and atherosclerosis

In a discussion on the relationship between milk components, blood lipids and atherosclerosis three divergent aspects deserve some attention. The first is the controversy regarding the role of milk in atherogenesis and its complications which arose after it was found that some African tribes consume a lot of their daily calories as milk but do not exhibit a high incidence of coronary heart disease. The second is the recently developed possibility to increase the linoleic acid content of ruminant fats. The third is the hypothesis that milk proteins may act as antigens and provoke antigen-antibody reactions in the human body, thus leading to damage of the arterial wall.

4.8.1 Absence of CHD in some milk-drinking African tribes

In members of the Samburu tribe in northern Kenya, Shaper (1962) found very low cholesterol values, with no increase with age. Their diet consists almost entirely of milk and meat. Milk fat in times of adequacy, may furnish more than 60% of total calories. Mann et al. (1964) have reported even lower serum cholesterol levels for men of the Masai from Tanzania, the dietary habits being similar to those of the Samburus. Both tribes have a high degree of physical activity. Biss et al. (1971) confirmed the findings in the Masai and concluded that there are very few atherosclerotic complications among them. Mann et al. (1972), however, observed a degree of atherosclerosis in the Masai, similar to that in the United States. They also observed an enlargement of their arterial lumen ascribed to the high physical activity, which would counteract the atherosclerosis.

Apart from a very high physical fitness, which is in the range of a habitual marathon runner (Mann, 1972), the Masai is also different from most American and probably European whites in that he apparently has a more efficient negative feedback control of endogenous cholesterol synthesis (Biss et al., 1971). This may account in part for the great tolerance of dietary fat by the Masai. These people are lean, active, their blood pressure is low and does not rise with age. They often carry heavy loads of parasites.

There seems good evidence that in these nomadic tribes irregularity of food supply, frequently resulting in periods of semi-starvation is of major importance in accounting for their cholesterol level (Shaper, 1970). Crude, non-quantitative dietary estimates, which is all available evidence, cannot be regarded as sufficient. The meat of an African cow is certainly not the same, as to fat content and fatty acid composition, as American beef (Crawford, 1968, 1972). Whereas Western bovids from grassland environment have only 2 to 5% of poly-unsaturated fatty acids in their fat, buffalo on parkland have about 10% and those in thick woodland even 30%. These differences may be partly related to different proportions of depot and structural fat, respectively. It remains to be elucidated whether microbial ruminal hydrogenation of the products of parkland and woodland occurs to the same degree as of the fats of grass.

4.8.2 Poly-unsaturated ruminant fats

The microbial hydrogenation of the poly-unsaturated fatty acids (pufa) of the diet in ruminants is, in fact, the cause of the low pufa content of their milk and meat. In non-ruminants, such as rats, swine, poultry, veal calves (Veen, 1973), and man changes in composition of body fat can be accomplished simply by changing the dietary fat source. In recent years a method has been developed to increase the pufa content of goat's milk and cow's milk (Scott et al., 1970) from about 2% to about 30%. By the same procedure it was possible to increase the pufa content of the body fat of cattle and sheep (Scott & Cook, 1972). The fatty acids are protected against bacterial action in the rumen because they are surrounded by formalin-treated casein. The casein will

be digested more distally and the fat will be liberated for digestion and absorption in the small intestine (Scott et al., 1971).

Two feeding experiments have been reported using these pufa-rich milk and meat. In both experiments (Nestel et al., 1973a, b) a 10% reduction in serum cholesterol was observed. Faecal sterol excretion was enhanced in the first short-term experiment (Nestel et al., 1973a). In the second experiment (Nestel et al., 1973b), which lasted six weeks serum triglycerides decreased with 15%. Cholesterol absorption was similar for the normal and pufa-rich fat. Increased sterol excretion was only observed during the first three weeks, while the change in serum cholesterol took place. This suggests that an increase of sterol output can only be measured during a period of change until a new steady state is reached.

It is difficult to see which advantages there are in increasing the pufa content of ruminant fats by such a cumbersome way as the one proposed by Scott et al. (1971). From a nutritional point of view supplementation of the milk fat with linoleic acid-rich oil would lead to about the same end result. From a technological point of view many problems related to oxidative deterioration, crystallization behaviour, etc. will have to be solved.

4.8.3 Milk protein antibodies and atherosclerosis

The third aspect which has to be mentioned in the relationship between milk consumption and atherosclerosis is the higher level of milk antibodies observed in patients with myocardial infarction. Annand (1967, 1971) drew attention to the possibility that the way milk is heated might have something to do with thrombosis and atherosclerosis. The possible damaging effect of antigen-antibody complexes on the structural integrity of the arterial lining has already been discussed (see Sections 2.1, 3.3.4). Davies (1969) and Poston & Davies (1974) reviewed all evidence up till now in regard to this problem and concluded that immune mechanisms may be involved in the pathogenesis of atherosclerosis and ischaemic heart disease. There is a disturbing lack of knowledge in this theory about the question how can large milk protein molecules pass through the intestinal wall. There is yet no direct proof of this in humans. However, indirect evidence suggests that intact protein must be absorbed.

Davies (1969) proved the presence of antibodies to cow's milk protein in the blood of man. Gunther et al. (1960, 1962), measuring serum antibody levels of normal infants to cow's milk protein, found antibody titres in the sera of almost 300 infants aged 7 to 97 weeks. These antibodies were specific for casein, α-lactalbumin and bovine plasma albumin. No antibody was found for β-lactoglobulin or human milk proteins. In man with myocardial infarction increased levels of milk antibodies were found compared with controls (Davies, 1969; Poston & Davies, 1974). In a study of persons with the presence of all kinds of manifestations of atherosclerosis, higher antibody titres were observed than in controls in whom atherosclerotic manifestations were absent (Oster et al., 1974).

A somewhat different theory, but depending upon the same principle of absorption

of macromolecules is the plasmalogen disease concept of Oster (1972). According to this theory xanthine oxidase from cow's milk would escape digestion, especially in homogenized milk, and if absorbed intact, would oxidize the fatty aldehyde of plasmalogen. This alters the integrity of cell membranes. Antibodies to bovine xanthine oxidase were demonstrated by Oster et al. (1974) in human blood with a higher titre in patients suffering from some atherosclerotic manifestation than in controls. It seems thus possible that large protein molecules are absorbed intact by the intestine.

The evidence that agents which damage and increase permeability of the arterial lining can induce the atherosclerotic process is strong (Section 2.1, 3.3.4). The importance of immune mechanisms in atherogenesis remains to be determined (Poston & Davies, 1974). In experimental animals antigen-antibody complexes are a potent adjuvant to cholesterol or lipid athergenesis. By the same way such complexes, provoked by dietary antigens, might play a role in human atherogenesis. Whether the high antibody titres to cow's milk protein in atherosclerotic patients really reflect some pathological mechanism or are merely indicators of a high milk protein consumption, perhaps influenced somewhat by the technological treatment of the milk, remains to be elucidated.

An interesting observation was the high anbibody production to milk protein and xanthine oxidase found in persons with gastroenteritis and peptic ulcer (Oster et al., 1974). This last kind of patient, consuming a milk and cream diet, now can be used to support the lipid theory, the sugar theory (Yudkin, 1972) and the immunological theory of atherosclerosis. In view of the widespread habit to bottle-feed new-born infants with formulas, based on cow's milk protein and of the fact that man is probably the only animal species which continues drinking milk after weaning, clarification of this question is urgently needed.

5 Conclusions from the literature and scope of the experiments

Ischaemic or coronary heart disease is the most important single cause of death in industrialized societies. Effective approach can only be based upon prevention of its underlying cause: atherosclerosis.

Epidemiological studies have yielded an enormous number of factors which are associated with an increased or decreased degree of atherosclerosis or incidence of coronary heart disease. Animal experiments have also yielded many factors by which the development of atherosclerosis can be modified (Chapter 2). Based on these two approaches, a theory on the pathogenesis of atherosclerosis has been developed, which accomodates all factors known at this moment to be involved in the development of the disease (Chapter 3). It is shown that a high content of blood lipids, hypertension, cigarette smoking, or thrombosis is not sufficient or a necessary cause of atherosclerosis.

Atherosclerosis should be considered as the result of arterial repair and restoration processes provoked by a multitude of factors pertaining to the blood, the structure and metabolism of the arterial wall or to haemodynamics. Structural and metabolic integrity of the endothelial lining appears an essential condition for successful prevention of atherosclerotic changes. Due to the many and diverse agents which may damage integrity it is unlikely that prevention can only be based on maintaining this integrity.

High blood pressure may damage the endothelial structure and increase plasma flux through the arterial wall. Cigarette smoking may cause increased carboxyhaemoglobin values. The carbon monoxide very probably damages the endothelium, rendering it more permeable for plasma constituents. Blood lipids, as lipoproteins, normally traverse the arterial wall but may become trapped as a consequence of altered composition, increased flow, or decreased metabolic activity of the arterial wall. The kind of lipid, e.g. cholesterol or triglyceride, the kind of cholesteryl esters, may determine the effect of the trapped lipid.

These three risk indicators, hypertension, cholesterol, and cigarette smoking, can therefore be regarded as causally related factors. Another important factor, usually not measured in epidemiological studies, is the thrombosis tendency. This factor, if enhanced, may contribute to the initiation and progression of atherosclerotic plaques. There seems to be an important relationship between dietary fat composition and thrombosis tendency, the poly-unsaturated fatty acids having a diminishing influence, the saturated fatty acids from myristic acid onwards an enhancing one. Prostaglandins, derived from linoleic acid metabolites, may play a decisive role.

Diet is frequently implicated as one of the most important factors in the causation and prevention of the disease. Dietary fat is intimately related to blood lipids, thrombosis tendency and atherosclerosis in experimental animals and man. Quality and quantity of dietary protein are important, as to blood lipids, in experimental animals. In man the effect of protein quality is minimal and of protein quantity is still dubious due to lack of information or poor experimentation. Milk fat provides in many industrialized countries 30 - 40 g per day or 25% of the calories used as fat (Renner, 1974: p. 17). In the Netherlands in 1972 the use of milk fat was about 27 g per capita per day and the daily grooss intake of fat 138 g (Muller, 1974). In Finland, however, about 70% of the total fat is derived from milk fat (Roine et al., 1968). Detailed knowledge of the properties of milk fat and the components from which it is made up is still fragmentary as to effects on blood lipids, thrombosis and atherosclerosis. Fractions from milk fat, obtained by crystallization, are not sufficiently different to induce differences in blood lipids. The cholesterol present in it may be the cause of almost half the hypercholesterolemic properties of the fat. The specific fatty acids with a chain length shorter than 12 carbon atoms seem to be neutral but this is mainly established with octanoic acid. The results in rabbits were equivocal.

The use of experimental animals in atherosclerosis research is necessary because effects of dietary factors on atherosclerosis can not be measured in the living human. Dietary changes can be more deliberately effectuated in animals than in man. The rabbit has been used in most experiments. Most objections against the use of this animal have disappeared since it has been demonstrated that the lesions, induced by diet, resemble human lesions very closely if enough time is allowed for progression and if the cholesterolemia is only moderate, within the range usually found in man.

Important progress has been made by the demonstration that rabbits may develop hypercholesterolemia and atherosclerosis when fed on a semi-synthetic diet devoid of cholesterol. Saturated fats enhance the atherogenic property of such a diet while polyunsaturated fats depress it. Fat-free semi-synthetic diets are also atherogenic, indicating that some component of this diet should be hypercholesterolemic. Casein, which is used as protein source in such diets, appears a very imbalanced protein for rabbits and it is tempting to suggest that this may be responsible for the properties of the semi-synthetic diet.

The experiments described in this report center around the establishment of the hypercholesterolemic principles of milk fat and of the semi-synthetic diet.

In the first experiment the effects of a large dose of butyric acid were compared with those of a saturated fat, coconut oil, and a carbohydrate, starch. The objective of the second experiment was to define which factor in the milk fat contributed most to the hypercholesterolemic property of this fat, i.e. the major fatty acids, the shorter-chain fatty acids or the cholesterol. Moreover, the atherogenic potential of milk fat and coconut oil were compared. These two experiments were done with the classical semi-synthetic diets. Most of the well-known complications reported in the literature for this experimental model were observed.

In the third experiment, therefore the improvement of the basal diet was the objective. The source of dietary protein seemed to be an important factor and a new protein source was devised. In the fourth experiment again an assessment of the hypercholesterolemic principle of milk fat was made, mainly with regard to its cholesterol and its low linoleic acid content. The diets contained either casein, as usual, or the newly devised protein. The type of protein seemed to be the overriding factor.

The influence of dietary protein source and its mode of action were further investigated in the fifth experiment by feeding casein or various protein mixtures in combination with coconut oil.

6 Material and methods

6.1 Animals

All experiments were performed with male rabbits. The experiments were started with Dutch Belt rabbits (Broekman Institute, Stiphout). Unfortunately if the experiments lasted more than six months many of this breed of rabbits died, mainly because of their sensitivity to snuffles disease. This disease clinically manifests itself by a mucus, or mucopurulent discharge from the nose. Additionally sneezing, coughing, and a snuffling sound are made. The disease is very probably caused by *Pasteurella multocida*, which may be carried by many rabbits asymptomatically in the nasal cavity. Some form of stress, such as experienced by experimentation, is evidently required to debiliate the host and to initiate clinical disease. The rabbits consuming a commercial rabbit chow did not exhibit signs of the disease. This *Pasteurella multocida* is also considered as the main cause of pneumonia in rabbits. At autopsy many of these Dutch rabbits showed pneumonia with severe abscesses from which *P. multocida* could be isolated. No effective remedy against 'snuffles' is known.

In the next experiments therefore White Viennese × Alaska crossbred rabbits were used (TNO Central Institute for breeding of laboratory animals). The general health status of these rabbits was satisfactory. The sensitivity of this breed for hypercholesterolemic agents appeared somewhat less than of the Dutch Belt rabbits.

All rabbits were delivered at the age of three to four months. The animals were fasted for 24 hours upon arrival and then got a diet of commercial rabbit pellets (Trouw & Co) for one month. All animals were treated twice prophylactically with 2 ml sulfamesathine (16%) per animal per day for three to five consecutive days As the change-over to the experimental diets took about six weeks, the animals were $6 \pm \frac{1}{2}$ months old at the beginning of the experimental period.

6.2 Housing

The animal experiments were carried out at the ILOB facilities Wageningen (Institute of Agricultural Research of Biochemical Products). The rabbits were individually housed in galvanized cages with floors of galvanized wirescreen. The cages were placed in an automatically heated, ventilated and illuminated room. The daylength was set at 15 hours. It was intended to keep the temperature at 18° C. Because of the absence of refrigerating facilities until the last experiment, this temperature was gradually increased in the summer to 22° C and kept at that level to avoid large temperature

fluctuations. However, it was inevitable that the temperature sometimes rose as high as 26 or 27° C, which resulted in an immediate drop in food consumption. The relative humidity was automatically adjusted at 60 – 70% and continuously recorded as was done with the room temperature.

6.3 Diet preparation and feeding procedure

The experimental semi-synthetic diets consisted of a basal diet, containing the protein, carbohydrate, fibre, vitamins and minerals and a part consisting of water and the fat under investigation. The basal diet was for the first experiment prepared every three months but for the other experiments every month, which resulted in improved acceptability. Maintenance of food consumption was taken as evidence for this. The final diets were prepared freshly three times a week by mixing the melted fats with the basal rations and by adding 40– 60 parts of water to 100 parts of diet. The water was added to change the flour-like structure of the diet into a paste-like structure to prevent spillage and make it more acceptable to the rabbit. This prepared food was stored in a refrigerator at 2° C.

Every day each animal got 100 – 125 g wet food which was slightly more than the consumption ad libitum. The food was supplied in a disposable polystyrene beaker, attached to the front of the cage with a special holdfast such that spilling was minimal and only the head of the animal could reach the food. Left food was collected every two days on a group basis, weighed to establish food consumption and disposed of. The animals were allowed to drink (soft) tapwater from drinking bottles ad libitum. The water was refreshed every day. The rabbits were weighed at arrival and thereafter minimally at fortnightly intervals.

After the first 4 weeks during which the animals were given a diet of commercial rabbit pellets to accustom them to the new environment, all rabbits were gradually changed to the semi-synthetic diet. This procedure took about four to five weeks. At first the pellets were ground and supplemented with water to accustom the rabbits to the structure of the diet. Subsequently part of the ground pellets was exchanged with the semi-synthetic diet. This exchange was completed in eight steps. All animals in a certain experiment were changed to a diet, containing the same source of protein and fat. After completion of this procedure the rabbits were distributed over the experimental treatments and during about one week gradually changed over to their experimental diets. After this change-over was completed the actual experimental period started.

Distribution of the animals over the different groups was based on body wieght gain and increase in serum cholesterol during the pre-experimental change-over period such that the frequency distribution and group means for body weight and serum cholesterol were similar. In some experiments also hereditary relationships of the animals were known and these were taken into account in the distribution procedure. In most experiments a small group of control rabbits was fed on a diet of commercial pellets.

6.4 Diets

The commercial rabbit diet was obtained from Trouw & Co (trade mark Cunicon I, size 3.5 mm). It was composed of corn, wheat, oats, carrob, extracted soya bean meal, sunflower seed meal, vegetable oil, vitamins and minerals. It provided 18.3% (w/w) crude protein, 4.3% crude fat, 9.4% crude fibre, 7.0% ash, 49.3% nitrogen-free extract, and 89.0% dry matter.

The semi-synthetic diets were prepared as described above (6.3) at ILOB. Its composition as described by Wigand (1959) is based on Wooley's (1954) diet 1436 for the growth of rabbits. All nutrients were calculated on a caloric basis to provide for differences in energetic density of diets differing in fat content. The sum of fat and starch calories always was kept nearly constant. Because of small differences in the diets used in the different experiments it is not possible to give a detailed composition here. This will be given with the respective experiments. Only a rough composition of the basal diets is given in Tables 2a, b and c. A standard diet provides 36 – 40% of the calories as fat, 23% as protein and 33 – 37% as starch.

Table 2a. Composition of the standard experimental diet.

	g/1000 kcal	g/100 g	kcal/100 kcal
Corn starch[1]	102	34.8	35.7
Casein[2]	62	21.2	23.0
Fibre[3]	63	21.5	
Soya bean oil[4]	4.3	1.48	40.1
Fat[5]	38.7	13.21	
Mineral mix[6]	5	1.71	0.6
Vitamin mix[7]	3	1.02	0.6
Potassium chloride	4	1.37	
Calcium phosphate	8.5	2.90	
Sodium chloride	2.5	0.85	
Total	293	100	100

1. Corn starch (maisamyl, Honig N.V.): fat, 0.4%; protein, 1.2%; dry matter, 88%; energy, 3.5 kcal per g.
2. Casein (acid casein, DMV Veghel): fat, 1.2%; protein, 88%; lactose, 0.5 – 1.0%; ash, 2.5%; energy, 3.7 kcal per g.
3. Fibre (saw dust, Broekman Institute, Stiphout): sterilized by radiation.
4. Soya bean oil added to improve texture and to supply essential fatty acids; only used in Experiment 1.
5. In all experiments refined coconut oil served as the control fat; in Experiment 1 starch was isocalorically substituted for the fat.
6. See Table 2b.
7. See Table 2c.

Table 2b. Composition of the mineral premix in the standard experimental diet (mg salts added per 1000 kcal).

$MgCO_3$	900	KBr	6
MgO	600[1]	NaF	2.5
Na Citrate $\cdot 2H_2O$	450	$NiSO_4 \cdot 6H_2O$	2.5
$FeSO_4 \cdot 2H_2O$	185	$Na_2B_4O_7 \cdot 10H_2O$	1.5
MnO	40.5	$CoSO_4 \cdot 7H_2O$	1.5
$KAlSO_4 \cdot 3H_2O$	30	$Na_2MoO_4 \cdot 2H_2O$	1.5
$ZnSO_4 \cdot 7H_2O$	60[1]	KJ	1.5
$CuSO_4 \cdot 5H_2O$	30[1]	As_2O_3	0.05

1. In Experiment 1 slightly different amounts were used: MgO, 1500; $ZnSO_4 \cdot 7H_2O$, 3; $CuSO_4 \cdot 5H_2O$, 8.

Table 2c. Composition of the vitamin premix in the standard experimental diet (as pure vitamin added per 1000 kcal).

Thiamine	17.6 mg
Riboflavine	6.6 mg
Niacin	44.6 mg
DL-Ca-Pantothenate	16.5 mg
Choline chloride	600 mg[1]
Inositol	293.5 mg
Folic acid	2.5 mg
Biotin	0.5 mg
Pyridoxine hydrochloride	6.6 mg
PABA	146.8 mg
Vitamin B_{12}	0.0045 mg[1]
α-Tocopheryl acetate	15.0 mg
Vitamin K_3	1.2 mg
Vitamin A	4500 iu[1]
Vitamin D_3	900 iu[1]
Vitamin C	125 mg[1]

1. In Experiment 1 slightly different amounts were used: Choline, 293.5; Vitamin B_{12}, 0.0024; Vitamin A, 1200 iu; Vitamin D_3, 400 iu; Vitamin C, absent.

6.5 The experimental period

All animals were weighed individually at the beginning of the experimental period and at fortnightly intervals afterwards. Though the rabbits were not housed in metabolism cages to measure food intake, food spillage was so minimal that the amounts of food ingested could be determined fairly accurately. This was done on a group basis for weekly periods and recalculated as weight of dry food ingested per day per

rabbit actually present. This procedure is only minimally influenced by mortality during the experiment.

Immediately before the animals were changed to the semi-synthetic diets and at completion of this accustoming period small blood samples of about 2 ml were taken from the marginal ear vein for serum cholesterol determinations. This bleeding procedure was repeated throughout the experimental period at about four weekly intervals. Any other determinations will be reported with the respective experiments.

At the end of the experimental period large samples of blood were taken from a marginal ear vein, or by heart puncture after an overnight fast of 16 hours. Animals were killed by exsanguination. As rapidly as possible thereafter the liver, spleen, adrenals, kidneys, in some experiments also the lungs, testes and pancreas, and the heart, together with the aorta extending to the bifurcation into the two iliac arteries were removed from each rabbit for chemical analysis or histological examination.

6.6 Chemical methods

All extraction, purification and analytical methods were those commonly used in lipidology. General literature on sampling, handling and storage of lipids can be found in Nelson (1972), Kates (1972), Henry et al. (1974) and Zöllner & Eberhagen (1965).

Serum lipids, lipoproteins and haemoglobin Serum cholesterol determinations were routinely made according to the method of Abell et al. (1952). Two pooled sera were used throughout as internal standards to guarantee constant levels of the determinations. If total lipids were determined during an experiment, the sulfo-phosphovanilline reaction was used (Zöllner & Eberhagen, 1965). In the last two experiments triglycerides were determined colorimetrically by the method of Soloni (1971). Haemoglobin was determined as cyanmethaemoglobin (Henry et al., 1974). Haematocrit was determined with a microtechnic. Lipoprotein distribution was established by polyacrylamide gel electrophoresis (apparatus: Canalco) according to Schaafsma & Van Oudheusden, 1974) followed by densitometry (Clifford densicomp 445). Lipoprotein fractions were obtained by ultracentrifugation (Christ omega 1) under conditions described by Van Gent (1972). Lipids from the isolated fractions were extracted (Folch et al., 1957), and analysed by thin-layer chromatography (tlc) followed by densitometry (Van Gent, 1968). Total lipids in the extracts were determined gravimetrically (Cahn microbalance) and used as the basis for the calculation of the lipid classes. Organic phosphorus was also determined in the extract (Böttcher et al., 1961). A factor of 25 was used to obtain the phospholipid content.

General health screening For general health screening, several parameters were used: alkaline phosphatase, SGOT, SGPT, γ-glutamyl transpeptidase (γ-GT), bilirubin, total serum protein, serum albumin. These determinations were performed with Boehringer Biochemica test combinations (Boehringer Mannheim) except albumin. The bromcresolgreen method of Doumas et al. (1971) was used to determine albumin.

Lipid composition For the determination of the lipid fractions at the end of the experiments, lipids were extracted from serum (Folch et al., 1951) or liver (Folch et al., 1957). Total lipids were determined gravimetrically, organic phosphorus (Böttcher et al., 1961) and total cholesterol colorimetrically in part of the extract. The rest of the extract was divided into two parts. One part was used for quantitative analysis of the lipid classes after separation by tlc. The other part was used for fatty acid analysis of the lipid classes, also after separation by tlc. The tlc plates, 0.5 mm thick layer of silicagel H, were first developed with chloroform for cleaning and, after sample application, developed with a mixture of petroleum ether (40 - 60°C)/ether/glacial acetic-acid (160:30:5) (Stahl, 1967). The lipid bands were visualized with a rhodamine-6G or 2,7-diCl-fluorescine spray, scraped off the plate and eluted. Free cholesterol and esterified cholesterol were determined in the eluate (Abell et al., 1952) as was the glyceride glycerol (enzymatic determination with Boehringer test combination).

Fatty acid composition Lipid bands from the other tlc plate were also scraped off and eluted. After preliminary experiments it appeared that triglycerides and phospholipids are readily hydrolysed, within 5 min at 80°C. Cholesteryl esters, and by consequence total lipids, require a saponification time of 30 min at 80°C. tlc was used to obtain information on the completeness of saponification and subsequent esterification. During esterification by BF_3-methanol (14% w/w) for at least 2 min at 80°C triglycerides and phospholipid fatty acids remained in the same vessel, a 2-ml flask. After completion, the flask was cooled in ice, saturated NaCl solution was added till the level of the fluid was in the narrow neck of the flask. The fatty acid methyl esters float on top of the salt solution. The methyl esters were extracted by shaking with 0.2 ml petroleum ether (30 - 40°C) and analysed by gas liquid chromatography (glc; Varian Aerograph 2100). This procedure is largely based on that of Metcalfe et al. (1966) with some modifications to prevent loss of shorter-chain fatty acids. The flask was always kept closed by teflon stoppers, and only opened to add salt and petroleum ether solutions. Petroleum ether was used to collect the esters as the amount of lipid was only about 2 mg. After saponification of cholesteryl esters and total serum or liver lipids, liberated cholesterol was removed by hexane extraction. The fatty acid solution was further treated as described above.

Gas liquid chromatography was performed on a Varian Aerograph 2100 dual column gas chromatograph with dual flame-ionization detectors with quartz flame tips. The columns (180 × 0.3 cm, all glass) were packed with 5% ethylene glycol adipate (EGA) on 80 - 100 mesh chromosorb-G. Temperature of the injection port and of the detector was 200°C. The samples were injected at an initial column temperature of 75°C, held isothermally for 1 minute and then temperature programmed at 4°C/min to 200°C. Gas flow rates were: hydrogen: 30 ml/min; air: 430 ml/min; and nitrogen: 20 ml/min. The chromatographic peak areas were calculated by an electronic digital integrator (Infotronics CRS-144). Recovery of fatty acid methyl esters was quantitative from a mixture of pure triglycerides down till C6:0. For butyric acid a correction factor was required of 1.49.

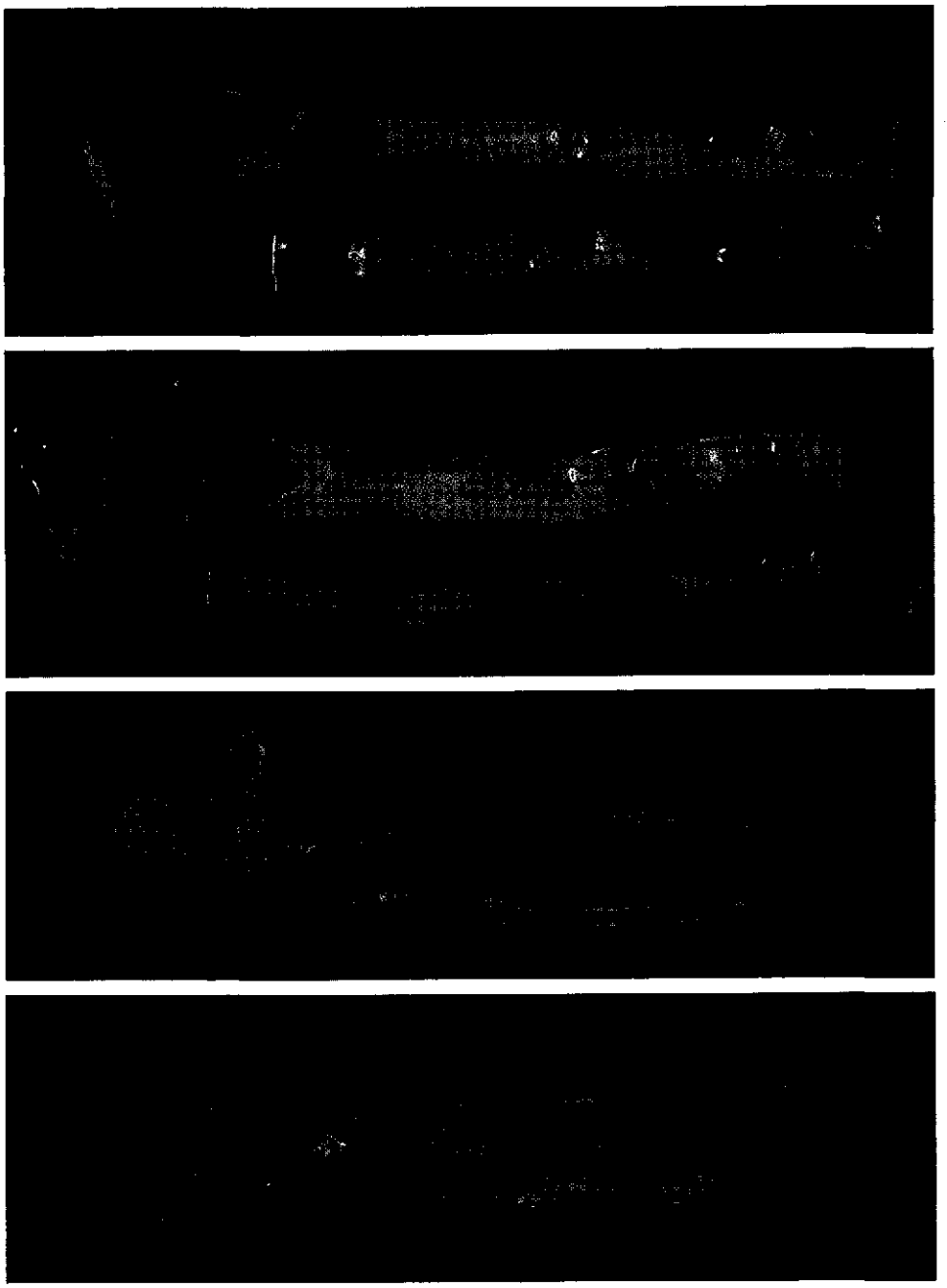

Plate. Characteristic examples of rabbit aortas with different extent of atherosclerosis.
From top to bottom: Grade 0, Grade 1, Grade 2 and Grade 3.

Cholesterol esterification rate In Experiment 5 cholesterol esterification activity was determined in serum of rabbits, both with its own (autologous, native) lipoproteins and with pooled (homologous) lipoproteins as substrate for the reaction. The method has been described by Stokke & Norum (1971) for autologous serum as substrate and by Stokke (1974) and Lacko et al. (1974b) for the use of heat-inactivated homologous serum. A tracer dose of 7 α-^{3}H-cholesterol was added to the sera, and after the reaction was stopped, free and esterified cholesterol were extracted, separated by tlc and activity in both fractions counted in a liquid scintillation counter. With the use of 'instagel' (Packard), it was not necessary to elute the lipids from the silicagel.

Cholesterol turnover rate Also in rabbits from Experiment 5 a cholesterol distribution and turnover study was performed according to the two-pool model as described by Goodman & Noble (1968) and Nestel et al. (1969). A 5-μCi dose of 4-^{14}C cholesterol per rabbit is injected into an ear vein and specific activity of serum cholesterol is determined after 4½ hours, every day during the first week, and every two days thereafter. After seven days a straight line for the specific activity is obtained which permits extrapolation to time zero. For details of calculation the reader is referred to the above mentioned literature and Section 11.6.

6.7 Histological methods

Immediately after death, organs were removed and weighed after examination for any gross pathological change. Specimens of kidneys, liver, spleen, lungs, and adrenals were fixed in 10% neutral formalin for histological examination. Heart and aorta were removed as a whole and cut longitudinally from the iliac bifurcation to the heart. Heart and aorta were flattened between glass plates, fixed in 10% neutral formalin, and stained. Staining was carried out, according to Kloeze et al. (1969), in equal quantities of dry Sudan III and Sudan IV, 2% solution in acetone and 70% ethanol (50:50, v/v). After staining and differentiation in 70% ethanol, the heart plus aorta were washed in water and kept in 10% formalin. From each preparation a coluor slide was made.

The degree of atherosclerosis was assessed macroscopically in accordance with an arbitrary scale ranging from 0 to 4, using size and thickness of the plaques as a criterion, by at least three independent observers (Duff & McMillan, 1949; Gottenbos & Thomasson, 1961). Typical examples of the aortas of grade 0 – 3 are shown in Plates 1 a, b, c, d. Aortas with Grade 0 exhibit no visible lesions while Grade 1 embraces all aortas with even the slightest fatty spots, mainly around intercostal orifices. Grade 2 comprises the aortas with more extensive lesions over the complete length of the aorta. Grade 3 is given to aortas with confluent plaques consisting of cushion-like swellings as a result of which the lumen is narrowed. Grade 4 would apply to complicated or very severe lesions which were not observed in our material. In Experiment 2 this grading was complemented with an assessment of the percentage surface area with sudan-positive staining. This was done by projection of the colour

slides on a screen with a grid of one-cm squares. The red and white coloured squares were counted and the percentage sudan-positive surface area calculated.

Frozen and paraffin sections were made from the removed organs for histological examination. Detailed results will not be published in this thesis. The observed pathology appeared similar to that reported by several investigators using rabbits in long-term experiments fed with semi-synthetic or purified diets (Wigand, 1959; Stormby & Wigand, 1963; Vles et al., 1964; Vles & Kloeze, 1967; Büller & Vles, 1966; Kloeze et al., 1969; Moore & Williams, 1964a; Funch et al., 1962).

6.8 Statistical methods

The response of rabbits on hypercholesterolemic diets is very variable. Some do not respond at all, some react moderately and others are extremely sensitive. This inherent variability causes some problems with the interpretation of results. Some investigators (Moore & Williams, 1964b; Krogh et al., 1961) found for serum lipids that the standard deviation within a group tended to be proportional to the group mean. After logarithmic transformation, heterogeneity among the within group variances was reduced and variances of the distributions became independent of the transformed values. This was confirmed by our observations. The Shapiro-Wilk test was used to test for normal distribution and the Bartlett test for independance of variances (Sachs, 1974). Even though serum lipid values did not appear to have a normal distribution, standard errors of the means are, nevertheless, reported in agreement with the common use in the literature and to provide an idea about variance.

Body and organ weights, which had a normal distribution and serum lipids, after log transformation, were tested for differences by the t-test for two groups or by one-way analysis of variance for more groups (Lindquest, 1953). If it could be decided that a group effect might be present, a sequential range test was performed to test which group means were different (Student-Newman-Keuls test: McCall, 1960). Differences in fatty acid composition were similarly analysed.

Sometimes the number of samples was too small to decide about a normal distribution of the values or was such an assumption evidently not valid. In these cases non-parametric techniques have been applied (Siegel, 1956). As a non-parametric analysis of variance the Kruskal-Wallis test has been used. To test differences between two groups, the Wilcoxon Q test was used. As a measure of correlation the Spearman rank correlation coefficient (r_s) was the method of choice. The χ^2 test was applied to test differences in degree of atherosclerosis.

In the text reference is mostly made to the method used.

Findings at $P < 0.05$ were regarded as significant. However, as pointed out by Simpson et al. (1974), when sample sizes are relatively small or when an exploratory study is undertaken, adherence to standard levels of significance such as 0.05 leads to excess risk of prematurely dismissing relationships between variables that may be genuine but weak: in statistical terms a type-II error. We have therefore accepted the level of $P < 0.10$ as indicative for a trend or possible effect.

7 Effects of tributyrate versus coconut oil or starch (Experiment 1)

7.1 Objectives

One of the characteristics of milk fat is the presence of fatty acids with a chain length of less than 12 carbon atoms, particularly butyric acid. As is demonstrated in Sections 4.6.4 and 4.6.5 these fatty acids are metabolized differently from the longer-chain fatty acids in all animal species studied. The net effects appear to resemble those effected by carbohydrates more than by fats. In Experiment 1 a diet supplying 36% of calories as coconut oil has been compared with diets in which half of the coconut oil calories were replaced by tributyrate or corn starch, respectively. Influence on body weight, food consumption, blood and liver lipids, degree of aortic atherosclerosis, and fatty acid composition of blood lipid classes and of perirenal adipose tissue was investigated.

7.2 Experimental details

Forty-five male Dutch Belt rabbits, about five months old, were gradually accustomed to the semi-synthetic diet with 36% of calories as coconut oil. Then they were distributed over three experimental groups of 15 rabbits each. Level of serum choles-

Table 3. Composition of diets (Experiment 1).

Components	Group 1		Group 2		Group 3	
	g/1000 kcal	% (cal/cal)	g/1000 kcal	% (cal/cal)	g/1000 kcal	%(cal/cal)
Basal diet	250.0	60	250.0	60	250.0	60
Soyabean oil[1]	4.3	4	4.3	4	4.3	4
Coconut oil[2]	38.7	36	19.4	18	19.4	18
Tributyrate[3]	–	–	25.7	18	–	–
Corn starch	–	–	–	–	51.4	18

1. Soyabean oil: fatty acid composition: 16:0, 10.6%; 18:0, 3.9%; 18:1, 23.9%; 18:2, 53.7%; 18:3, 8.1%.
2. *Coconut oil: fatty acid composition: 6:0, tr.; 8:0, 8.5%; 10:0, 6%; 12:0, 50%; 14:0, 18%; 16:0,* 7.5%; 18:0, 2.5%; 18:1, 6%; 18:2, 1.5%.
3. Tributyrate: Merck, Darmstadt. Does not contain other fatty acids by g.l.c. Caloric value assessed with a bomb calorimeter as 7.0 kcal per g, compared with coconut oil as 9.3 kcal per g.

terol and body weight and the increase in these two parameters during the pre-experimental period were used as criteria for distribution. The rabbits were then changed to their experimental diets, by replacing part of the coconut oil by starch or tributyrate. The experimental period lasted 23 weeks. Four animals were kept on a diet of commercial rabbit pellets as controls. The diets are schematically represented in Table 3. The basal diet as well as other experimental details are given in Chapter 6.

7.3 Results

7.3.1 Food consumption

At the start of the experiment the rabbits were 6 – 7 months old and thus full-grown (Bragdon, 1952). For maintenance of body weight Wigand (1959) calculated and actually observed that 25 g of his pellets, containing 26% of calories as fat, per kg body weight per day were sufficient. In Table 4 the food consumption data are reported as g per day per rabbit or as kcal per animal per day. No individual food intake was established. As the weight of the rabbits at the beginning of the experiment was about 2 kg the data indicate that the food intake is in accordance with the figures of Wigand (1959) and should be just sufficient to cover the rabbit's energy requirements. Rabbits appear to eat tributyrate as readily as coconut oil or starch.

7.3.2 Body weights

The very slight changes of the mean body weights (Fig. 1 and Table 5) are also evidence for the observation reported above, that food consumption was just sufficient to maintain body weight. The observed trend for a somewhat higher food intake when starch replaces part of the coconut oil appears to be reflected in the slightly better performance as to body weight. In Table 5 the mean initial weights and the mean body weights at 20 weeks are shown for those animals which completed the full 23 weeks duration of the experiment. Data of the three animals which died during the experiment (see below) are omitted because of possible weight changes just before

Table 4. Food consumption data (Exp. 1).

Group	Food consumption	
	g/day[1]	kcal/day
1 (36 % (cal/cal) coconut oil)	44.4	151
2 (18 % (cal/cal) coconut oil + 18 % (cal/cal) tributyrate)	46.4	155
3 (18 % (cal/cal) coconut oil + 18 % (cal/cal) starch)	52.0	160

1. Dry matter.

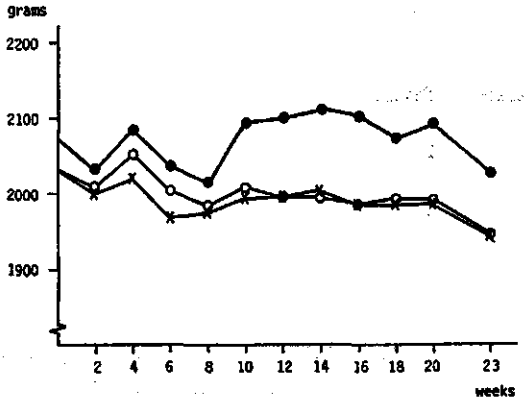

Fig. 1. Exp. 1. Mean body weight of rabbits, surviving the experiment, as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, tributyrate; Group 3: ●, starch.

Table 5. Mean body weight changes (± s.e.) in g of rabbits, surviving Experiment 1, between 0 and 20 weeks.

Group	n	Initial body weight	%	Final body weight	%	Change
1	13	2035 ± 47	100	1987 ± 52	100	− 48 ± 29
2	15	2036 ± 33	100	1994 ± 48	100	− 42 ± 48
3	14	2075 ± 40	102	2092 ± 48	105	+ 17 ± 44
Controls[1]	4	2202		2688		+486

1. Fed on commercial rabbit pellets.

death. Fig. 1 shows the group means for body weight during the 23 weeks experiment. Neither the differences between the mean body weights of the three groups at the end of the experiment nor the changes in body weight were significant ($P > 0.2$). The drop in body weight for all groups at 23 weeks is probably caused by the overnight fast as rabbits usually eat most of their food during the night. Compared with the controls, fed on laboratory chow, weight gain is, however, seriously depressed.

7.3.3 Mortality

Many animals showed symptoms of snuffles disease during the experiment, having a wet nose and a purulent discharge from the nose. Normally this did not interfere seriously with the course of the experiment. However, combined with a high environmental temperature during the summer months (see Fig. 1), a decline in appetite and weight reduction occurred. In July and August three animals died, two from Group 1 (coconut oil), one from Group 3 (starch). At autopsy they all showed seriously affected lungs with hardly any normal lung tissue left. From these animals *Pasteurella multocida* could be isolated and this may be held partly responsible for the troubles.

Table 6. Occurrence and severity of snuffles disease (Experiment 1).

Group	Not affected	Slight	Moderate	Severe
1	2	5	6	2
2	7	2	5	1
3	6	5	4	0
Controls	4	0	0	0

Antibiotics did not relieve the symptoms. The disease was spread over all groups but Group 1 had the highest frequency of affected animals and also the most severe symptoms, see Table 6. Absence of manifest symptoms among the control rabbits suggests that resistance against infection is reduced in the animals consuming the fat-rich semi-synthetic diets.

The deleterious influence of high temperatures (above 22°C) was not only visible through an aggravation of the signs attributed to the snuffles disease. Warmth appeared also to cause a decrease of food consumption. The graph depicting the course of the temperature almost mirrors the graph showing food consumption.

7.3.4 Serum cholesterol

Fig. 2 shows the mean serum cholesterol content of the rabbits from the three groups during the experiment. The group means are calculated from the values of the individual rabbits which survived till the end of the experiment. The curves of the tributyrate and starch groups are almost identical. The coconut oil group shows a

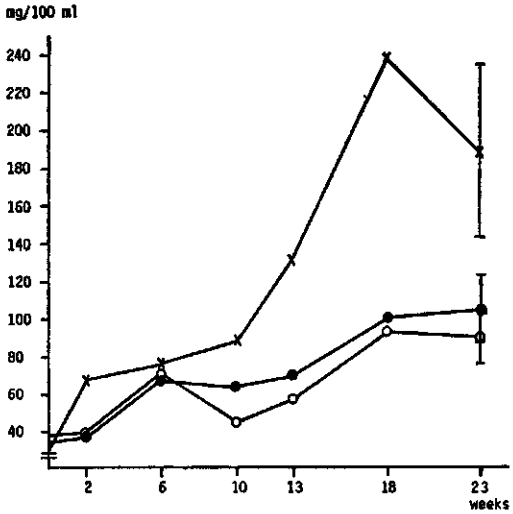

Fig. 2. Exp. 1. Mean serum cholesterol content of rabbits, surviving the experiment, as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, tributyrate; Group 3: ●, starch. Vertical bars denote s.e. mean.

Table 7. Mean serum cholesterol content (± s.e.) of those animals surviving Experiment 1.

Serum cholesterol	Group 1 (n = 13)		Group 2 (n = 15)		Group 3 (n = 14)	
Weighed average cholesterol content	138	± 37	71	± 10	81	± 11
Terminal cholesterol						
arithmetic mean	188	± 46	90	± 14	105	± 17
mean log	2.1393	± 0.0963	1.8765	± 0.0729	1.9434	± 0.0744
geometric mean	138		75		88	

different pattern, particularly from 10 weeks onwards. They have mean cholesterol values double those from the other two groups. Within each group the individual cholesterol values scatter widely. The standard deviation is almost as great as the group mean. Also intra-individual variability is great. The sharp peak in Group 1 at 18 weeks is caused by a tremendous increase in one animal followed by a similar decline at 23 weeks. Such changes cannot be attributed to technical errors but show real biological variability.

Large fluctuation of serum cholesterol values is a phenomenon often observed in rabbits, the cause of which is unknown (see e.g.: Wigand, 1959; Vles & Kloeze, 1967). To avoid misinterpretations caused by incidentally high or low values of some individual rabbits, the weighed average cholesterol value over the entire experimental period was calculated. The results are shown in Table 7. For comparison also the values at the end of the experiment, called terminal values, are given.

Another way to avoid possible misinterpretation is indicated by Moore & Williams (1964b). They applied logarithmic transformation to reduce heterogeneity among the within-group variances. The same procedure was successfully applied by Carlson & Lindstedt (1968) and Truswell & Mann (1972) for the statistical evaluation of serum triglycerides in man, which appeared also not normally distributed but skewed to the right. The average cholesterol concentration, calculated over the entire experimental period is lower than the terminal content, because of the low values at the beginning. The variability is somewhat reduced as compared with the terminal values. Logarithmic transformation, however, reduces variability most. The mean log cholesterol values are shown in Fig. 3. The serum cholesterol content of the control rabbits remained constant during the entire period between 20 and 40 mg per 100 ml. The differences between the groups did not reach significance during the experiment at the 5% level. The differences between the mean terminal values, the log terminal values and the log average cholesterol values had a $P \geqslant 0.1$. With the non-parametric Wilcoxon test, differences between the groups for terminal or average cholesterol values were also not significant with one exception. The difference between Groups 1 and 2 at the last bleeding point after 23 weeks just reached 5% significance with the Wilcoxon test.

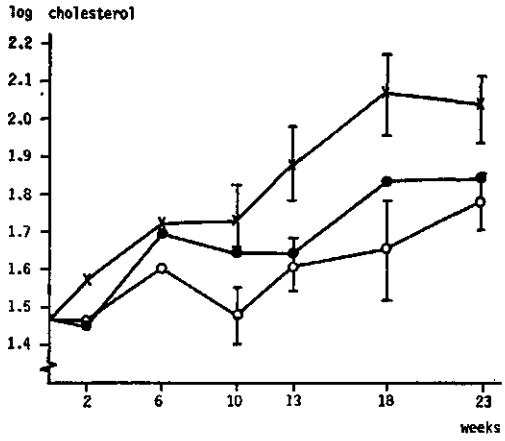

Fig. 3. Exp. 1. Mean serum cholesterol content, log transformed values, of rabbits, surviving the experiment, as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, tributyrate; Group 3: ●, starch. Vertical bars denote s.e. mean.

7.3.5 Observations post mortem

After the animals were killed and the organs removed as described in Section 6.7, the aorta was graded using an arbitrary scale from 0 to 4. The results are shown in Table 8. No clear differences can be observed between the groups. The tributyrate group tends to have a somewhat lower incidence of lesions but this is very difficult to interpret as even the slightest red spot is graded as degree 1. The distance between Grade 0 and 1 is much smaller than between 1 and 2 etc. The relative absence of animals with a serum cholesterol content of higher than 100 mg over the entire experiment in Group 2 as compared with Group 3 may explain the lower incidence of Grade 1 in Group 2 than in Group 3.

The weight of the lungs, spleen, liver, kidneys, adrenals, testes and pancreas were recorded and are shown in Table 9. The relative weights are computed on the basis of g per 1000 g body weight and shown in Table 10.

No great differences were present between the groups in either absolute or relative

Table 8. Aortic atherosclerosis (number of animals per atheroma grade); Experiment 1.

Group	n	Grade[1]				Incidence[2]
		0	1	2	3	
1 coconut oil	13	7	5	1	0	6/13
2 tributyrate	15	11	4	0	0	4/11
3 starch	14	6	8	0	0	8/14
Controls	4	4	0	0	0	0/4

1. Mean atheroma grade not different between the three experimental groups (χ^2 test, $P > 0.2$).
2. Number of animals with atheroma/total number of animals.

Table 9. Organ weights at the end of Experiment 1 (g ± s.e.)[1].

Organ	Group 1 (n = 13)	Group 2 (n = 15)	Group 3 (n = 14)	Controls (n = 4)
Lungs	6.8 ± 0.4	6.4 ± 0.2	7.3 ± 0.4	7.7 ± 0.5
Spleen	0.68 ± 0.06	0.64 ± 0.07	0.71 ± 0.06	0.89 ± 0.04
Liver	58 ± 5	55 ± 4	56 ± 4	65 ± 12
Kidneys	12 ± 0.4	12 ± 0.4	14 ± 0.7	15 ± 1.4
Adrenals	0.68 ± 0.05	0.62 ± 0.05	0.55 ± 0.06	0.55 ± 0.03
Testes	3.7 ± 0.3	3.9 ± 0.2	3.9 ± 0.3	4.4 ± 0.4
Pancreas	1.6 ± 0.1	1.6 ± 0.1	1.2 ± 0.2	1.7 ± 0.2
Body weight	1942 ± 42	1944 ± 48	2029 ± 47	2471 ± 236

1. None of the differences was found significant by analysis of variance at the 5% level.

Table 10. Relative organ weights at the end of Experiment 1 (g/1000 g body weight ± s.e.)[1].

Organ	Group 1 (n = 13)	Group 2 (n = 15)	Group 3 (n = 14)	Controls (n = 4)
Lungs	3.6 ± 0.3	3.3 ± 0.1	3.6 ± 0.2	3.2 ± 0.4
Spleen	0.35 ± 0.03	0.34 ± 0.04	0.35 ± 0.03	0.37 ± 0.03
Liver	30 ± 3	28 ± 2	28 ± 2	26 ± 4
Kidneys	6.4 ± 0.3	6.3 ± 0.2	6.7 ± 0.3	6.2 ± 0.2
Adrenals	0.34 ± 0.01	0.33 ± 0.03	0.28 ± 0.04	0.23 ± 0.02
Testes	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
Pancreas	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1

1. Only relative pancreas weights of Group 3 statistically different ($P < 0.05$) from those of Groups 1 and 2.

organ weights. The differences in absolute weight of the kidneys, adrenals and pancreas were not statistically significant ($P > 0.05$). From the relative weights only the pancreas of Group 3 was significantly different ($P < 0.05$) from Groups 1 and 2. In all three groups one animal had a grossly fatty liver, weighing more than 85 g. Relatively hypertrophied spleens were also observed in one animal per group. Adrenals were enlarged in one animal of Group 1 and one of Group 3.

7.3.6 Serum lipids

At the end of the experiment a complete analysis was performed of the various lipid classes of serum. The results are shown in Table 11.

The change in serum lipids when a commercial rabbit diet is replaced by a purified diet is in complete agreement with the observations of Wigand (1959), i.e. a decrease in triglycerides, except when coconut oil is fed, a rise in cholesterol, accompanied by an increase in phospholipids, though somewhat less, resulting in a decreased phospholipid: cholesterol ratio.

Table 11. Concentration of lipid classes in serum (mg/100 ml ± s.e.), the percentage distribution and some ratios (× 100); Experiment 1.

	Group 1 (n = 13)		Group 2 (n = 15)		Group 3 (n = 14)		Controls (n = 4)	
	mg	%	mg	%	mg	%	mg	%
Total lipids	599 ± 147	100	301 ± 42[1]	100	331 ± 40	100	318 ± 58	100
Triglycerides	108 ± 32	18 ± 2	47 ± 9[1]	15 ± 2	58 ± 9	18 ± 2	153 ± 38	45 ± 5
Phospholipids	165 ± 34	30 ± 2	85 ± 11[1]	30 ± 2	98 ± 11	31 ± 2	75 ± 12	23 ± 8
Cholesterol	186 ± 49	29 ± 2	81 ± 13[1]	26 ± 1	97 ± 14	28 ± 1	33 ± 4	11 ± 1
Free cholesterol	54 ± 13	9 ± 1	27 ± 4[1]	9 ± 1	29 ± 4	9 ± 1	15 ± 3	5 ± 0.1
Esterified cholesterol	125 ± 35	21 ± 1	54 ± 10[1]	17 ± 1	66 ± 11	19 ± 1	17 ± 1	6 ± 1
NEFA	23 ± 2	4	23 ± 1	8	22 ± 1	7		
Free/total cholesterol	29 ± 2		34 ± 3		32 ± 2		43 ± 3	
Phospholipids/cholesterol	105 ± 8		113 ± 8		116 ± 11		227 ± 16	
Phospholipids/free cholesterol	349 ± 30		351 ± 27		375 ± 33		518 ± 25	
Phospholipids/esterified cholesterol	157 ± 12		177 ± 15		174 ± 18		432 ± 51	

1. Significantly different from Group 1 ($P < 0.05$) by analysis of variance on the log values.

Though the total lipids of the coconut oil group are twice as high as of the other groups, the distribution over the lipid classes is rather constant. The absolute rise in cholesterol may therefore mainly be attributed to an absolute increase in the esterified cholesterol fraction. This confirms the findings of Gottenbos & Thomasson (1961) and Vles et al. (1964). The free cholesterol forms only one third of the total cholesterol. The tendency of the decrease in the ratio free: total cholesterol which is seen between the groups is also present within the groups. In Group 1 the cholesterol level is inversely correlated with the free: total cholesterol ratio ($r = -0.489$; $P \leqslant 0.05$). Wigand (1959) observed after butterfat feeding a similar relative increase of esterified cholesterol and decrease of the free: total cholesterol ratio. After coconut oil feeding, however this ratio was again increased, indicating a relative increase of free cholesterol. His rabbits fed on coconut oil had much higher cholesterol values than the rabbits I studied.

The agreement between the total lipids, as determined gravimetrically, and the total lipids calculated as the sum of determined lipid classes, is in all cases satisfactory. In view of the many analytical steps involved which can only lead to losses of material, the tendency of the gravimetric method to yield somewhat too high values and the fact that no monoglycerides and diglycerides were determined, it may be concluded that the recovery of slightly more than 90% was satisfactory. While all lipid classes of Group 2 except the non-esterified fatty acids were significantly different from those of Group 1 ($P < 0.05$) none of the differences between Groups 3 and 1 reached significance though some were borderline. Neither did the differences between Group 2 and 3. None of the calculated ratios of Table 11 differed between the experimental groups.

7.3.7 Fatty acids of serum lipids

Tables 12 – 15 show the fatty acid composition of the serum lipid classes at the end of the experiment. In some cases the number of samples is not quite in agreement with the number of animals reported in Table 11 because of technical difficulties.

The total blood lipids fatty acids (Table 12) are a mixture of mainly triglyceride, steryl and phospholipid fatty acids. Differences in fatty acid composition between the dietary groups are only present ($P < 0.05$) for lauric, palmitic and oleic acid. The differences however are small and probably unimportant. Differences between Groups 2 and 3 never reached significance for any fatty acid.

Table 13 shows the fatty acids in serum triglycerides, Table 14 in cholesteryl-esters and Table 15 in phospholipids.

It is evident from the Tables 12 – 15 that fatty acids with a chain length shorter than 12:0 (lauric acid) could not be recovered from the serum lipids. Despite the enormous amount of 12:0 in the diet this was only a major fatty acid in the triglycerides and even then in a moderate amount. The greater amount of 12:0 in the diet of Group 1 was reflected in the percentage of this fatty acid in the serum triglycerides and cholesteryl-esters of Group 1 as compared to Group 2 and 3. The much higher concentrations of 14:0 (myristic acid), particularly in the triglycerides and steryl esters of serum, as

Table 12. Fatty acid composition of the serum total lipids at the end of Experiment 1 (weight % ± s.e.).

Fatty acid code[1]	Groups[2]			Significance of differences[3]
	1 (n = 13)	2 (n = 13)	3 (n = 14)	
4:0 – 8:0	–	–	–	
10:0	tr[4]	–	tr[4]	
12:0	5.5 ± 0.9	3.3 ± 0.4	2.6 ± 0.4	1 2 3
14:0	7.8 ± 0.9	6.6 ± 0.6	6.4 ± 0.6	1 2 3
16:0	21.9 ± 0.8	25.5 ± 0.7	25.5 ± 1.1	3 2 1
18:0	8.3 ± 0.7	8.1 ± 0.6	6.4 ± 0.8	1 2 3
16:1	3.2 ± 0.3	4.3 ± 0.5	5.1 ± 0.8	3 2 1
18:1	26.8 ± 1.2	29.8 ± 0.8	30.6 ± 1.1	3 2 1
18:2	21.7 ± 1.2	17.8 ± 1.3	18.9 ± 0.9	1 3 2
18:3	0.8 ± 0.2	0.8 ± 0.3	0.7 ± 0.3	1 2 3
20:4	1.0 ± 0.4	0.3 ± 0.2	0.6 ± 0.1	1 3 2

1. Number of carbon atoms : double bonds.
2. Dietary groups: 1: 36% (cal/cal) coconut oil; 2: 18% (cal/cal) coconut oil and 18% (cal/cal) tributyrate; 3: 18% (cal/cal) coconut oil and 18% (cal/cal) starch.
3. The mean values differ significantly ($P < 0.05$) except when the group numbers share a common underlining.
4. Trace amounts, < 0.2%.

Table 13. Fatty acid composition of the serum triglycerides at the end of Experiment 1 (weight % ± s.e.)[1].

Fatty acid code	Groups			Significance of differences
	1 (n = 13)	2 (n = 15)	3 (n = 14)	
4:0 – 8:0	–	–	–	
10:0	trace	trace	–	
12:0	6.1 ± 0.9	3.3 ± 0.7	2.4 ± 0.3	1 2 3
14:0	14.0 ± 0.8	11.6 ± 0.8	11.3 ± 1.5	1 2 3
16:0	30.2 ± 1.0	33.4 ± 1.1	33.9 ± 1.6	3 2 1
18:0	4.1 ± 0.3	3.7 ± 0.2	3.3 ± 0.2	1 2 3
16:1	3.5 ± 0.4	4.3 ± 0.2	5.0 ± 0.4	3 2 1
18:1	28.1 ± 0.5	28.7 ± 0.8	29.6 ± 1.0	3 2 1
18:2	9.4 ± 0.9	10.1 ± 0.9	9.5 ± 0.7	2 3 1
18:3	0.6 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	2 3 1

1. For symbols and diets, see Table 12.

Table 14. Fatty acid composition of the serum cholesteryl esters at the end of Experiment 1 (weight % ± s.e.)[1].

Fatty acid code	Groups			Significance of differences
	1 (n = 11)	2 (n = 15)	3 (n = 13)	
4:0 – 10:0	–	–	–	
12:0	1.3 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	1 2 3
14:0	6.5 ± 0.2	4.3 ± 0.3	4.0 ± 0.4	1 2 3
16:0	17.9 ± 1.3	17.8 ± 0.7	20.4 ± 1.2	3 1 2
18:0	2.1 ± 0.7	3.9 ± 0.4	3.0 ± 0.3	2 3 1
16:1	5.5 ± 0.4	5.6 ± 0.5	7.4 ± 0.9	3 2 1
18:1	35.7 ± 1.5	38.7 ± 0.8	37.6 ± 2.9	2 3 1
18:2	26.7 ± 1.6	23.8 ± 1.5	22.5 ± 2.3	1 2 3
18:3	0.8 ± 0.3	1.1 ± 0.4	0.8 ± 0.3	2 3 1
20:4	trace	trace	0.2 ± 0.1	3 1 2

1. For symbols and diets, see Table 12.

Table 15. Fatty acid composition of the serum phospholipids at the end of Experiment 1 (weight % ± s.e.)[1].

Fatty acid code	Groups			Significance of differences
	1 (n = 13)	2 (n = 15)	3 (n = 14)	
4:0 – 10:0	–	–	–	
12:0	0.2 ± 0.03	0.2 ± 0.1	0.2 ± 0.1	1 2 3
14:0	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1 2 3
16:0	22.3 ± 0.5	24.9 ± 0.8	25.5 ± 0.7	3 2 1
18:0	23.3 ± 0.7	21.9 ± 0.4	20.2 ± 1.0	1 2 3
16:1	0.8 ± 0.1	1.0 ± 0.2	1.5 ± 0.2	3 2 1
18:1	20.3 ± 1.0	21.2 ± 1.1	22.5 ± 1.1	3 2 1
18:2	26.3 ± 0.8	22.5 ± 1.2	23.5 ± 0.8	1 3 2
18:3	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	2 1 3
20:4	2.3 ± 0.3	2.0 ± 0.4	1.9 ± 0.3	1 2 3

1. For symbols and diets, see Table 12.

compared with 12:0 may indicate that 14:0 is much less readily metabolized or more easily incorporated into body lipids. This is the more striking because coconut oil contains about three times as much lauric as myristic acid.

The substitution of starch for half of the coconut oil calories causes hardly any change in the fatty acid pattern of the serum triglycerides, cholesteryl esters and phospholipids. This agrees with the findings of Vles et al. (1964). They found with coconut oil 7% myristic acid in the steryl esters, 16% in the triglycerides and 2% in the phospholipids. In this study 6.5%, 14% and 1.5%, respectively were found, which indicates the constancy of these characteristics.

The substitution of tributyrate for coconut oil caused exactly the same, though small, changes as the replacement of coconut oil by starch. This observation holds not only for the fatty acid composition of the serum lipids but for the absolute amounts of the lipid classes as well.

7.3.8 Liver lipids

Table 16 shows the composition of the liver lipids. To allow for differences in liver weight and liver composition the data are expressed as mg per g nitrogen. The results of Groups 1 and 2 were somewhat obscured by the presence of at least one grossly fatty liver in each group. This was mainly caused by triglyceride accumulation. The results of these two livers were omitted.

The results indicate that phospholipids and free cholesterol in liver are rather constant. These results agree exactly with those from Wigand (1959) who found in his controls and in rabbits fed on coconut oil for phospholipids 820 and 702 mg/g N, respectively and for free cholesterol 57 and 104 mg/g N, respectively. An increase in liver cholesterol can therefore be attributed to an increase in the steryl ester fraction. Moore & Williams (1963) also noted an absence of effect on liver phospholipids of

Table 16. The lipid composition of the livers as mg/g nitrogen (± s.e.); Experiment 1.

Lipid	Groups			Controls
	1 (n = 11)[1]	2 (n = 13)[2]	3 (n = 11)[3]	(n = 3)
Triglycerides	1338 ± 487	494 ± 74[4]	640 ± 114	798 ± 347
Phospholipids	887 ± 72	788 ± 63	729 ± 40	978 ± 164
Cholesterol	196 ± 29	155 ± 13	197 ± 24	82 ± 15
Free cholesterol	84 ± 4	91 ± 2	94 ± 4	58 ± 15
Esterified cholesterol	106 ± 27	65 ± 13	104 ± 23	21 ± 4

1. The results of one grossly fatty liver (7900 mg triglycerides/g N) and one technical failure have been omitted.
2. The results of one grossly fatty liver (15493 mg triglycerides/g N) and one technical failure have been omitted.
3. The results of three technical failures have been omitted.
4. Significantly different from Group 1 ($P < 0.05$); Wilcoxon test.

Table 17. Fatty acid composition of the total liver lipids of rabbits at the end of Experiment 1 (weight % ± s.e.)[1].

Fatty acid code	Groups 1 (n = 12)	2 (n = 13)	3 (n = 10)	Significance of differences
12:0	10.4 ± 1.6	3.3 ± 0.7	3.1 ± 1.0	1 2 3
14:0	13.4 ± 1.1	7.6 ± 0.9	7.8 ± 1.3	1 3 2
16:0	19.3 ± 1.1	22.6 ± 0.9	21.8 ± 1.2	2 3 1
18:0	6.8 ± 0.8	10.3 ± 1.1	8.1 ± 1.0	2 3 1
14:1	1.2 ± 0.2	0.7 ± 0.1	1.0 ± 0.3	1 3 2
16:1	3.9 ± 0.3	3.9 ± 0.3	5.3 ± 0.6	3 1 2
18:1	19.9 ± 1.6	23.3 ± 1.3	24.7 ± 1.3	3 2 1
18:2	19.4 ± 1.6	21.4 ± 2.0	22.3 ± 2.2	3 2 1
18:3	1.3 ± 0.3	1.5 ± 0.3	1.1 ± 0.2	2 1 3
20:4	2.4 ± 0.3	3.5 ± 0.5	3.5 ± 0.6	3 2 1

1. For symbols and diets, see Table 12.

several fats. With cholesterol-fed rabbits, Albrecht & Schuler (1965) noted no increase of liver phospholipids and only a moderate increase of free cholesterol compared with the esterified cholesterol. The ratio of free/esterified cholesterol decreased from about 1 to about 0.16. Swell et al. (1962) reported a decrease in the ratio of free/esterified cholesterol in the liver of cholesterol-fed rabbits from 3 to 0.15.

The liver triglycerides appear to be the main variable lipid. High triglyceride levels were found in the controls. This level was further increased by the 36%-coconut oil diet. The same observation was made by Wigand (1959). Moore & Williams (1963) also noted the highest triglyceride values on coconut oil diet. The tributyrate-fed group had significantly lower liver triglycerides than the rabbits fed on coconut oil ($P < 0.05$). The fatty acid composition of the total liver lipids is shown in Table 17. Only the major fatty acids are reported which account for about 98% of the fatty acids present. The remaining 2% consists of: 14:2, 15:0, 16:2 and 17:0.

Lauric and myristic acid were significantly more present in the liver lipids of Group 1 than of 2 and 3 ($P < 0.01$). Other, small differences were noted for palmitic acid, palmitoleic acid, stearic acid and oleic acid.

7.3.9 Fatty acids in adipose tissue

In Table 18 the fatty acid composition of the perirenal adipose tissue is given. Fatty acids with a chain length of less than ten carbon atoms could not be found in this tissue. Capric acid (10:0) in hardly detectable amounts was present in all three groups. The greater amount of dietary lauric acid in Group 1 as compared with Groups 2 and 3 is reflected in the fatty acid pattern of the adipose tissue ($P < 0.01$). However, myristic acid, though much lower in the diet was present in even higher

Table 18. Fatty acid composition of perirenal adipose tissue of rabbits at the end of Experiment 1 (weight% ± s.e.)[1].

Fatty acid code	Groups 1 (n = 12)	2 (n = 15)	3 (n = 14)	Significance of differences
10:0	0.2 ± 0.04	0.2 ± 0.02	0.1 ± 0.03	1 2 3
12:0	12.9 ± 1.1	7.6 ± 0.4	8.1 ± 0.8	1 3 2
14:0	18.9 ± 1.0	16.6 ± 0.5	16.6 ± 1.1	1 2 3
16:0	22.7 ± 0.4	25.9 ± 0.4	25.3 ± 0.4	2 3 1
18:0	4.1 ± 0.3	4.5 ± 0.2	4.2 ± 0.2	2 3 1
16:1	2.3 ± 0.2	2.9 ± 0.2	3.5 ± 0.2	3 2 1
18:1	22.2 ± 0.6	24.5 ± 0.5	23.9 ± 0.7	2 3 1
18:2	13.3 ± 1.0	13.8 ± 0.5	13.5 ± 1.0	2 3 1
18:3	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	3 2 1

1. For symbols and diets, see Table 12.

amounts than lauric acid. No significant differences at the 5% level between the groups were present for myristic acid. Palmitic acid was somewhat lower in the coconut oil fed group than in the other two groups ($P < 0.01$). Group 1 had a lower oleic acid content than Group 2 ($P < 0.01$) but was not lower than the starch-supplemented Group 3 ($P < 0.05$). No differences in linoleic acid content between the three groups were detected.

Compared with the serum triglyceride fatty acids (Table 13), more 12:0, 14:0 and 18:2 and less 16:0 and 18:1 were deposited in the adipose tissue of all groups. Tributyrate did not specifically influence the composition of fatty acids in adipose tissue as compared with starch. The difference for palmitoleic acid was the only one which reached significance ($P < 0.05$).

Though many differences were statistically significant one may wonder whether these differences are also of physiological significance because of their small size.

7.4 Discussion

The observation of many investigators (see Section 4.2.1) that serum cholesterol increases when rabbits are fed on semi-synthetic diets, supplemented with saturated fat, is confirmed in this experiment. The increase of serum cholesterol is, however, very variable. Inspection of the individual data shows that the onset of the cholesterol increase is very different for different animals. Some animals react immediately, others have a lag period of several months. As the cholesterol values were still increasing at the end of the experiment after 23 weeks, the standard error of the mean was very great, preventing differences to become significant at the 5% level. Logarithmic transformation of the data as proposed by Moore & Williams (e.g. 1966) yields reduced standard deviations, but not yet significance below the 10% level. The dura-

tion of the experiment was evidently too short and the sensitivity for induced hypercholesterolemia too low in these rabbits to reach significance.

The low sensitivity of the rabbits from this study is evident when the cholesterol levels are compared with those from other investigators (Wigand, 1959; Gottenbos & Thomasson, 1961). A smaller increase of serum cholesterol in Dutch rabbits as compared with New Zealand Whites upon feeding 0.25% cholesterol in the diet was reported by Adams et al. (1972). Strain and breed differences are reported by several authors (Moore & Williams, 1966; Marquié, 1970; Adams et al., 1972).

Roberts et al. (1974) concluded from a controlled breeding trial from selected hyper-responding and hypo-responding parents that the cholesterolemic response to dietary cholesterol is heritable. The heritability (h^2) is 50 $\pm$ 4.7%. They likewise observed a relationship between initial and final cholesterol level on cholesterol feeding. No such study has been done with purified diets but we could find no consistent relationship between initial serum cholesterol level and cholesterolemic response. The small and slow increase of serum cholesterol may also be related to the inclusion in the diet of 4% of calories as soya bean oil. Moore (1969) reported a lowering of serum cholesterol and degree of atherosis in New Zealand white rabbits when small amounts of linoleic acid were included in a diet containing butterfat. The first 4g linoleic acid per 100 g diet almost completely abolished the hypercholesterolemic effect of butterfat. Gottenbos & Thomasson (1961) fed coconut oil without a linoleic acid supplement and observed within 8 weeks a serum cholesterol content of about 500 mg/100 ml. They used White Viennese rabbits. Though small amounts of linoleic acid have a profound effect, the strain of rabbits is possibly of even greater importance in the induction of hypercholesterolemia.

The body weights of the animals behaved in the same way as those of Wigand (1959), Moore & Williams (1964a) and Funch et al. (1962), when feeding coconut oil or cacao butter. Gottenbos & Thomasson observed a weight gain of about 1000 g with 3-month-old rabbits. However the diet which they used was more complex than the diet used by the other investigators mentioned. They supplied large amounts of grass meal (30% w/w or 29% cal/cal), rice (51% cal/cal), and yeast (10% cal/cal) and only 6.2% (cal/cal) casein. The better growth on this diet may be regarded as a result of yeast supplementation (see Section 4.3) or casein reduction (see Sections 4.3 and 4.7.2). The same applies to the better growth observed by Vles et al. (1964), and Vles & Kloeze (1967). The growth observed by Kloeze et al. (1969), feeding hydrogenated coconut oil, is more in agreement with the results presented here. Though they also used yeast, the amount of rice was much less, the grass meal was completely eliminated and casein was tripled. The composition of the basal diet may, therefore, have a strong influence on the body weight gain of rabbits fed on purified diets.

Feeding coconut oil at 18% or 36% of calories had no different influence on body weight. Neither had tributyrate. Organ weights and pathology were not different for the three groups.

Though the differences of serum lipids only reached significance ($P < 0.05$) at the end of the experiment between rabbits fed on coconut oil and those fed on tributyrate

the trend towards higher cholesterol values with the coconut oil diet (36%) was obvious. Replacement of part of the coconut oil with starch or tributyrate induced similar serum lipids, liver lipids, and fatty acid composition of serum lipid classes, of liver lipids and of perirenal adipose tissue.

The degree and incidence of atherosclerotic changes in the aorta was almost the same but very little. This is probably due to the low serum cholesterol content over the entire period and the relatively short duration of the experiment. Constantinides (1965) reasoned that atherosclerosis is related to the intensity and duration of hypercholesterolemia. Moore & Williams (1964c) demonstrated that a high ratio linoleic: oleic acid in the plasma phospholipids of their rabbits was related to less extensive atheromatous degeneration of the aorta. No difference in this ratio was found between the rabbits fed on tributyrate or starch. The ratio in these groups was even less than in the group fed on 36% coconut oil. Another indicator for the degree of aortic atherosclerosis is the ratio phospholipid: free cholesterol (Moore & Williams, 1964b). The results, shown in Table 11 also fail to denote a difference between the groups in this respect.

The results of the tributyrate-fed animals which were similar to those of the starch-fed rabbits indicate that shorter-chain fatty acids like butyric acid have about the same function in the body as glucose, i.e. providing energy and acetyl units for synthesis. Malmros et al. (1964) also observed no effect of tributyrate, given as 26% of calories on serum cholesterol of rabbits.

The results of this study, together with those collected in many animal species with MCT-fat (medium chain triglyceride, see Sections 4.6.4 and 4.6.5) support the theory that fatty acids with a chain shorter than 12 carbon atoms do not influence serum cholesterol. Also no effect, compared with starch, could be detected on serum triglycerides or any other lipid class in serum or liver. The presence of butyric acid and other fatty acids with a relatively short chain length in milk fat will therefore probably not influence, either positively or negatively, the effect of this fat on blood lipids. An investigation about which factors in milk fat contribute most to its hypercholesterolemic properties is reported in Chapter 8.

8 Effects of simulated butter oil versus coconut oil and butter oil (Experiment 2)

8.1 Objectives

Review of the literature, reported in Sections 4.5 and 4.6, has yielded controversial information about the hypercholesterolemic and atherogenic properties of the components which constitute milk fat. It is suggested that these effects may be partly related to its fatty acid composition and partly to its cholesterol content. This was further investigated in this experiment by comparing effects induced by feeding butter oil with effects induced by feeding a fat mixture which was stepwise adjusted to resemble the butter oil more and more with every step. To be able to compare the different experiments a group fed on coconut oil was included. This also allows a comparison of degree of atherosclerosis between rabbits fed on butter oil and those fed on coconut oil. By this procedure it is possible to determine for both fats whether the degree of atherosclerosis is correlated with and therefore predictable from the serum lipid values, or whether the degree is at variance with serum lipid levels. This may provide a clue to the mechanism of atherogenesis induced by the butter oil.

8.2 Experimental details

Sixty male Dutch Belt rabbits, about 5 months old, were gradually accustomed to the semi-synthetic diet with coconut oil as dietary fat (see Section 6.3). The rabbit population consisted of 20 litters of three each, so that hereditary factors in cholesterolemic response could be determined. After the change-over to the semi-synthetic diet was completed, the animals were distributed over the groups. The groups were matched for serum cholesterol and body weight. No litter mates were assigned to the same experimental group. The experiment started at the beginning of the summer (1971) and the experimental period lasted 58 weeks. The diets were composed of the basal diet as described (Section 6.4) and a fat source.

Group 1 received 36% of calories as coconut oil (13.8% by weight), Group 2 36% (cal/cal) butter oil. These two groups got the same diet throughout the entire experiment. Group 3 was fed on a fat mixture which was gradually in four steps adjusted to match the butter oil as to fatty acid composition and cholesterol content. Table 19 gives a summary of the experimental design and composition of the fat mixtures in the consecutive periods.

In Period 1, lasting 11 weeks, the dietary fat of Group 3 resembled roughly the composition of butter oil regarding the distribution in saturated, mono and poly

Table 19. Design of Experiment 2 and composition of the dietary fats (weight %). G = group; P = period; SG = subgroup. In brackets number of experimental weeks.

	G 1 (58)	G 2 (58)	G 3					
			P 1 (11)	P 2 (22)	P 3		P 4	
					SG A (12)	SG B (12)	SG A (13)	SG B (13)
Coconut oil	13.8	–	4.6	0.6	0.6	0.5	0.6	0.5
Butter oil	–	13.8	–	–	–	–	–	–
Tallow	–	–	9.2	10.6	10.6	9.4	10.6	9.4
Palm oil	–	–	–	1.3	1.3	1.3	1.3	1.3
Trimyristate	–	–	–	1.3	1.3	1.1	1.3	1.1
MCT oil	–	–	–	–	–	0.9	–	0.9
Tributyrate	–	–	–	–	–	0.6	–	0.6
Cholesterol added (mg/kg)	–	–	–	–	–	–	–	296

unsaturated fatty acids. In period 2 the composition of the individual fatty acids was somewhat adjusted to resemble butter oil more closely. After 22 weeks on this diet Group 3 was divided into two sub-groups A and B. The division of Group 3 into similar sub-groups was based on several parameters, i.e. body weight, serum cholesterol at 33 weeks, the average serum cholesterol over the entire preceding 33 weeks, and serum triglyceride content. Sub-group 3A continued on the same diet as was supplied in Period 2 for Periods 3 and 4. The fat of sub-group 3B was supplemented in Period 3 with tributyrate and a medium chain triglyceride (MCT) to make up the deficit in 4:0 to 10:0 as compared with butter oil. In Period 4 the diet of sub-group 3B was supplemented with cholesterol to simulate the cholesterol content of the butter oil containing diet. The cholesterol, already present in the tallow was taken into account. The cholesterol was dissolved in the palm oil. The fatty acid composition and cholesterol content of the diets are shown in Table 20.

Food consumption was determined weekly on a group-basis. Body weight was recorded every two weeks and as a rule blood was taken every four weeks. If triglycerides were determined the animals were fasted for 16 hours. During the experiment serum cholesterol was determined every four weeks, triglycerides at Week 33 in all groups and at Week 45 in Group 3 before Period 4 started. At the end of the experiment organs were removed, judged macroscopically, and weighed. The heart plus aorta was graded as described in Section 6.7. Complete analysis of the serum and liver lipids was performed according to methods described in Section 6.6.

Table 20. Fatty acid composition of the dietary fats (weight %) and cholesterol content of the diets (mg/kg) (Experiment 2). G = group; P = period; SG = subgroup. In brackets number of experimental weeks.

Fatty acid code	G 1 (58)		G 2 (58)		G 3[1] P1 (11)		P2[2] (22)		P3[3,4] (12)	
4:0	0.0		4.0		0.0		0.0		4.0	
6:0	0.0		2.0		0.0		0.0		0.1	
8:0	7.0	13.0	1.4	6.5	2.4	4.5	0.5	0.8	3.9	6.6
10:0	6.0		3.1		2.1		0.3		2.6	
12:0	46.0		3.6		16.3		3.2		3.0	
14:0	18.5		10.3	39.9	8.2	44.3	11.3	39.1	10.2	35.3
16:0	9.5		26.0		19.8		24.6		22.1	
18:0	3.0		12.7		15.6		17.4		15.7	
16:1	0.0		2.9		2.3		2.6		2.4	
18:1	7.0		26.1		26.9		32.1		28.9	
18:2	2.0		4.4		4.5		4.6		4.8	
18:3	trace		3.0		1.2		0.0		0.0	
20:4	0.0		0.3		0.0		0.0		0.0	
Saturated	90.0		63.1		64.4		57.3		61.6	
Monoene	7.0		29.0		29.2		34.7		31.8	
Diene	2.0		4.4		4.5		4.6		4.1	
Cholesterol	–		428		129		148		132	

1. Subgroup 3A got the same diet during Periods 2, 3 and 4. Subgroup 3B got the same diet during Period 4 as during Period 3, supplemented with 296 mg cholesterol. It contained 428 mg cholesterol per kg (0.04%).
2. Trimyristate (Fluka, Buchs); fatty acid composition: 12:0, 10%; 14:0, 80.7%; 16:0, 9.3%.
3. Tributyrate (Merck, Darmstadt); contained only 4:0.
4. MCT oil was kindly provided by Dr R. Beerthuis, Unilever Research Vlaardingen. Composition: 8:0, 60%; 10:0, 38%; 12:0, 0.8%; 16:0, 0.4%; 18:0, 1%; 18:1, 0.3%; 18:2, 0.2%.

8.3 Results and discussion

8.3.1 Food consumption and body weight

The data about food consumption were gathered per group. This implies that no correction can be made for moribund animals which often display decreased appetite and weight loss. This may seriously hamper the interpretation of results with regard to food consumption and body weight. The high death rate in this experiment (see below) caused sharp fluctuations in body weight. To avoid these fluctuations the body weight data are only presented for those animals surviving the entire experiment, or for Group 3, for each separate period. The general trend of the results is, however, not changed by omission of the data of the dead animals.

Food consumption data are given in Table 21 and body weight in Fig. 4. Roughly

Table 21. Food consumption data during the four periods and entire Experiment 2.

Groups	Food consumption (g/day)[1]				
	periods				whole experiment
	1	2	3	4	
1 (coconut oil)	38.7	41.5	39.1	40.1	40.2
2 (butter oil)	41.6	50.3	47.4	44.1	47.1
3 (simulated butter oil)	40.8	49.0			
3A (simulated butter oil)			48.4	42.6	
3B (simulated butter oil)			50.1	45.8	

1. Dry matter.

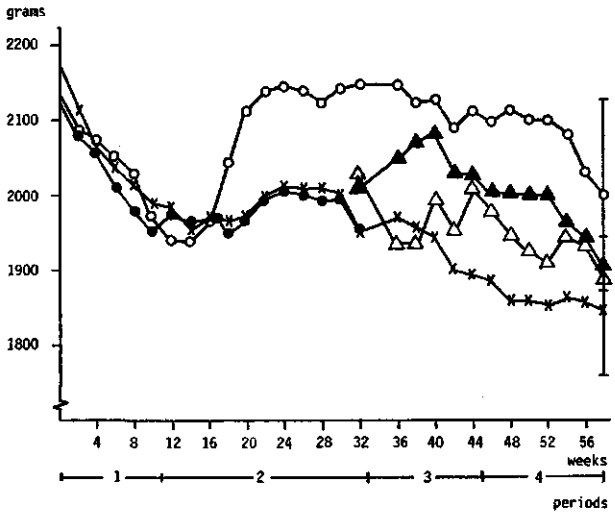

Fig. 4. Exp. 2. Mean body weight of rabbits, surviving the experiment (Groups 1 and 2) or the respective periods (Group 3), as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, butter oil; Group 3: ●, simulated butter oil; Subgroup 3A: △; Subgroup 3B: ▲. Vertical bars denote s.e. mean.

the same picture is seen as was reported in Experiment 1. None of the differences in body weight between the different groups was significant ($P > 0.2$). This also applies to the differences between the sub-groups A and B or Group 3 after Periods 3 and 4. The decrease in body weight immediately after the start of the experimental period may be partly caused by the high temperatures from June till August. An inverse relationship between food intake and temperature was often observed. The food intake during the summer months was for all animals together about 42 g per day per animal and during the winter months 48 g per day.

8.3.2 Mortality

The mortality in this experiment was very high, amounting to about 45%. The animals were as in Experiment 1, severely infected with *Pasteurella multocida*,

causing snuffles disease. The much higher mortality in this experiment compared with the first experiment may be ascribed to the longer duration. After a comparable time as Experiment 1 lasted, i.e. 23 weeks, only 5 animals had died against 3 in Experiment 1 which is a similar percentage.

Mortality was equally distributed among the three groups. Apart from severely affected lungs with pus-containing nodes, also macroscopically abnormal livers were found at autopsy. All livers had a pale yellowish tan colour, indicating lipid infiltration. Some had an irregular surface and a firm indurated consistency, indicating replacement of the parenchymal cells by fibrous tissue. Some had a spongy consistency. The kidneys of several animals of all groups showed radial white linings in the external part of the renal medulla. In all these animals and some others, the cortex was scarred indicating necrosis. Two animals, one in Group 1 and one in Group 3 had to be killed because of a twisted neck (torticollis), prohibiting eating. Two animals, one of Group 1 and one of Group 3 were killed because of paralysis of the hind legs. These animals had a spinal fracture. One animal of Group 3 showed severely ulcerated feet and had to be killed.

8.3.3 Serum cholesterol

Fig. 5 shows the mean serum cholesterol content of the groups during the 58 weeks of the experiment. It is evident from this figure that coconut oil induces the highest serum cholesterol levels, followed by butter oil, which is again higher than the simulated butter oil till Period 4. The values of the simulated butter oil are low and almost

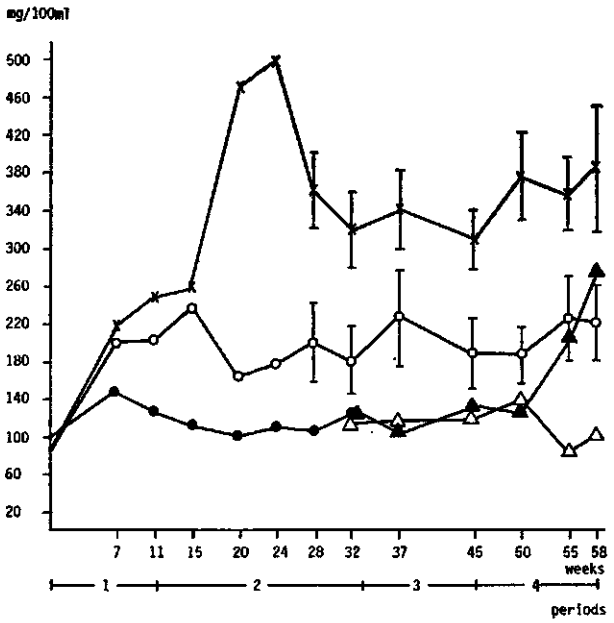

Fig. 5. Exp. 2. Mean serum cholesterol content of all rabbits as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, butter oil; Group 3: ●, simulated butter oil; Subgroup 3A: △; Subgroup 3B: ▲. Vertical bars denote s.e. mean.

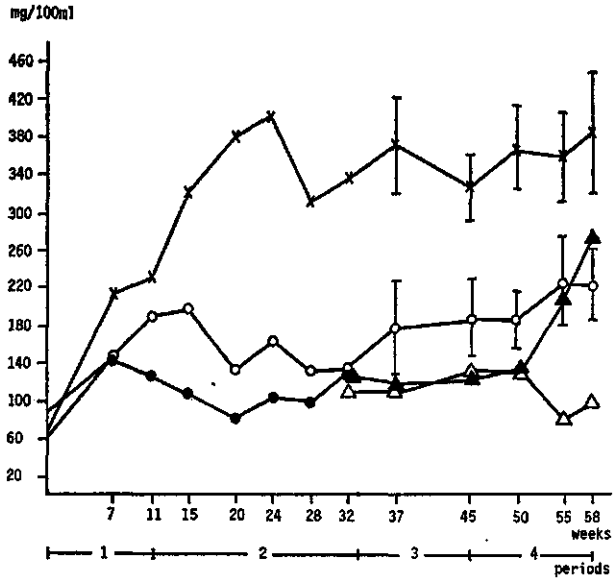

Fig. 6. Exp. 2. Mean serum cholesterol content of rabbits, surviving the experiment (Groups 1 and 2) or the respective periode (Group 3), as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, butter oil; Group 3: ●, simulated butter oil; Subgroup 3A: △; Subgroup 3B: ▲. Vertical bars denote s.e. mean.

do not increase during the experiment up till Period 4. The picture may be influenced by the high mortality rate, which implies that not all means apply to the same animals. In Fig. 6 therefore the means of the groups are shown, which were calculated for those animals only, which survived the whole 58 weeks. The result is not really different, indicating that those animals which died were average animals, behaving similarly to the surviving rabbits.

The differences in serum cholesterol, calculated as log cholesterol to normalize the distribution, were significant after 32 weeks duration of the experiment, if the analysis of variance was carried out on the results of all animals alive at that moment. These numbers were for Group one 16, for Group two 15 and for Group three 16. The log cholesterol values for Group 1 were also significantly higher than for Groups 2 and 3 ($P < 0.01$). The difference between Groups 2 and 3 did not reach the 5% level.

After 58 weeks of the experiment, the difference of serum cholesterol between Groups 1 and 2 did not reach significance ($0.05 < P < 0.10$), due to the small number of animals left (10 and 11). At that moment only the difference between Groups 1 and 3A was significant ($P < 0.05$). Changes in cholesterol during Period 4 occurred in all dietary groups except 3A. None of these changes was significant ($P > 0.2$). The steep rise visible for Group 3B from 110 till 277 during this period was mainly caused by three hyper-responding rabbits. The number (seven) of surviving animals in this sub-group was too small to draw reliable conclusions.

By calculating at each bleeding point the average serum cholesterol over the entire preceding experimental period a cumulative average serum cholesterol concentration may be obtained, which is much less dependent on individual incidental findings. The mean of the cumulative average serum cholesterol is shown in Fig. 7. This cumulative average value over the entire period should correlate much better with the degree of

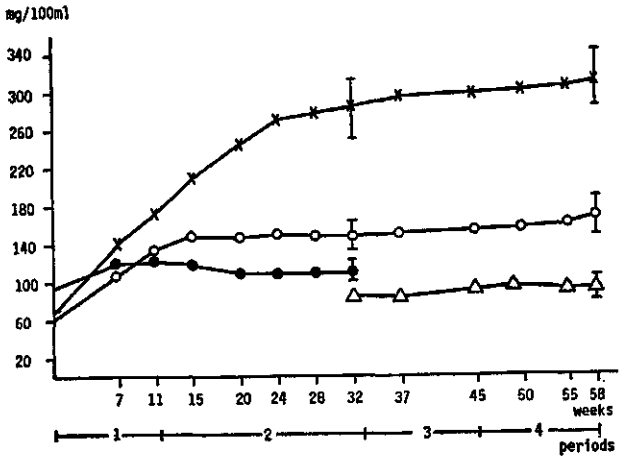

Fig. 7. Exp. 2. Mean of the average serum cholesterol content of rabbits, surviving the experiment (Groups 1 and 2) or the respective periods (Group 3), as a function of experimental time. Group 1: x, coconut oil; Group 2: ○, butter oil; Group 3: ●, simulated butter oil; Subgroup 3A: △. Vertical bars denote s.e. mean.

atherosclerosis than one incidental finding at an arbitrary moment. Analysis of variance on the log cumulative average of serum cholesterol concentration yielded a significant group effect at 32 weeks. All differences between the groups were highly significant ($P < 0.01$). At 58 weeks the difference between Groups 1 and 2 also was highly significant ($P < 0.001$). The difference between Groups 2 and 3A was slightly less significant ($P < 0.05$) and between 2 and 3B not significant ($P > 0.1$).

8.3.4 Observations post mortem

Immediately after killing the animals, the organs were processed as described in Section 6.7. The aortas were graded on a scale from 0 - 4. taking into account the size and thickness of the plaques. The results are presented in Table 22.

The grading of the aortas showed evidently more severe involvement than observed in Experiment 1. This is not unexpected as both the duration of this experiment was longer and hypercholesterolemia more pronounced. The mean degree of atherosclerosis seems to parallel the serum cholesterol value which is most clear if Fig. 7 is compared with Table 22. Almost all animals of the dietary Groups 1 and 2 have some degree of atherosclerosis. The difference between these two groups is gradual, the rabbits fed on coconut oil having more severe aortic involvement. Those animals which died or were killed during the experiment showed the same picture as given in Table 22 for the survivors. All animals from Groups 1 and 2 which died in the second half of the experiment had at least Grade 1.

The weights of the liver, kidneys, adrenals and spleen were recorded and are shown in Table 23. The relative weights of these organs were calculated on the basis of g per kg body weight at the moment of death. They are shown in Table 24.

Differences present in absolute organ weights between Groups may be partly explained by differences in body weight. The higher relative liver weights of rabbits

Table 22. Aortic atherosclerosis (number of animals per atheroma grade) at the end of Experiment 2.

Group	Grade						Incidence
	0	1	2	3	4	mean[1]	
1 (n = 10)	0	4	1	5	0	2.10	10/10
2 (n = 11)	1	6	3	1	0	1.36	10/11
3A (n = 5)	2	2	1	0	0	0.80	3/5
3B (n = 7)	4	1	1	1	0	0.86	3/7

1. The difference in atheroma grade between Groups 1 and 2 was not significant ($\chi^2 = 3.11$; $P < 0.10$). The difference between Groups 1 and 3A was significant ($\chi^2 = 5.63$; $P < 0.05$).

fed on coconut oil (Group 1) are significant compared with other rabbits ($P < 0.05$). The results of Group 3A tend to be different from the other groups as for the spleen and adrenals. They approach more the values found in Experiment 1.

Hypertrophy of spleen (Vles et al., 1964) and adrenals (Albrecht & Schuler, 1965; Marquié, 1970) is often observed in hypercholesterolemic animals. Though lipid acumulation by the spleen may be important, hypertrophy is not always the consequence (Marquié, 1970). It may be relevant that the rabbits of 3A had the lowest serum cholesterol levels throughout the experiment.

All livers were somewhat heavier when compared with those from Experiment 1. This is also a common observation in prolonged hypercholesterolemia (Vles et al.,

Table 23. Organ weights (g ± s.e.) at the end of Experiment 2.

Organ	Group 1 (n = 10)	Group 2 (n = 11)	Group 3A (n = 4)	Group 3B (n = 7)
Spleen	0.70 ± 0.08	0.89 ± 0.13	0.65 ± 0.14	0.79 ± 0.11
Liver	79 ± 5	67 ± 5	66 ± 16	62 ± 8
Kidneys	13 ± 0.8	12 ± 0.6	14 ± 0.6	13 ± 1.2
Adrenals	0.84 ± 0.08	0.94 ± 0.04	0.80 ± 0.10	0.98 ± 0.16

Table 24. Relative organ weights at the end of Experiment 2 (g/1000 g body weight ± s.e.)[1].

Organ	Group 1 (n = 10)	Group 2 (n = 11)	Group 3A (n = 4)	Group 3B (n = 7)
Spleen	0.42 ± 0.04	0.45 ± 0.06	0.33 ± 0.06	0.43 ± 0.06
Liver	45 ± 3	35 ± 2	34 ± 3	34 ± 2
Kidneys	8 ± 0.5	6 ± 0.3	7 ± 0.3	7 ± 0.5
Adrenals	0.47 ± 0.05	0.50 ± 0.04	0.40 ± 0.05	0.55 ± 0.09

1. Liver weights of Group 1 are significantly different from the other groups ($P < 0.05$). Other differences not significant.

1964; Funch et al., 1962; Marquié, 1970). Marked hypertrophied liver was found in one animal of Group 1, weighing 67 g per kg body weight. Also one animal of the same group had a large spleen (0.63 g relative) as had three animals of Group 2 (0.61, 0.72 and 0.73 g relative) and two animals of 3B (0.61 and 0.62 g relative weight). These very animals also happened to have high serum cholesterol values.

Macroscopic inspection of the organs revealed the same pathology as described for the animals which died during the experiment. The combined mortality data and the pathological findings post mortem indicate no gross differences between rabbits fed on coconut oil and those fed on butter oil. The findings in Group 3, fed on the simulated butter oil, tend to be somewhat less severe and frequent, especially in Group 3A, which got no dietary cholesterol supplemented, than in the other two groups. Their serum cholesterol over the entire experiment is much lower and it is tempting to speculate that this might be related to the reduced incidence of disease.

8.3.5 Serum lipids at the end of the experiment

At the end of the experiment a larger blood sample was taken to analyse the content of the different lipid classes in serum. Total lipids, total cholesterol, free and esterified cholesterol, triglycerides and phospholipids were determined. The results are shown in Table 25 together with some calculated ratios.

Coconut oil induced higher serum lipid values than butter oil. However the distribution over the lipid classes remained similar, indicating the same mechanism. The calculated ratios between phospholipids and other classes tended to be lower for Group 1 than for Group 2. This is in agreement with the results of Experiment 1. These ratios decreased when serum lipids increased. This means that the rise of phospholipids is less than of esterified or free cholesterol. The ratio free/total cholesterol was constant at 28% when total cholesterol was increased. The same ratio was found in Experiment 1 for the rabbits fed on coconut oil. When total cholesterol was low as in Group 3A this ratio was much higher. Group 2 from Experiment 1 had about the same serum cholesterol content as Group 3A from this experiment. They also had about the same ratio free/total cholesterol. The same applies for the other lipid ratios of these two groups.

The differences between Groups 3A and 3B are difficult to interpret because of the small number of survivors in Group 3A. The tendency of a low cholesterol content seen during the experiment (Fig. 6 and 7) is also apparent at the end of the experiment. The high lipid values found in Group 3B are caused by half of the animals which may be called 'hyper-responders'. At the moment of division of Group 3 into sub-groups 3A and 3B both sub-groups had identical values. During Period 3, when tributyrate and MCT were added to 3B, no increase of serum cholesterol occurred (Fig. 5 and 6). However, during Period 4 when cholesterol was added in an amount to matcht he butter oil diet, serum cholesterol of some animals increased tremendously. The duration of Period 4 of 13 weeks was clearly too short to reach stability of serum cholesterol values of the responders. The period may have been too short also

Table 25. Concentration of lipid classes in serum (mg/100 ml ± s.e.), (in parenthesis the percentage distribution) and some ratios × 100 (Exp. 2).

	Group 1 (n = 10)	Group 2 (n = 11)	Group 3A (n = 4)	Group 3B (n = 7)
Total lipids	934 ± 161	599 ± 93	355 ± 104[1]	754 ± 229
Triglycerides	110 ± 26 (12)	82 ± 26 (14)	148 ± 78 (42)	114 ± 34 (15)
Phospholipids	222 ± 26 (24)	160 ± 18[1] (27)	102 ± 7[1] (29)	204 ± 48 (27)
Total cholesterol	348 ± 67 (37)	206 ± 38 (34)	79 ± 12[1,3] (22)	252 ± 87 (33)
Free cholesterol	92 ± 16 (10)	55 ± 9[1] (9)	28 ± 4[1] (8)	70 ± 23 (9)
Esterified cholesterol	252 ± 50 (27)	160 ± 31 (27)	54 ± 7[1,3] (15)	187 ± 67 (25)
Free/total cholesterol	28 ± 1	28 ± 2	36 ± 4[1,2]	28 ± 1[3]
Phospholipids/total cholesterol	74 ± 7	93 ± 11	136 ± 17[1]	103 ± 13[1]
Phospholipids/free cholesterol	264 ± 16	329 ± 30	377 ± 21[1]	364 ± 47[1]
Phospholipids/esterified cholesterol	105 ± 13	126 ± 17	197 ± 20[1,3]	145 ± 22

1. Significantly different from Group 1 ($P < 0.05$).
2. Significantly different from Group 2 ($P < 0.05$).
3. Significantly different from Group 3A ($P < 0.05$).

to allow all animals to react. The trend is evident that the greatest part of the effects of simulated butter oil can be seen during Period 4 when the cholesterol was supplemented.

The high triglyceride level shown for Group 3A was caused by one animal. The other groups did not differ in this respect. Increased serum triglyceride levels are commonly observed in hyper-cholesterolemic rabbits (Wigand, 1959). Huang & Kako (1970) investigated the mechanism of this hypertriglyceridemia in rabbits. They demonstrated a slow increase of triglycerides and a rapid rise of serum cholesterol upon cholesterol feeding. The fractional turnover rate of triglycerides was depressed within a week. From experiments *in vitro* and *in vivo* they concluded that the presence of free cholesterol in the substrate of lipoprotein lipase competitively inhibits the enzyme activity and therefore causes triglyceride accumulation in the blood.

It is not possible from the data of this study to conclude whether the same mechanism is also acting in this case. Comparison of the triglyceride values of the animals with their free cholesterol values did not yield a correlation. One conclusion may be drawn, however, from that comparison. At high free-cholesterol levels in serum, above 100 mg per 100 ml, triglyceride contents are always high. Some animals have already increased triglyceride levels at lower serum free cholesterol values. It should be remarked that in this experiment no cholesterol was determined in the lipoproteins of very low density. Huang & Kako's rabbits had generally much higher concentrations of total and free cholesterol.

True hypercholesterolemia (> 300 mg/100 ml) in the rabbits of this study was always accompanied by triglyceridemia. All animals with triglyceridemia had always at least moderate cholesterolemia (100 – 300 mg/100 ml). From these findings and Huang & Kako's (1970) results it is suggested that also in the semi-synthetic, saturated fat model the increase of triglycerides is secondary to an increase of cholesterol.

8.3.6 Liver lipids

Similar determinations as were made of the serum lipids were done on the liver lipid classes. To correct for compositional changes the nitrogen content was determined as well. The results are shown in Table 26, expressed as mg lipid per g nitrogen. The percentage distribution is also given. The results for Groups 1 and 3A were obscured by the presence of some livers with a high lipid content. In Group 1 one liver had total lipids over 4 g and two livers over 5 g per g nitrogen. These high total lipid values were mainly caused by triglycerides, which were 2.0, 2.3, and 3.4 g per g nitrogen, respectively. Cholesterol was concomitantly increased in these livers. The same applies to one animal of Group 3A with 5.9 g total lipids, 3.8 g triglycerides and 0.6 g cholesterol. When these livers are omitted all means become similar and not statistically different.

The phospholipids show a constant pattern, even in the fatty livers. This confirms the results of Experiment 1 and is in agreement with the data of Moore & Williams (1963). They observed no difference in liver phospholipids when rabbits were fed on

Table 26. Liver lipid composition as mg/g nitrogen (± s.e.); Experiment 2 (percentage composition between brackets). For diets see Tables 19 and 20.

Lipid	Group 1[1] (n = 10)	Group 2 (n = 11)	Group 3A[1] (n = 4)	Group 3B (n = 7)	Without fatty livers	
					Group 1 (n = 7)	Group 3A (n = 3)
Total lipids	3366 ± 373	2559 ± 121[2]	3236 ± 891	2459 ± 202	2709 ± 177	2348 ± 98
Triglycerides	1312 ± 314 (39)	653 ± 54[2] (26)	1434 ± 795 (44)	726 ± 160 (30)	760 ± 131 (28)	647 ± 162 (28)
Phospholipids	1305 ± 41 (39)	1346 ± 25 (53)	1491 ± 40[2,3] (46)	1497 ± 79[2] (61)	1261 ± 32 (47)	1451 ± 7 (62)
Cholesterol	486 ± 69 (14)	338 ± 58[2] (13)	250 ± 116 (8)	198 ± 35[2,3] (8)	413 ± 64 (15)	134 ± 8 (6)
Free cholesterol	143 ± 11 (4)	119 ± 5 (5)	104 ± 10[2,3] (3)	105 ± 5 (4)	136 ± 10 (5)	95 ± 6 (5)
Esterified cholesterol	367 ± 70 (19)	229 ± 58[2] (9)	162 ± 122 (5)	92 ± 36[2,3] (4)	290 ± 64 (11)	40 ± 5 (2)

1. Group 1 includes 3 and Group 3A 1 fatty liver.
2. Significantly different from Group 1 ($P < 0.05$).
3. Significantly different from Group 2 ($P < 0.05$) by Wilcoxon ranks test and the Kruskal-Wallis one way analysis of variance.

diets containing corn oil, butter fat, coconut oil or starch. The liver cholesterol content tends to reflect the serum cholesterol concentration, being highest in rabbits on the coconut oil diet, intermediate for the butter oil diet, and lowest for the simulated butter oil diet. Moore & Williams (1963) observed the same trend but could not detect significant differences due to the large variation within the groups. The same large variation is also present in the rabbits of the present study. A significant correlation within a group between serum cholesterol and either total, free or esterified liver cholesterol was not present (Kendall test). Wigand (1959) reported for rabbits fed on butterfat a liver total cholesterol content of 409 mg/g nitrogen which is close to the 338 mg reported in this study. He reported about the same value for rabbits fed on coconut oil with a greater relative increase of free cholesterol than the butter-fed rabbits. Moore & Williams (1963) also found a relatively more pronounced increase of the free cholesterol fraction in their animals fed on coconut oil. This tendency is also seen in the livers of group 1 fed on coconut oil (Table 16).

The liver triglycerides are similar after omission of the fatty livers and are in the range found in Experiment 1. Even with inclusion of the fatty livers differences between groups are only of borderline significance due to the great within-group variation. The same level of liver triglycerides was reported by Wigand (1959) for butterfat-fed animals and by Moore & Williams (1963). In this study coconut oil induced the same or higher triglyceride values than butter oil. Wigand observed lower liver triglyceride contents and Moore & Williams (1963) higher triglyceride contents by comparing coconut oil with butterfat. The level of fat in the diet may be of significance as Wigand (1959) fed 26% of the calories as fat, Moore & Williams (1963) 35% and in this study 36%. Substitution of half of the coconut oil in Experiment 1 for starch also halved the liver triglyceride content to the control level (Table 16).

The results for Groups 3A and 3B are difficult to interpret as the number of animals in Group 3A is too small to be reliable and the last period for Group 3B probably too short to affect liver lipids considerably. All livers of this last group had still low total and esterified cholesterol contents and normal or even low triglyceride levels while simultaneously serum cholesterol content increased tremendously in some.

It may be suggested that the increase of liver lipid content is secondary to increased blood lipid content. This suggestion is supported by the study of Albrecht & Schuler (1965). They fed rabbits on 3% cholesterol for periods of different length and observed a less pronounced increase of liver cholesterol than of serum cholesterol. This rise is slower and somewhat delayed in the liver. It appears as if maximum levels are attained in the serum of 1800 mg/100 ml and in the liver of about 1600 mg/g nitrogen. When cholesterol feeding is withdrawn the liver responds in somewhat the same manner as the serum, although the rate of decrease is slower than in the serum. It is probable that the increased liver cholesteryl esters observed upon cholesterol feeding and on feeding a saturated fat-supplemented semi-synthetic diet, represent in both cases a storage form.

8.4 Relationship between serum lipids and degree of atherosclerosis

The third objective of Experiment 2 was to establish whether there is a relationship between blood lipids and atherosclerosis. More specifically, whether rabbits fed on butter oil show a similar relationship between their blood lipids and degree of atherosclerosis as rabbits fed on coconut oil. If this would be so, the degree of atherosclerotic involvement of the aorta would be merely a function of blood lipids and might be predicted from the blood lipids. It would save time in future experiments if it is not necessary to wait until macroscopically visible atherosclerosis has evolved.

The high mortality rate in Experiment 2, however, forms a serious drawback for the establishment of such a relationship. Fortunately at the same time a separate experiment (Experiment 2A) was run, with rabbits from the same age, strain, breed and supplier, which was not a part of the study described here but independent. The basal diets fed in that Experiment 2A were identical to those in Experiment 1. One group was fed also on coconut oil at 36% of calories, another group 36% coconut oil and 4% soya bean oil. The duration of Experiment 2A was 59 weeks compared with 58 weeks for Experiment 2. The body weight, mortality and serum cholesterol pattern of the rabbits was exactly the same during Experiment 2A as in Experiment 1 and 2. Also the degree of atherosclerosis was comparable in both experiments for rabbits fed on coconut oil. It appears therefore justified to combine the results of these two experiments to increase the number of data.

8.4.1 Serum cholesterol and atheroma grade

The relationship between serum cholesterol level and atherosclerosis grade is shown in Fig. 8. Kakita et al. (1972) observed in cockerels a direct relationship between the degree of coronary artery involvement and the logarithm of the serum

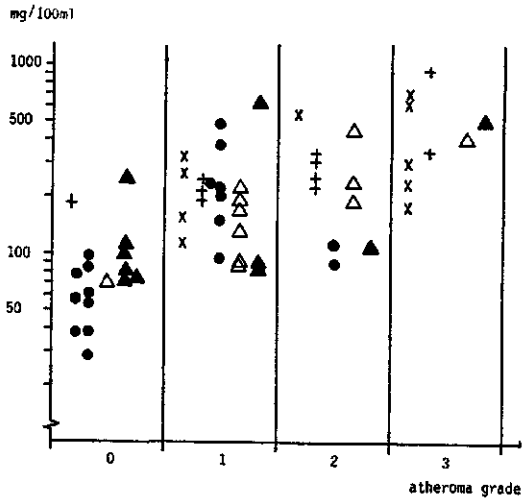

Fig. 8. Exp. 2. Final serum cholesterol levels (log scale) of rabbits, divided to atheroma grade. Combined results of Exps 2 and 2A (see text). x: coconut oil, Exp. 2; +: coconut oil, Exp. 2A; ●: coconut oil plus soya bean oil, Exp. 2A; △: butter oil, Exp. 2; ▲: simulated butter oil, Exp. 2.

cholesterol values.

In Fig. 8 serum cholesterol as present at termination is presented on a logarithmic scale. This measuring point has been discussed earlier as arbitrary. If atherosclerosis is the net outcome of a long-lasting process of injury and repair, comparison of the degree of atherosclerosis with the serum lipids over the whole experimental period would be recommendable and more logical. The relationship between average serum cholesterol and atheroma grade is presented in Fig. 9, again on a logarithmic scale. It is evident that the variation of serum cholesterol of rabbits, exhibiting the same degree of atherosclerosis, is great, but is not unimportantly reduced by using the average cholesterol values over the entire duration of the experiment.

The discrimination between rabbits with Grade 0 and 1 appears very sharp at about 100 mg cholesterol/100 ml serum. No animals with a serum cholesterol level below 80 mg/100 ml ever exhibited the slightest sudanophilia. This agrees remarkably with the data of Kakita et al. (1972). They analysed the regression between coronary lesions versus plasma cholesterol for more than 2000 individual cockerels. They found a curvilinear relationship, indicating a greater effect on atherogenesis of the cholesterol levels between 100 and 400 than above. No coronary lesions were observed if plasma cholesterol was below 150 mg/100 ml. From their regression equation, however, it was calculated that the threshold, below which no lesions are to be expected, lies at 80.4 mg cholesterol. A value very similar to the lowest values observed among the animals with Grade 1 or more in this experiment, i.e. 78 and 84 mg.

The question whether rabbits fed on butter oil were different from the other rabbits is difficult to answer. Their number was rather small. Rabbits fed on butter and those fed on simulated butter oil, with Grade 1, 2 or 3, displayed a tendency to have somewhat lower average serum cholesterol levels than the other rabbits with the same grade (Fig. 9). Gottenbos & Thomasson (1961) noted also some dissimilarity between blood cholesterol and degree of aortic atherosis when atherosclerosis was induced by

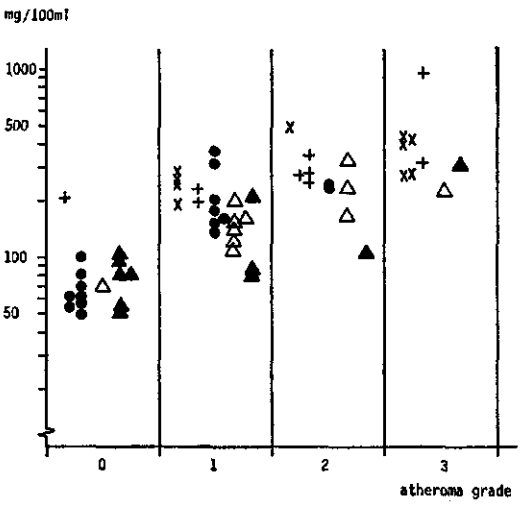

Fig. 9. Exp. 2. Average serum cholesterol level (log scale) of rabbits, divided to atheroma grade. For details see Fig. 8.

cholesterol or coconut oil. They observed with coconut oil higher serum cholesterol levels but less atherosclerosis than with diets containing 0.08% cholesterol. As the hypercholesterolemia of rabbits fed on butter oil is partly caused by the cholesterol present in the butter oil, the tendency for somewhat more atherosclerosis than expected from serum cholesterol may be correct.

Both in the study of Gottenbos & Thomasson (1961) and in this study grading was performed on a scale from 0 till 4. Within each grade a large variation of serum cholesterol and extent of atherosclerosis is present.

8.4.2 Serum cholesterol and percentage surface area affected

It has been discussed earlier (Section 6.7 and 7.3.5) that a division of atheroma grade into 4 non-equidistant classes is arbitrary and may obscure relationships with serum lipids. To meet these drawbacks the percentage area of the whole aorta and of the aortic arch which showed sudanophilia was established. The relationship between average serum cholesterol of the individual rabbits and their percentage surface

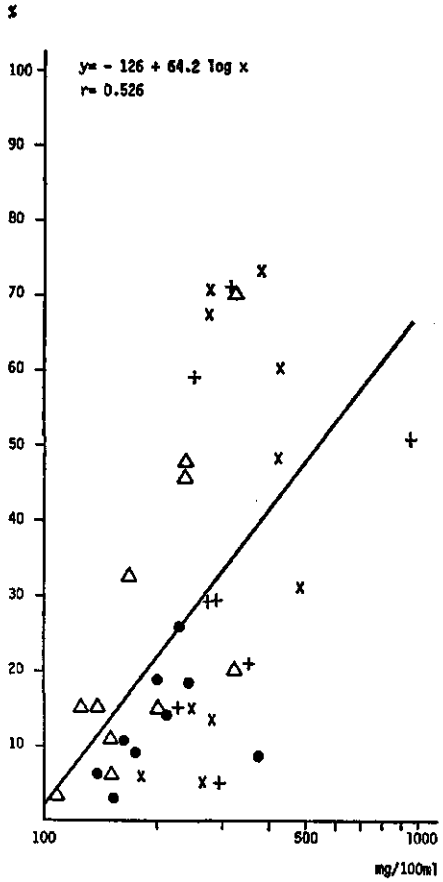

Fig. 10. Exp. 2. Regression analysis of percentage surface area of whole aorta, involved with sudanophilia, versus average serum cholesterol content (log scale) of rabbits : $y = a + b. \log x$ (for details see Fig. 8).

area involved is presented in Figs 10 and 11 for the whole aorta and the aortic arch, respectively. It can be seen that a direct relationship exists between serum cholesterol and extent of aortic involvement. This relationship is somewhat obscured for the whole aorta because most of the sudanophilia is concentrated in the aortic arch and thoracic aorta. The rest of the aorta therefore weakens the relationship.

Fig. 11 depicting the percentage of the aortic arch which is involved, shows a much better correlation with serum cholesterol. The correlation coefficients for the relationship between percentage involvement of the whole aorta or aortic arch with the log average serum cholesterol are 0.526 and 0.670, respectively. Both are highly significant ($P < 0.001$). It may, however, be seen from the Figs 10 and 11 that individual values differ markedly at a certain degree of atherosclerosis. From the correlation coefficients it can be calculated that in the first case (Fig. 10) only 27.5% from the variance in extent of atherosclerosis can be explained with variations in serum cholesterol. In the second case (Fig. 11) this explained variability is about 45%. It is evident that many more animals are needed per dietary group to draw reliable conclusions. The tendency for the rabbits fed on butter oil, as may be seen in Fig. 11, is again the

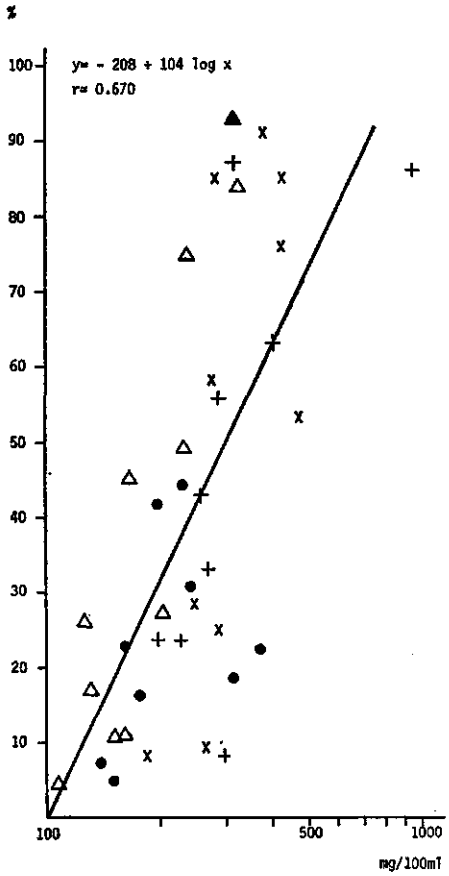

Fig. 11. Exp. 2. Regression analysis of percentage surface area of thoracic aorta, involved with sudanophilia, versus average serum cholesterol content (log scale) of rabbits (for details see Fig. 8).

same as in Fig. 9. They tended to require a lower serum cholesterol level to attain the same level of atherosclerosis.

From the regression equations the serum cholesterol level can be calculated below which no atherosclerosis is expected, the threshold value. For the whole aorta this threshold value is 90 mg/100 ml, for the aortic arch 99 mg/100 ml. These values compare favourably with the threshold calculated by Kakita et al. (1972) for cockerels at 80.4 mg and for rodents and primates at 145 mg or lower (Constantinides, 1965; p. 67). These regression equations and threshold values are, of course, only valid in this species in this strain and breed, at this age, over this length of experiment etc. The similarity of the values obtained with several species under divergent experimental conditions, may indicate however their general applicability.

8.4.3 Amount of serum lipid and extent of atherosclerosis

The means for terminal and average serum cholesterol levels of rabbits with the same degree of atherosclerosis are presented in Table 27. The values for terminal serum triglycerides and the ratio phospholipids: free cholesterol are also included. At last the percentage surface area of whole aorta and aortic arch with sudan-positive staining are presented. The trend for all serum lipids to increase with degree of atherosclerosis is evident. It is also clear that the different grades are more homogeneous as to average cholesterol than to terminal cholesterol. The discrimination between the grades becomes sharper as the average cholesterol values are used. This appeared also from Fig. 8 and 9.

8.4.3.1 Ratio phospholipid: free cholesterol

The ratio phospholipid: free cholesterol tends to decrease as severity of atherosclerosis increases. However, between Grade 2 and 3 no further decrease was observed. This is in contrast with the findings of Moore & Williams (1964b) who observed a decreased ratio at higher degrees of atherosis, a ratio of 2.00 being the point below which more severe atherosclerosis starts to be present. The value for the ratio phospholipid: free cholesterol seems to approach a lower limit. The degree of aortic involvement and the absolute serum cholesterol levels were much less in the experiments of Moore & Williams (1964a, b) than in this study. This may explain why the ratio approached the limit more closely in this study, resulting in no difference between Grades 2 and 3. A steep increase of aortic atherosclerosis was found with ratios phospholipid: free cholesterol below 3.

The divergence with Moore & Williams' results may be due to strain and diet differences. Moore & Williams (1964b) noted that different diets caused different parameters of the curve, fitting the data.

At cholesterol levels above 100 – 150 mg/100 ml the ratio free: total cholesterol remained constant at about 28 – 29% (Experiments 1 and 2). As the ratio phospholipid: total cholesterol simultaneously decreased, the ratio phospholipid: free choles-

Table 27. Serum lipids (mg/100 ml ± s.e.) and extent of atherosclerosis in rabbits with different grades of atherosclerosis (number of animals between brackets).

	Grade 0	Grade 1	Grade 2	Grade 3	Significance of differences[1]
Terminal cholesterol	89 ± 14 (17)	221 ± 28 (23)	263 ± 44 (11)	479 ± 84 (9)	3 2 1[2] 0[2]
Average cholesterol	80 ± 9 (17)	195 ± 16 (23)	270 ± 29 (11)	401 ± 72 (9)	3 2 1[2] 0[2]
Triglycerides	67 ± 20 (17)	108 ± 19 (23)	104 ± 24 (11)	229 ± 103 (9)	3 2 1 0
Phospholipids/free cholesterol	451 ± 34 (13)	336 ± 16 (22)	227 ± 20 (10)	231 ± 14 (8)	0[2] 1[2] 2 3
Whole aorta sudan positive		10.8 ± 1.1 (20)	31.0 ± 4.0 (10)	62.3 ± 3.5 (8)	
Aortic arch sudan positive		17.9 ± 2.2 (20)	52.8 ± 5.5 (10)	78.9 ± 5.1 (9)	

1. Differences are significant ($P < 0.05$) except when the grade numbers share a common underlining.
2. Differences between grade 0 and 1 for terminal cholesterol, average cholesterol and ratio phospholipids/free cholesterol significant at 1%.

terol decreased as well. During the first 100 mg increase of serum cholesterol from the control level of 40 – 50 to 100 – 150 mg both ratios free cholesterol: total cholesterol and phospholipid: cholesterol decreased. The decrease of the ratio free: total cholesterol was much greater, resulting in a greatly decreased ratio phospholipid: free cholesterol (Experiments 1 and 2). This might explain why Moore & Williams (1964a, b), with serum cholesterol levels between 50 and 150 mg/100 ml noted a better correlation between the degree of atherosis and the ratio phospholipid: free cholesterol than with serum cholesterol values. However at higher levels of serum cholesterol, as in our experiments, the ratio phospholipid: cholesterol approaches its lower limit and loses its predictive power. In that case a better correlation of serum cholesterol levels with degree of atherosclerosis is obtained.

8.4.3.2 Serum triglycerides

Comparison of animals with different atheroma grades also tends to direct attention to the serum triglycerides. It might be concluded from Table 27 that the higher the serum triglycerides, the higher the atheroma grade. It has already been discussed in Section 8.3.5 that increased serum cholesterol levels in rabbits are often accompanied by elevated serum triglycerides (Huang & Kako, 1970). The apparent relationship between triglycerides and atheroma grade may be actually caused by a relationship between cholesterol and triglycerides.

To unravel this possible interaction the rabbits have been divided into four groups, based on serum cholesterol and triglyceride levels. These groups were compared as to percentage surface area of whole aorta or aortic arch with sudanophilia. The

Table 28. Relationship between level of serum cholesterol and triglycerides (mg/100 ml) and aortic atherosclerosis (mean ± s.e.).

	Serum lipid group[1]			
	0	A	B[2]	C
Number	19	20	3	16
Mean atheroma grade	0.38	1.69	0.33	1.82
Mean cholesterol	80 ± 7	255 ± 17	97 ± 6	319 ± 45
Mean triglycerides	43 ± 4	53 ± 5	207 ± 84	252 ± 51
Percentage whole aorta	2.2 ± 1.1	27.5 ± 5.4		31.1 ± 5.7
Percentage aortic arch	2.9 ± 1.6	36.9 ± 6.0		50 ± 7.7

1. Rabbits have been allocated into four different groups according to serum lipid levels (Constantinides, 1965 p. 67):

	0	A	B	C
cholesterol (mg/100 ml)	⩽ 145	> 145	⩽ 145	> 145
triglycerides (mg/100 ml)	⩽ 100	⩽ 100	> 100	> 100

2. From these three animals two showed Grade 0 and one minimally Grade 1. No percentage surface area could be calculated.

results are shown in Table 28.

It is evident from Table 28 that the rabbits with both low cholesterol and triglyceride levels display a low atheroma score and very little atherosclerosis. If only cholesterol is elevated above 145 mg/100 ml the mean atheroma grade and percentage area of the aorta with atherosclerosis are both increased. Rabbits with pure triglyceridemia are rare. Their serum is milky and the triglycerides are probably mainly present as chylomicrons. The three animals with isolated elevated triglycerides showed minimal atherosclerosis. The fourth group with both cholesterolemia and triglyceridemia demonstrates slightly more atherosclerosis. This may be easily ascribed to the somewhat higher mean cholesterol level present in this group. It has already been noted that very high cholesterol levels are almost always accompanied by increased triglyceride levels (Section 8.3.5). This is probably the reason for the somewhat higher mean cholesterol in the high triglyceride group. From Table 28 it may be concluded that in this rabbit model high triglyceride levels are commonly present at high cholesterol levels but do not aggravate atherosclerosis.

8.4.4 Atheroma grade and surface area involvement

From Table 27 it can be concluded also that grading aortas on a scale from 0 – 4 is as adequate in establishing the degree of atherosclerosis as measuring the positively stained percentage surface area of whole aorta or aortic arch. For more detailed calculations the last method perhaps presents some advantages as a scale from 0 – 100% can be used and absolute figures are obtained. With the first method a qualitative division is obtained in non-equidistant classes. This can be seen in Table 27 as the distance between Grade 0 and 1 is only 10 or 18% while the distance between Grade 1 and 2 is 20 or 30% and between Grades 2 and 3 is 30 and 25% for the whole aorta and the aortic arch, respectively. In the first method also attention is paid to the severity of atherosclerosis, i.e. protrusion in the lumen of the vessel of, and consequently luminal narrowing by the atherosclerotic plaque. Both grading systems have their advantages and should be applied next to each other.

8.5 Conclusion of Experiment 2

Experiment 2 was done to find out which constituent of milk fat is mainly responsible for the rise in serum cholesterol and increase in atherosclerosis when fed to rabbits. A second objective was whether the degree of atherosclerosis, induced by butter oil feeding, would be comparable with the degree of atherosclerosis, induced by coconut oil feeding, at similar serum cholesterol concentrations.

The second question has been discussed in Section 8.4. It is concluded that a strong correlation exists between serum cholesterol level, as measured over the whole duration of the experiment, and degree and extent of atherosclerosis of the aorta. However, unexplained variability is great and it is difficult to predict individual atherosclerosis from individual serum cholesterol concentrations.

The same observation has been reported by Krogh et al. (1961). They concluded that some rabbits were more susceptible than others at the same cholesterol level. Mean serum cholesterol on a group basis is a more reliable index. Unfortunately, mortality, probably due to *Pasteurella* infection and other 'normal' rabbit diseases, was very high. This interferes seriously with drawing far-reaching conclusions from this experiment. From the Figures 8 – 11 the suggestion is derived that the rabbits fed on butter oil exhibit slightly more atherosclerosis than could be expected on the basis of their serum cholesterol level.

In this experiment it has been found that almost cholesterol-free simulated butter oil induces low serum cholesterol levels, whereas cholesterol supplementation to this fat induces an increase of serum cholesterol till the level attained by butter oil. Addition of fatty acids of short and medium chain length to the simulated butter oil had not the slightest effect, which confirms the results of Experiment 1. These findings tend to confirm the observations by Funch et al. (1962) that cholesterol added to cocoa butter in an amount equal to that found in butterfat increases serum cholesterol of rabbits in a similar way to butterfat itself. They also found the same degree of atherosclerosis with both fas.

The importance of the cholesterol component of milk fat in inducing hypercholesterolemia and atherosclerosis is now well established for rabbits. In relation to the literature discussed in Section 4.5 it is also likely that dietary cholesterol is of importance in human nutrition as far as the establishment of serum cholesterol levels is concerned.

The degree of atherosclerosis induced by butter oil seems to be more extensive than with coconut oil feeding. Gottenbos & Thomasson (1961) observed that atherosclerosis was more easily produced by cholesterol than by coconut oil feeding. This would confirm that the butter oil effect is mainly a cholesterol effect in rabbits. The absence of effect of feeding 0.04% cholesterol in the diet, which is exactly the cholesterol content of our butter oil diet, reported by Gottenbos & Thomasson (1961), may be explained by the low-fat diet used by these investigators. The importance of the cholesterol vehicle in experimental hypercholesterolemia and atherosclerosis has been studied by Kritchevsky (1970). He concluded that cholesterol added to a low-fat diet induces lower levels of serum cholesterol than when added as a suspension or solution in oil.

An investigation of the role of dietary cholesterol in inducing atherosclerosis in rabbits at amounts present in milk fat and with a vehicle resembling milk fat in its fatty acid composition is clearly needed. As such a study would demand a duration of at least one year and with the general health status of our animals in mind it was decided to try to improve first the properties of the diet. It was hoped that by such an improvement the rabbits would increase food consumption, gain weight or at least stop losing weight and ultimately would not prematurely die. At last the model should remain intact.

9 Modification of the basal diet (Experiment 3)

9.1 Objectives

It appeared from the first two experiments that the general health status of rabbits fed on the semi-synthetic diets with inclusion of coconut oil or butter oil was not satisfactory. Rabbits lost weight or maintained body weight at levels that were too low compared with controls fed on commercial laboratory diets. Infections occurred more frequently and were more often fatal. Mortality was high if the experiments were longer than six months.

As it is not certain whether such suboptimal conditions in rabbits influence the results it was decided to try to improve the diets. Two approaches were chosen. One to improve acceptability, the other to alter the composition.

In this section the results are given of these small experiments without many experimental details. The following experiments were performed:
- a study of the effects of a large dose of coconut oil, added to a commercial diet;
- a study of the effects of supplementation of the semi-synthetic diet with grass meal;
- a study of the effects caused by the addition of varying amounts of quinine sulphate to the diet to affect acceptability;
- a study of the effects of replacing the casein in the diet by a protein mixture, consisting of casein, fish protein concentrate, soya bean protein and gelatin. The mixture was chosen as to resemble the amino acid composition of the commercial diet.

9.2 Supplementation of a commercial rabbit diet with coconut oil (Experiment 3A)

To investigate whether our rabbits could endure large amounts of fat, an experiment was performed with 8 Dutch Belt rabbits, divided into two groups of 4 animals each, which were fed either on a commercial rabbit diet (Cunicon I, Trouw & Co) or on the same diet supplemented with 14.6% (by weight) of coconut oil. The diet contained already 6% fat. The fat-rich diet therefore contained 20.6% fat (by weight) or 50% of calories as fat. The crude protein content of the diet was decreased by this procedure from 19.0% till 16.2% by weight. This experiment took 7 months. Diet preparation and feeding procedure were the same as described in Section 6.3.

Results In Table 29 a summary of the results is shown. Both diets induced similar weight gain and food intake. The serum cholesterol level tended to increase a little on the high fat diet but the difference was not significant nor important. Coconut oil per

Table 29. Influence on body weight, food intake and serum cholesterol level of adding 36% (cal/cal) coconut oil to a commercial rabbit diet (± s.e.).

	Diets	
	low fat	high fat
Starting weight (g)	2113 ± 114	2149 ± 175
Final weight (g)	2343 ± 127	2428 ± 200
Weight change (g)	231 ± 46	279 ± 45
Food intake (g)	86	64
Food intake (kcal/day)	259	243
Final serum cholesterol (mg/100 ml)	24 ± 3	35 ± 6

se can therefore not be considered as the cause of the poor performance of the rabbits in the Experiments 1 and 2.

9.3 Supplementation of the basal diet with grass meal and substitution of saw-dust by grass meal (Experiment 3B)

In two small experiments the influence of the addition of two amounts of grass meal was studied. Grass meal has been used by Gottenbos & Thomasson (1961), Vles et al. (1964) and Vles & Kloeze (1967). In these experiments satisfactory growth was reported.

In the first experiment, 10 parts grass meal were simply added to a semi-synthetic diet containing 40% of calories as coconut oil. In the second experiment, which followed the first and was performed with the same animals, 20% sawdust was replaced by 20% grass meal leaving only 1.5% sawdust in the diet.

The first experiment was started with 12 Dutch Belt rabbits. After 5 months they were supplemented with the animals from the experiment described in Section 9.2. The results of these extra animals are only included in the second part of this experiment. The first experiment lasted 7 months. The second one 5 months.

The change-over procedure, diet preparation and feeding were as described in Section 6.3.

Results A summary of the most important findings is shown in Table 30. The addition of 10% grass meal had no effect as compared with controls which received no grass meal. Weight loss occurred equally as did the increase of serum cholesterol. Symptoms of snuffles disease were present in both groups. Mortality was absent in the first grass meal period. During the second period, after sawdust was substituted for by 20% grass meal, three animals died in both groups. Some weight increase occurred. No differences between the groups were present at autopsy. Relative organ weights were similar. In both groups fatty livers, lungs affected by Pasteurella infection, and scarred kidneys with necrosis were equally present.

Table 30. Influence of 10 or 20% grass meal in semi-synthetic diets fed to rabbits (± s.e.).

	Diets			
	controls 1	10% grass meal	controls 2	20% grass meal
Number	6	6	9	9
Months	7	7	5	5
Mortality	2	–	3	3
Weight change (g)	−144	−145	−128	+178
Serum cholesterol (mg/100 ml)				
at start	36 ± 6	20 ± 3	196 ± 39	205 ± 39
at end	219 ± 67	221 ± 64	369 ± 58	141 ± 25
Atheroma grade			1.0	1.0

The most striking observation was the decrease of serum cholesterol on the 20% grass meal diet, which was highly significant compared with the controls ($P < 0.01$). The controls behaved in the same way as the Groups 1 of the Experiments 1 and 2. It is not clear which factor in the grass meal is responsible for this effect on serum cholesterol. The general performance of the rabbits fed on grass meal appeared slightly better, but not sufficiently so as to include such a complex product in a semi-synthetic diet. Moreover, the cholesterol depressing effect of grass meal, which is possibly comparable with the same effect elicited by spinach (Iritani & Nogi, 1972) in rats, would be a serious drawback in this model.

Replacement of the sawdust by cellulose (Akuflock) did not favourably influence the results either.

9.4 Influence of added quinine sulphate (Experiment 3C)

In this Experiment 3C the effect on food consumption of a bitter tasting substance, quinine sulphate, was investigated. Food was presented to 10 rabbits in two food cups. One cup contained the usual semi-synthetic coconut oil diet, the other cup the same diet with added quinine sulphate. The cups were exchanged twice per 24 hours to exclude a learning effect as related to place. Five animals got a constant dose of 100 mg/kg quinine sulphate, the other five got increasing doses of 300, 600 and 1000 mg/kg. The experiment lasted 14 days. Every day, consumption of food from each cup was established per animal.

Results Food consumption from the cups with and without quinine addition is shown in Table 31. Food selection does not seem to occur. From both cups similar amounts were consumed. Also within groups no preferences were present. The addition of a taste enhancing substance such as quinine sulphate is evidently without effect on food consumption at amounts below 600 mg/kg. Whether the 1000 mg/kg level

Table 31. Consumption of quinine sulphate supplemented diets (g/day) ± s.e. Number of animals between brackets.

Dose (mg/kg)	Diets[1]		
	without quinine	with quinine	total
100 (5)	27.7 ± 3.3	30.9 ± 3.2	58.6 ± 6.4
300 (5)	25.3 ± 4.4	26.7 ± 3.3	52.0 ± 7.1
600 (5)	24.0 ± 3.4	26.8 ± 4.8	50.8 ± 7.3
1000 (5)	23.9 ± 6.8	18.7 ± 1.4	42.6 ± 6.6

1. Usual semi-synthetic diet with 40% (cal/cal) coconut oil.

depresses food consumption is not certain, though some animals in this comparison evidently preferred the quinine free diet.

9.5 Replacement of casein from the basal diet (Experiment 3D)

From Experiment 3A it has been concluded that coconut oil per se has no deleterious effects on rabbits. In Sections 4.3 and 4.7.2 the important role played by the protein in the diet of rabbits has been discussed. The general conclusion was that casein at the 20% (w/w) level in the diet might not be an ideal source of protein to promote growth. Wooley (1954) recommended to increase the potassium level of a casein diet till 0.8% to promote growth. This level was exactly present in our diets.

To investigate whether the protein source might be the cause of the poor performance of the rabbits, an experiment was initiated in which the effects of diets containing casein or a protein mixture were compared. The protein mixture was prepared from roughly four parts gelatin, six parts casein, six parts fish protein concentrate and four parts soya bean protein. The proportions of each protein were chosen in such a way that the amino acid composition of the mixture resembled the composition of the commercial rabbit diet. For a more detailed composition of the diet, see Section 10.2. This commercial diet has been proven to promote growth in rabbits efficiently.

This preliminary trial was performed with 10 Dutch Belt rabbits divided into two groups of five each. The animals were the same as used in Experiment 3C (9.4) and were accustomed to the semi-synthetic diet with casein and coconut oil. As they had been several months on this diet, their serum cholesterol levels at the start of this experiment had already increased to about 200 mg/100 ml. Before the experimental period started a preference study was carried out as described in Section 9.4. One food cup contained the casein diet, one the protein mixture diet (PM). Both diets were supplemented with 36% of calories as coconut oil. From the 10 animals two preferred the casein diet, four the PM diet and four displayed no preference for the 14 days. The mean consumption of the PM diet was 10 g per day more than of the casein diet, 34 versus 24 g. Animals with a particular preference were equally distributed over the two groups. This experiment lasted 18 weeks.

Table 32. Effects of replacement of casein in a semi-synthetic diet for rabbits by a protein mixture.

	Diets	
	casein	protein mixture
Number	5	5
Mortality	2	0
Weight change (g)	−288	+14
Food consumption (g/day)	32	48
Serum cholesterol (mg/100 ml)		
at start	203	177
after 18 weeks	316	135

Results The changes in body weight, food consumption and serum cholesterol are shown in Table 32. All animals fed on the casein diet lost weight between 200 and 460 g. From the PM-fed rabbits two lost 25 – 30 g, while the other three gained 5 – 65 g body weight. The difference between the two groups was highly significant ($P < 0.01$). Food intake parallelled the body weight changes and was also significantly different between the two groups ($P < 0.05$). No relationship was seen between food intake and the preferences as studied before.

The serum cholesterol levels, already moderately increased at the start of this experiment, increased further while on the casein diet. On the PM diet, however, the levels tended to decrease.

Two rabbits from the casein group died at 13 and 18 weeks, respectively, while none died from the PM group. At autopsy all animals fed on casein had a grossly fatty liver while only two out of five showed a fatty liver when fed on the mixed protein. The casein-fed rabbits had relatively enlarged spleens. Two animals from the casein group showed symptoms of snuffles disease while none of the PM-fed animals showed such signs.

From these combined data it is concluded that the kind of protein in the semi-synthetic diet may profoundly affect the results.

9.6 Conclusion of Experiment 3

Several small experiments have been performed to find the component in the semi-synthetic diet which may be responsible for the poor condition and high mortality of the animals. The addition of coconut oil to a commercial diet had no deleterious effect on the rabbits. By this procedure, however, serum cholesterol does not increase. This confirms the many data in the literature (see: e.g. Kritchevsky, 1964; Carroll, 1971) that fats added to a stock diet do not influence blood lipids or degree of atherosclerosis in rabbits. This was the very reason for the development of the semi-synthetic diet or for the use of large amounts of dietary cholesterol.

The use of grass meal in the diet at the 10% level did not influence weight change or

serum cholesterol level. Increasing the amount to 20% improved growth considerably but reduced at the same time serum cholesterol levels. Grass meal contains a multitude of substances which may be responsible for this effect. In view of the lower cholesterol levels induced by a protein mixture compared with casein, it is tempting to speculate that the grass meal effect is partly a protein effect. The growth-promoting effect of grass meal may be related to the effects reported by Wooley (1954) for kale or alfalfa meal or forage juice. However, Wooley (1954) ascribed these effects partly to the cations present in these products and recommended that a 20% casein diet should contain at least 0.8% potassium. This amount exactly was present in our diets. The possibility that grass meal has an appealing taste for rabbits and stimulates growth through non-specific appetite effects should also be considered.

An attempt to improve acceptability of the diet by adding a bitter-tasting substance, quinine sulphate, was without effect.

There are many advantages in using a well-defined source of protein in the diet. Casein, however, appears an unsuitable protein for rabbits as discussed in Sections 4.3 and 4.7.2. The results of Experiment 3D (Section 9.5) tend to confirm this hypothesis. It has not been established yet whether the cholesterolemic properties of the semi-synthetic diet are due to the casein component. Kritchevsky & Tepper (1968) and Howard et al. (1965) tried to define the acting principle in this kind of diet without success. Howard et al. (1965) abolished the hypercholesterolemia by substituting soya bean meal for casein. They reduced the protein content of the diet by this procedure from about 20 to about 10%. Carroll (1971) was the first to directly assign casein as the cause of hypercholesterolemia in rabbits fed on semi-synthetic diets. This may be an important point as the fat-free and low-fat semi-synthetic diet is also hypercholesterolemic and atherogenic (Malmros et al., 1964; Moore & Williams, 1964a, b).

Different fats added to an otherwise atherogenic diet can only modify the basic atherogenic properties caused by the casein. It is important to study the effects of different fats added to a semi-synthetic diet in which the protein source is not only casein. Such a study may provide a clue as to whether the effects of different fats are only gradually or fundamentally altered by the kind of dietary protein. This will be the subject of the next two experiments.

10 Effects of butter oil versus simulated butter oil and butter oil supplemented with linoleic acid fed with different dietary proteins (Experiment 4)

10.1 Objectives

From a review of the literature (Sections 4.3 and 4.7.2) and from the preliminary experiment reported in Section 9.5 it is evident that the protein component in a semi-synthetic diet fed to rabbits may influence the results profoundly. From Experiment 2 (Ch. 8) it was concluded that the main effect of butter oil might be contributed by its cholesterol content. The literature also suggests that the low content of linoleic acid of butter oil might be responsible for its hypercholesterolemic effect when supplied in semi-synthetic diets.

In this experiment the hypercholesterolemic properties were studied of diets containing either coconut oil, butter oil, butter oil supplemented with 10% of calories as soya bean oil, and simulated butter oil with and without added cholesterol in an amount similar to that present in the butter oil. These fats were fed in diets containing except Group 1 a mixture of proteins as nitrogen source resembling the amino acid composition of laboratory chow. It was meant to study the differing effects of the fats on serum lipids and extent of atherosclerosis.

After several months no or only small effects on blood lipids were noted. The dietary groups were then divided into two comparable subgroups. One subgroup continued on the protein mixture diet, the other subgroup was changed over to casein as dietary protein. This procedure allowed a comparison of the influence of two protein sources on serum lipids as induced by several fats.

10.2 Experimental details

One hundred and twenty White Viennese × Alaska crossbred rabbits were used in this study. They were purchased from another supplier as in the former experiment, who claimed the absence of manifest snuffles disease (see Section 6.1). Unfortunately, this supplier could not provide the same breed of rabbits as used in Experiment 1 and 2. However, this breed is also susceptible to hypercholesterolemia and atherosclerosis (Kloeze et al., 1969) on this kind of diet.

All animals were gradually accustomed to a semi-synthetic diet containing coconut oil and the protein mixture. Based on serum cholesterol and body weight, the animals were distributed over six similar dietary groups of 20 animals each. During one week the protein in Group 1 was gradually replaced by casein. In the same period the coconut oil in four other groups was replaced by the dietary fat to be studied. The

Table 33. Ingredients and analysed nutrients of diets (Experiment 4) (weight %, mg/kg respectively).

	Dietary groups[1]					
	1 cas./coc.	2 PM/coc.	3 PM/BO	4 PM/SBBO	5 PM/IBO–C	6 IBO+C
Corn starch[5]	35.8	36.8	36.8	27.4	36.8	36.8
Casein[5]	21.16	6.0	6.0	6.0	6.0	6.0
Fish protein concentrate[2]	–	6.0	6.0	6.0	6.0	6.0
Soyabean protein concentrate[3]	–	4.0	4.0	4.0	4.0	4.0
Gelatin[4]	–	4.18	4.18	4.18	4.18	4.18
Sawdust[5]	21.5	21.5	21.5	27.3	21.5	21.5
Vitamin premix[5]	1.2	1.2	1.2	1.2	1.2	1.2
Mineral premix[6]	1.2	1.2	1.2	1.2	1.2	1.2
Calcium phosphate (di-)	2.9	2.9	2.9	2.9	2.9	2.9
NaCl	0.85	0.85	0.85	0.85	0.85	0.85
KCl	1.37	1.37	1.37	1.37	1.37	1.37
$MgCO_3$	0.3	0.3	0.3	0.3	0.3	0.3
Coconut oil	13.8	13.8	–	–	0.5	0.5
Butter oil	–	–	13.8	13.8	–	–
Soyabean oil	–	–	–	3.6	–	–
Beef tallow	–	–	–	–	9.4	9.4
Trimyristate[7]	–	–	–	–	1.1	1.1
MCT[7]	–	–	–	–	0.9	0.9
Tributyrate[7]	–	–	–	–	0.6	0.6
Palm oil	–	–	–	–	1.3	1.3
Cholesterol (mg/kg) added	–	–	–	–	–	280
Methionine	0.60	0.38	0.38	0.38	0.38	0.38
S-amino acids	0.70	0.49	0.49	0.49	0.49	0.49
Lysine	1.53	1.12	1.12	1.12	1.12	1.12
Arginine	0.98	1.00	1.00	1.00	1.00	1.00
Crude protein	18.7	18.9	18.9	18.9	18.9	18.9
Iron (mg/kg)	642	701	701	701	701	701
Calcium	0.86	1.07	1.07	1.07	1.07	1.07
Phosphorus	0.76	0.84	0.84	0.84	0.84	0.84
Cholesterol (mg/kg)	–	–	380	380	100	380

1. cas./coc. = casein/coconut oil.
   PM/coc. = protein mixture/coconut oil.
   PM/BO = protein mixture/butter oil.
   PM/SBBO = protein mixture/soya bean + butter oil (1:4).
   PM/IBO–C = protein mixture/simulated butter oil without cholesterol.
   IBO+C = simulated butter oil with cholesterol added.
2. Protanimal (Astra): crude protein: 88%; fat: 0.4%; Ca: 3.7%; P: 2.2%; Fe: 600 mg/kg.
3. Promosoy-100 (Central Soya Chemurgy, Chicago): crude protein: 65.5%; fat: 0.3%; ash: 5.8%; Ca: 0.2%; P: 0.64%; Fe: 100 mg/kg.
4. Gelatin standard quality 1 (Lijm- en Gelatinefabriek, Delft): crude protein: 90%.
5. As in Table 2a and c.
6. As in Table 2b but Mg separately added.
7. As in Table 20; composition MCT: 6:0, 2.7%; 8:0, 57.6%; 10:0, 37.1%; 12:0, 1.7%; 16:0, 0.6%; 18:1, 0.4%.

Table 34. Fatty acid composition of several fats used in Experiment 4 (weight %).

Fatty acid code	Dietary fats			
	coconut oil	soybean oil	butter oil[1]	simulated butter oil[2]
4:0	–	–	4.0	4.0
6:0	trace	–	2.8	0.2
8:0	7.0	–	1.4	3.8
10:0	6.0	–	2.4	2.5
12:0	46.0	–	2.6	2.9
14:0	18.5	–	8.9	8.9
14:1	–	–	2.1	0.7
16:0	9.5	11.2	21.4	21.3
16:1	–	–	2.6	2.2
18:0	3.0	4.3	12.4	16.6
18:1	7.0	24.0	30.5	29.0
18:2	2.0	53.4	3.9	4.1
18:3	trace	7.2	2.0	1.1
20:0	–	–	0.7	0.8

1. The tallow contained 106 mg cholesterol per 100 g. The butter oil 275 mg per 100 g.
2. The simulated butter oil was composed of: tributyrate, 4%; MCT, 6%; coconut oil, 4% ; trimyristate, 7%; palm oil, 7%; beef tallow, 72%.

composition of the diets is shown in Table 33, the fatty acid compositions in Table 34.

After 14 weeks on these diets many groups except Group 1 showed not even the slightest change in serum lipids. The groups receiving the protein mixture were therefore divided into two subgroups. In subgroup A the protein was replaced by casein, while subgroup B continued on the protein mixture. The subgroups 3B and 5B were removed from the experiment after a further 12 weeks because of lack of response.

During the experiment, body weights were determined fortnightly. Blood was taken generally every four weeks for cholesterol determinations. If total lipid was also determined, bleeding was done after at least 14 hours fasting. Haemoglobin levels were determined irregularly. At the end of the experiment a more detailed lipid analysis was performed of blood and liver. As anaemia developed during the experiment, also the iron content of liver and spleen was determined at termination.

10.3 Results and discussion

10.3.1 Food consumption and body weight

Food consumption figures per group are shown in Table 35. The experiment was subdivided into three periods. During the first period of 14-weeks all groups of 20

Table 35. Food consumption per group (g dry matter/rabbit/day) Experiment 4.

Groups[1]	Periods		
	1 (14 weeks)	2 (13 weeks)	3 (24 weeks)
1 cas./coc.	45	42	43
2A cas./coc.		38	39
2B PM/coc.	61	61	61
3A cas./BO		38	44
3B PM/BO	77	72	
4A cas./SBBO		39	46
4B PM/SBBO	75	72	73
5A cas./IBO−C		41	40
5B PM/IBO−C	75	77	
6A cas./IBO+C		40	47
6B PM/IBO+C	75	69	74

1. See Table 33.

animals each except of Group 1, were fed on protein mixture. The second period, beginning with the division of Groups 2 – 6 into equal subgroups A and B, lasted 13 weeks. The final period was 24 weeks. Between the first and second period one week was needed for the change-over procedure to the new diets of half of the animals of Groups 2 – 6, so that the experiment lasted 52 weeks in all.

During the first period, food consumption was much higher when the nitrogen in the diet was supplied by a protein mixture rather than by casein. The kind of fat did not exert much influence except in the case of coconut oil. This fat evidently depressed food intake. After the change-over of half of the animals to a casein diet, food consumption decreased immediately to the level of Group 1 or even lower. Coconut oil seemed to cause, an additional decrease of food intake, also with casein as protein (2A) though the differences were small. The high food intake of the PM-fed rabbits was maintained completely till the end of the experiment. The very high fat content of the diet of Group 5 (50% cal/cal) had no deleterious effect on food intake.

The data of body weight parallel the food intake pattern. They are shown in Fig. 12 a-f. During Period 1 the rabbits fed on casein and coconut oil immediately started losing weight (Fig. 12a), while the rabbits fed on the same fat with a protein mixture (Fig. 12b) maintained body weight or even regularly increased their body weight slightly. This difference is a reflection of their higher food intake. The much higher food consumption of the other four groups is also reflected in the weight gain of these animals, amounting to 500 g. By analysis of variance it was concluded that a significant treatment effect existed ($P < 0.001$) related to body weight. By the sequential linear contrast analysis according to Student, Newman & Keuls (see Section 6.8), it could be shown that the differences between Group 1 and the other groups were significant ($P < 0.01$). Also the differences between Group 2 and the other groups

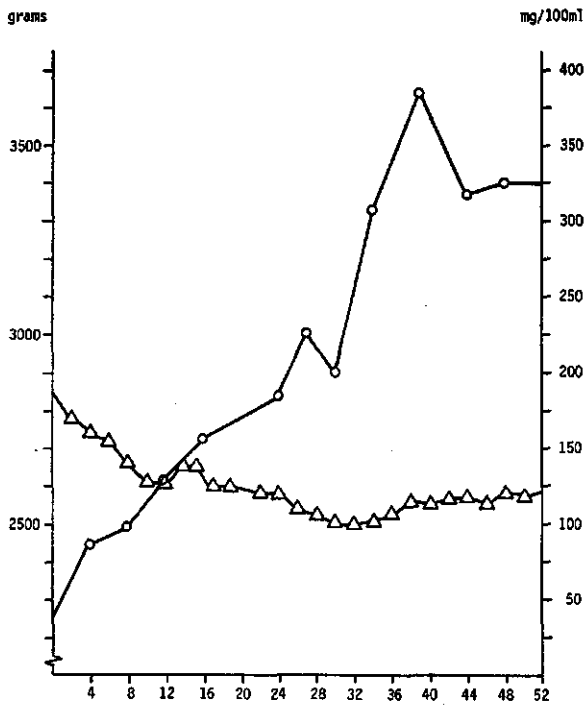

Fig. 12a. Exp. 4. Mean body weight and serum cholesterol content of rabbits, surviving the experiment, as a function of experimental time. Group 1: △, body weight; ○, serum cholesterol; casein/coconut oil.

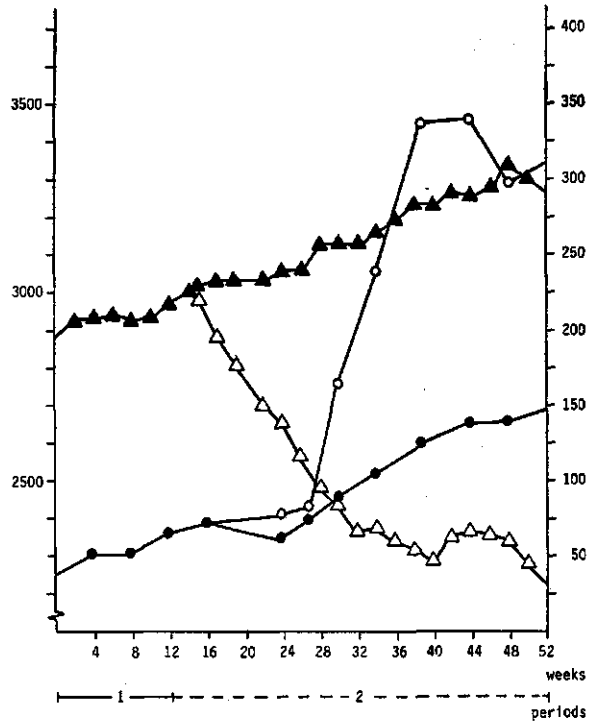

Fig. 12b. Exp. 4. Mean body weight and serum cholesterol content of rabbits, surviving the experimental periods, as a function of experimental time. Group 2: ▲, body weight; ●, serum cholesterol (protein mixture/coconut oil); △, body weight; ○, serum cholesterol (casein/coconut oil).

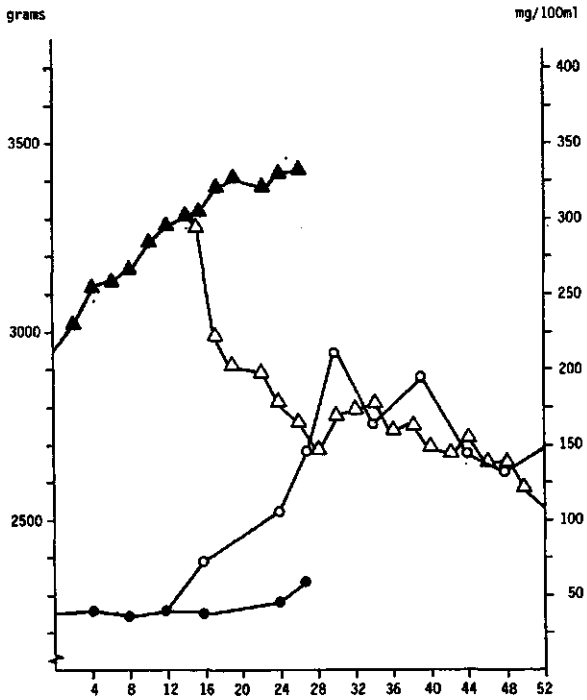

Fig. 12c. Exp. 4. Mean body weight and serum cholesterol content of rabbits, surviving the experimental periods, as a function of experimental time. Group 3: ▲, body weight; ●, serum cholesterol (protein mixture/butter oil); △, body weight; ○, serum cholesterol (casein/butter oil).

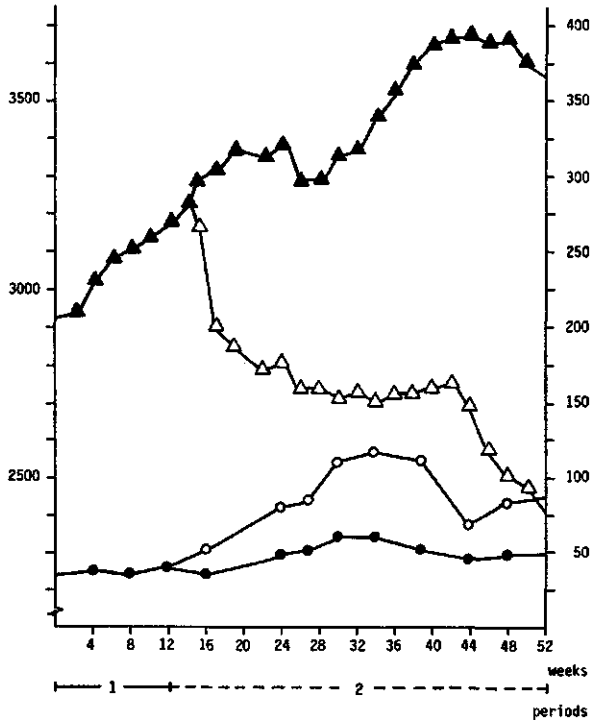

Fig. 12d. Exp. 4. Mean body weight and serum cholesterol content of rabbits, surviving the experimental periods, as a function of experimental time. Group 4: ▲, body weight; ●, serum cholesterol (protein mixture/butter oil supplemented with soya bean oil); △, body weight; ○, serum cholesterol (casein/butter oil supplemented with soya bean oil).

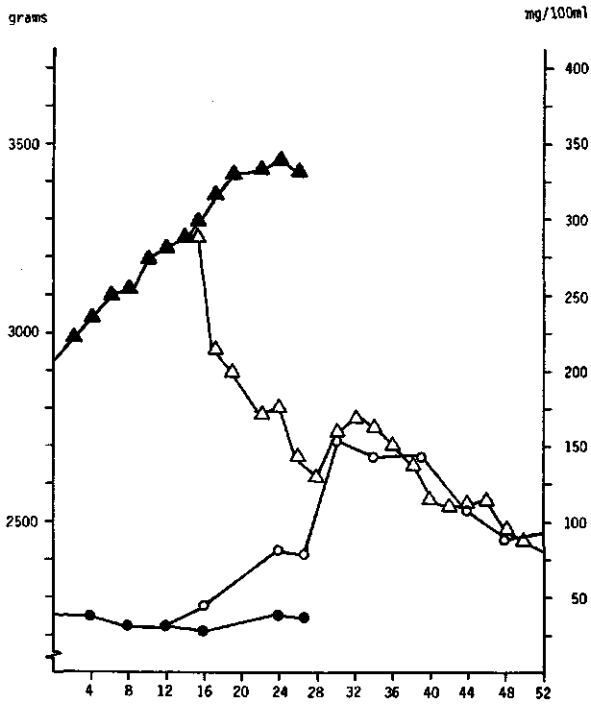

Fig. 12e. Exp. 4. Mean body weight and serum cholesterol content of rabbits, surviving the experimental periods, as a function of experimental time. Group 5: ▲, body weight; ●, serum cholesterol (protein mixture/simulated butter oil without cholesterol); △, body weight; ○, serum cholesterol (casein/simulated butter oil without cholesterol).

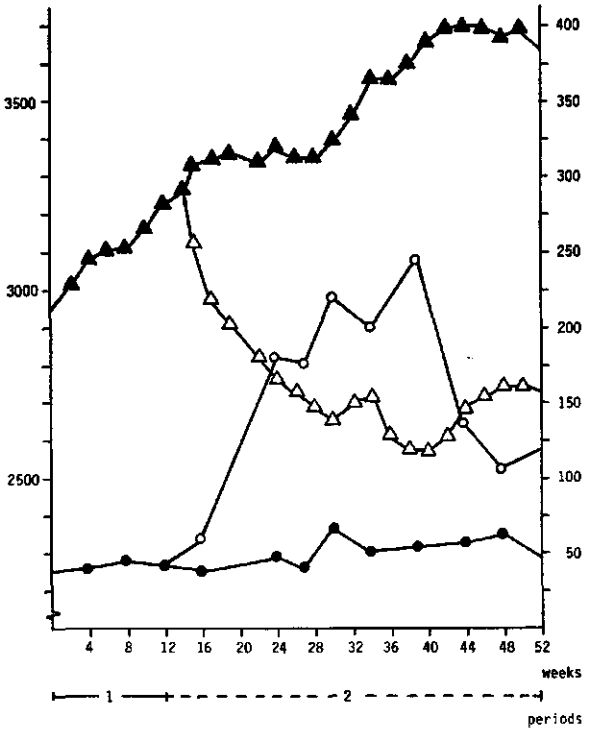

Fig. 12f. Exp. 4. Mean body weight and serum cholesterol content of rabbits, surviving the experimental periods, as a function of experimental time. Group 6: ▲, body weight; ●, serum cholesterol (protein mixture/simulated butter oil with cholesterol); △, body weight; ○, serum cholesterol (casein/simulated butter oil with cholesterol).

were significant ($P < 0.01$) as was the difference between groups 1 and 2 ($P < 0.01$). The other groups did not differ significantly.

The immediate drop in food intake by the animals of the A groups, fed on casein, is directly reflected in their body weight. Almost at the end of Period 2, another analysis of variance was performed. At that moment almost all animals were still alive. The two protein treatments, the five fat treatments and the protein-fat interaction were considered as source of variance in body weight. The difference between the groups fed on the A (casein) diets and the B (protein mixture) diets was highly significant ($P < 0.001$). Also the fat treatments resulted in significant differences ($P < 0.001$). The protein $\times$ fat interaction had no significance (n.s.).

The fat effect could be ascribed to the coconut oil supplementation as the weight change of Group 2 differed significantly from the change in Groups 3 – 6. These last groups did not differ between themselves. The casein and coconut oil effects are therefore additional and should be considered both as causes of the poor performance of rabbits on semi-synthetic diets as reported by Wigand (1959), Funch et al. (1960, 1962) and Moore & Williams (1964a). This also explains the poor results of food intake and body weight, observed in the previous experiments.

10.3.2 Mortality

Mortality was absent during Period 1. This was not unexpected, as in the previous studies mortality did not occur until later in the experiment. After subdivision of the large groups into two subgroups which were fed on different proteins, mortality remained low among the rabbits fed on protein mixture. From the Groups 2B, 4B and 6B each, one rabbit died or had to be killed before the end of the experiment at 45, 48 and 50 weeks, respectively. From the Groups 3B and 5B, not one animal died during the 13 weeks these animals were on the B diet. The pattern in the rabbits fed on casein was quite different. Group 1, which got the same diet throughout the 52 weeks showed a relatively favourable pattern compared with the previous experiments and with the groups which were fed on an A diet. Only 4 out of 20 rabbits died prematurely in this group, at 27, 29, 41 and 46 weeks, respectively. The mortality in the subgroups 2A – 6A was 3, 4, 5, 5 and 6 respectively. These figures are in line with the mortality rate in Experiment 2 of about 45%. Most animals died after about 30 – 35 weeks after body weight was reduced till the lowest levels attained.

The causes of the mortality in this experiment appear to differ considerably from those in the previous experiments. Lung disease was rarely seen. Several rabbits in this experiment, and all those in the B subgroups died or were killed because of paralysis of the back due to a fracture in the vertebral column. Weakness of the bones has been reported repeatedly in this kind of experiment (Kritchevsky, 1964) and is ascribed to the minimal body exercise of the rabbits. This, together with the higher body weight of these crossbred rabbits compared with the Dutch Belt rabbits, may explain the higher frequency of this ailment in this experiment.

The cause of the mortality of the A groups is not clear. It was observed fortuitously

that many rabbits of the A groups showed low haemoglobin values (see below). At the moment of starting Period 2, after 12 weeks on the diet, the Hb values of the rabbits from Group 1 were already somewhat lower than of Groups 3 - 6. Group 2 was intermediate. The iron content of the diets hardly differend. The haematocrit values reflected the Hb values. At 17 weeks the differences were somewhat smaller, but at 34 weeks the Hb levels of the A groups had decreased to 10.5 - 11 g/100 ml, while the B groups were still in the normal range of 13.5 - 14 g/100 ml, the lowest values being found in Group 2B fed on coconut oil. At that moment already 9 rabbits from A groups had died from unknown cause but none of the B groups.

The mortality might be related to the decrease of the Hb content. Mortality was not related to body weight of the individual. At the last bleeding of the rabbits before they died the Hb values indicated very low levels in most of the animals, dying from unknown causes. It seems that rabbits do not tolerate a Hb level below 7 g/100 ml. These animals also had generally moderate to high serum cholesterol levels. It is suggested that in the rabbits that died spontaneously in this experiment, the low Hb level might have contributed to mortality. No increase was observed during this experiment of SGOT, γ-GT, or bilirubine. Total serum protein nor serum albumin content were changed throughout this experiment.

10.3.3 Serum cholesterol and total lipids

The course of the serum cholesterol level during the experiment is shown in Figs 12 a-f. During Period 1 an important increase was noted only in Group 1 fed on coconut oil in a casein diet. Group 2, fed on coconut oil in a mixed protein diet, showed a small, but very consistent increase of the serum cholesterol level. All other groups had very low levels which do not appear to change. Analysis of variance after Period 1 indicated a fat effect on serum cholesterol level ($P < 0.001$). The differences between Group 1 and the other groups, and also between Group 2 and all other groups were significant ($P < 0.01$). If the cumulative average serum cholesterol levels were analysed over the entire period, the same significant differences resulted.

In blood samples from fasting rabbits, total lipids were determined monthly by a colorimetric method (see Section 6.6). The results closely parallel the serum cholesterol values. A high correlation was found between serum total lipids and serum cholesterol, indicating that no extra information is obtained by the total lipid determination. This has also been concluded from Experiment 2 for the triglyceride determinations. At a later stage in this experiment, after cholesterol levels had risen to higher values, a coefficient of correlation was calculated of $r = 0.977$ between cholesterol and total lipids. A characteristic example of this relationship is shown in Fig. 13. The differences found to be significant for cholesterol at 14 weeks were also significant at the same level for total lipids.

The pattern of the serum cholesterol during the second part of this experiment is seriously disturbed by the mortality in some A groups. The B groups show a very consistent pattern throughout the entire 52 weeks for the serum cholesterol and total

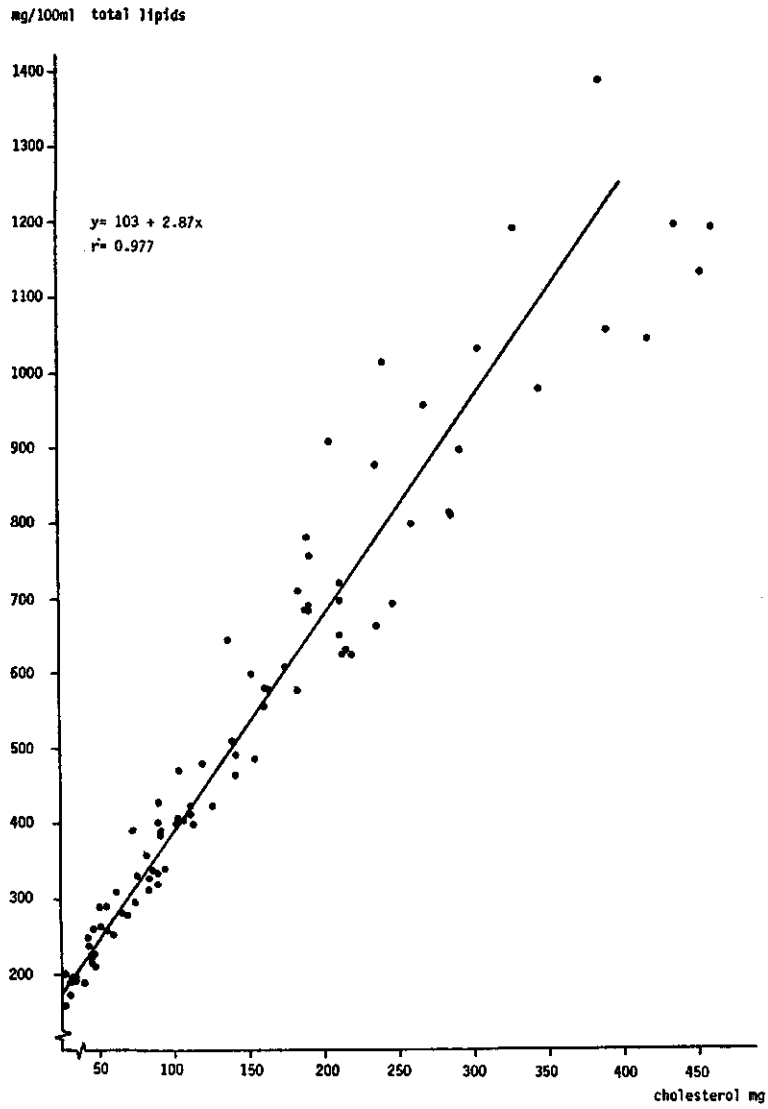

Fig. 13. Exp. 4. Regression analysis of serum total lipids versus serum cholesterol in rabbits (at Week 44) : $y = a + b.x$.

lipids (see Figs 12 a–f). At 27 weeks, by which time Groups 3B and 5B had been removed from the experiment, only the mean serum cholesterol of Group 2B differed significantly from the other B groups ($P < 0.05$). During the rest of the experiment until 52 weeks, 4B and 6B remained constant while 2B increased steadily, though moderately (Fig. 12b). The differences at 52 weeks between Group 2B and Groups 4B and 6B were significant at the 1% level.

The cholesterol curve for Group 1, with a low mortality rate in this experiment, was

about the same as for Group 1 in Experiments 1 and 2 (Fig. 12a). This indicates no great breed difference in sensitivity for cholesterolemia induction between Dutch Belt and these crossbred rabbits.

Immediately after the change-over of the B groups to a casein diet serum cholesterol as well as total lipids started to rise. Both of these increases for the groups fed on butter oil and for those fed on simulated butter plus cholesterol were more rapid than for the coconut oil group. This slower increase of serum cholesterol with coconut oil versus butterfat confirms a similar observation by Moore & Williams (1964a). The simulated butter oil without added cholesterol also shows a slower increase. The difference between the two simulated-butter oil groups was nearly significant at 27 weeks ($P = 0.05$). None of the other differences was significant at that point. At the end of the experiment at 52 weeks, comparison of A groups is misleading because in several groups only hypo-responding rabbits were left and because of the extreme variability within groups. Group 2A showed a great increase during the second part of the experiment, approaching the level of Group 1 which got the same diet as 2A throughout the experiment. Analysis of variance of the cumulative average serum cholesterol values of the A groups showed an almost significant fat effect ($P = 0.05$). None of the differences between the groups reached significance.

From the combined data in this section it is evident that the kind of protein fed to rabbits may influence serum cholesterol levels strongly. It cannot be concluded from this experiment whether proteins in general affect lipid metabolism in rabbits. The observed effect might be due to some non-specific effect of casein as related to acceptability. It seems as if casein affects metabolism of rabbits unfavourably in a general way, leading to reduction of food intake, body weight loss, anaemia and reduced resistance against infection. Concomitantly an increased sensitivity for fats was established. The increase of serum cholesterol on a mixed protein diet, induced by coconut oil, appears more physiological, without side effects. The time required to approach atherogenic levels may be too long, however, to be a useful method.

The decreased sensitivity for fat effects is also clearly illustrated among the B groups, fed on different fats. Only coconut oil induced an increase of serum lipids while the other fats were evidently below the sensitivity threshold. This trend confirms the results obtained with the casein diets where coconut oil induced the highest lipid levels. For technical reasons, it would be worthwhile to find a protein which would promote growth and maintain health without destroying the sensitivity of rabbits for dietary fats as related to blood lipids and atherogenesis.

In this experiment a negative correlation between body weight and serum cholesterol was found ($r = -0.48$). In many other experiments, however, (e.g. Weigensberg et al., 1964; Kobernick et al., 1964) no such relationship was found and it is suggested that both body weight and cholesterol level are influenced by some common mechanism. Liberally induced body weight reduction by food restriction is even reported as a way of lowering cholesterol level (Firstbrook, 1950). It is therefore unlikely that the inverse relationship between cholesterol and body weight observed in our rabbits, is a causal one.

10.3.4 Haemoglobin and anaemia

As already mentioned before in the section on mortality, sometimes very low Hb levels were observed in the rabbits. After this experiment started, Hb determinations have been performed routinely. At the end of the first period of this experiment all values were similar at about 14 g/100 ml. Two weeks after the change-over to the A diets, the levels were still around 14 g/100 ml. Between 17 and 34 weeks on the diets (between Week 2 and 19 after the change-over) the Hb levels decreased significantly in animals fed on the A diets to about 10 – 11 g/100 ml. Of the rabbits fed on the mixed protein only those fed on coconut oil showed slightly reduced Hb levels. The other B groups remained normal.

The pattern of the mean Hb levels was disturbed by the death of rabbits with the lowest Hb content. This means that all A groups levelled at a mean value of about 10 – 11 g/100 ml. The haematocrit values changed similarly, maintaining a constant MCHC between 30 and 35%. This suggests a normochromic anaemia. Thorn-apple formed cells were observed in peripheral blood smears of both anaemic and normal rabbits. The proportion of this kind of cells was, however, increased in anaemic rabbits. This thorn-apple form is characteristic for rabbits.

Reticulocytes are normally found in rabbits as 2 – 3% of erythrocytes. In our anaemic rabbits reticulocytosis was very pronounced indicating accelerated formation of erythrocytes. Together with the finding of increased iron content in liver and spleen (see below), it is suggested that the lifespan of erythrocytes is shortened. It is very tempting to speculate that this observed anaemia syndrome is similar to the anaemia in guinea pigs and rabbits by cholesterol feeding (Okey & Greaves, 1939; Silver et al., 1964; Westerman et al., 1970).

Anaemia by itself is long known to cause lipaemia as well (Bloor, 1921; Horiuchi, 1920; Lewis & Iammarino, 1971). It has been demonstrated that the lipaemia, caused by repeated bleeding, in rats is actually a hypertriglyceridemia (Lewis & Iammarino, 1971). Recently it has been reported for rabbits by Kazi et al. (1972) that anaemia does not influence cholesterol levels while cholesterol feeding induces reduced Hb values. A significant negative correlation was calculated between serum cholesterol over the range of 30 – 1500 mg/100 ml and Hb content. The increased triglyceride level, observed by Lewis & Iammarino (1971) in rats in iron-deficiency anaemia is ascribed to a decrease of serum and tissue lipoprotein lipase. A low serum thyroxine was also previously shown in cholesterol-fed rabbits by Bernick et al. (1962) and Marquié, (1970).

Huang & Kako (1970) suggested that the triglyceridemia observed in cholesterol-fed rabbits is caused by inhibition of lipoprotein lipase by the high free cholesterol content of VLDL. The present observations suggest a similar sequence of events in rabbits fed on semi-synthetic diets. An increase of serum cholesterol leads to anaemia and an increase of lipoprotein-free cholesterol. Perhaps both these mechanisms cause a decrease of lipoprotein lipase action, resulting in increased triglyceride levels. It may be significant that taking all rabbits in this experiment together, a negative corre-

lation between serum cholesterol and Hb was found of $r = -0.48$ ($P < 0.001$). Also a strong negative correlation ($r = -0.77$; $P < 0.01$) was found between the means for the different groups of cholesterol and Hb.

An explanation for the cholesterol-induced anaemia is still lacking. Westerman et al. (1970) concluded from their study in rabbits that both intracorpuscular and extracorpuscular factors were related to a shortened red-cell lifespan. The (free) cholesterol content of the red cells was increased. Osmotic fragility was increased in some and decreased in others. It is suggested that when the relation between red cell and plasma cholesterol is altered, so that red-cell concentrations increase, red-cell survival becomes shortened. Because of this, increased haemolysis tendency would occur. Exchange of free cholesterol between serum lipoproteins and erythrocytes has been demonstrated by Cooper & Jandl (1969).

It has been postulated that the enzyme lecithin: cholesterol acyltransferase (LCAT) plays an important role in the transfer of free cholesterol from peripheral tissues and from lipoprotein residues to the liver for breakdown (Glomset & Norum, 1973; Norum, 1974). In cases of familial LCAT deficiency, also increased cholesterol content of erythrocytes and haemolytic anemia are observed (see: Glomset & Norum, 1973), comparable with the cholesterol induced anaemia in quinea pigs. Lacko et al. (1974a) observed a rectilinear relationship between LCAT activity in human serum and free cholesterol content in the serum between 20 - 100 mg/100 ml, while the fractional esterification rate showed only minor fluctuations. Such a relationship was also established between the free-cholesterol level of man, rat, guinea pig, pig and dog and their rate of cholesterol esterification (Lacko et al., 1974b). The results for rabbits were not clear as the free-cholesterol range in normal rabbits is very narrow.

It has already been reported earlier that LCAT levels of rabbit plasma do not reflect the rise of plasma cholesterol resulting from feeding an atherogenic diet (Hashimoto & Dayton, 1971). The lipid metabolism of rabbits differs also from another aspect of that in man. Its cholesterol esters are largely synthesized by the liver (Rose, 1972) and not by the LCAT mechanism in the blood stream. This might explain the absence of LCAT response to cholesterolemia. Free cholesterol accumulation may be a result of (relative) deficiency of LCAT activity in rabbits. It was concluded that in the next experiment LCAT activity should be determined to elucidate its relationship to serum cholesterol, lipoproteins and Hb.

10.3.5 Liver and spleen iron

To establish whether the anaemia was indeed of haemolytic origin iron contents of liver and spleen were determined. The results for the liver iron content are shown in Table 36. It is evident that those groups with a low mean Hb value also display high iron contents, i.e. Group 1 and the A groups. Group 2B, with intermediate Hb values is also intermediate for iron content between the A and other B groups. The iron contents of the spleen show the same pattern as those of the liver. The A groups have a content of about 600 μg/g, the B groups of about 210 μg/g. A strong negative corre-

Table 36. Liver content of total lipids, total cholesterol (mg/100 g) and iron (mg/kg ± s.e.); Experiment 4.

Groups[1]	Total lipids[2]	Cholesterol[2]	Iron[3]
1 (n = 16)	6649 ± 283	807 ± 93	1127 ± 110
2A (n = 7)	6102 ± 437	699 ± 119	935 ± 113
2B (n = 9)	6872 ± 313	524 ± 92	539 ± 97
3A (n = 6)	9287 ± 597	1451 ± 321	677 ± 86
4A (n = 5)	6762 ± 355	431 ± 98	718 ± 159
4B (n = 9)	8092 ± 357	404 ± 33	237 ± 22
5A (n = 4)	7351 ± 642	528 ± 79	623 ± 45
6A (n = 4)	7906 ± 955	1382 ± 655	567 ± 125
6B (n = 9)	7905 ± 215	502 ± 87	197 ± 12
Controls (n = 7)	7523 ± 497	502 ± 120	430 ± 74

1. For diets see Table 33; controls fed on commercial laboratory chow.
2. mg per 100 g fresh tissue.
3. mg/kg.

lation was present between Hb values and liver iron ($r = -0.83$) or spleen iron ($r = -0.74$). The difference between the Groups 2A and 2B for liver and spleen iron was significant ($P < 0.05$) as between 4A and 4B for liver ($P < 0.01$) and spleen ($P < 0.05$) iron. Between 6A and 6B liver and spleen iron was significantly different ($P < 0.01$). Groups 2B and 4B differed also in liver iron ($P < 0.01$) and spleen iron ($P < 0.05$). These chemical data confirm the histochemical data of Marquié for cholesterol-fed rabbits (1970).

10.3.6 Liver lipids

In previous experiments it was established that phospholipid and free cholesterol content are rather constant. Liver lipid determinations were therefore limited to total lipids and total cholesterol. The results are shown in Table 36. The close similarity between the A and B groups is strikingly related to total lipids. These total lipid levels are also similar to the values reported in Experiment 2. It should be borne in mind that in this experiment values are expressed as mg/100 g and in Experiment 2 as mg/g N. The nitrogen content of fresh rabbit liver was about 3%. The liver cholesterol content was a reflection of the serum cholesterol levels. The A groups again demonstrated generally the highest levels, 2B intermediate and the other B groups very low levels. The low cholesterol content of both 4A and 4B groups demonstrated that supplementation of a butter oil diet with an oil rich in linoleic acid depressed liver cholesterol content in rabbits. This is in agreement with the finding of Funch et al. (1962), who substituted 20% of the butter oil for corn oil. Wigand (1959) observed a decrease of liver cholesterol when linoleic acid was added to trilaurate.

10.3.7 Serum lipids

At the end of the experiment, in a larger blood sample, total lipids, total cholesterol, free and esterified cholesterol were determined. For some groups the samples were pooled. The fatty acid composition of cholesteryl esters and phospholipids was also assessed for some groups. The results are shown in Tables 37 and 38 for serum lipids and cholesteryl ester fatty acids, respectively.

The small difference between Group 1 and 2A for serum total lipids was not significant ($P > 0.1$). Group 1 showed a great variation within the group, perhaps due to the longer duration of hypercholesterolemia in Group 1, leading to hypertriglyceri-

Table 37. Concentration of lipid classes in serum: Experiment 4 (mg/100 ml ± s.e.).

Groups[1]		Lipid class[2]				
		total lipids	cholesterol			
			free	esterified	total	free/total cholesterol x100%
1 cas./coc.	(16)	904 ± 171	96 ± 24	181 ± 30	285 ± 51	31 ± 2
2A cas./coc.	(7)	689 ± 127	84 ± 18	173 ± 26	255 ± 46	31 ± 1
2B PM/coc.	(9)	-	35	89	124	28
3A cas./BO	(6)	359 ± 62	37 ± 7	111 ± 22	139 ± 27	27 ± 1
4A cas./SBBO	(5)	301 ± 41	22 ± 5	63 ± 16	86 ± 17	25 ± 2
4B PM/SBBO	(9)	-	10	38	51	20
6A cas./IBO+C	(4)	-	28 ± 11	96 ± 35	118 ± 41	23 ± 2
6B PM/IBO+C	(9)	-	10	36	47	-

1. For diet codes see Table 33.
2. If no s.e. is shown, analysis was performed on pooled samples; number of animals between brackets.

Table 38. Fatty acid composition of serum cholesteryl esters (weight %); Experiment 4.

Groups[1]	Fatty acid (code)					
	14:0	16:0	18:0	16:1	18:1	18:2
1 cas./coc	7.0	17.6	2.5	9.1	45.2	17.8
2A cas./coc	7.1	17.1	2.4	8.5	46.5	18.4
2B PM/coc	6.1	17.7	2.1	7.8	46.0	21.0
3A cas./BO	1.3	20.4	5.6	6.0	60.5	6.1
4A cas./SBBO	1.2	17.6	4.0	3.5	37.0	35.9
4B PM/SBBO	1.3	18.7	3.7	4.3	35.7	36.3
6A cas./IBO+C	1.3	16.8	6.9	4.8	62.9	7.3
6B PM/IBO+C	1.3	17.2	5.1	5.8	54.1	16.4

1. For diet codes see Table 33.

demia. The level of blood lipids being higher with coconut oil than butter oil confirms the results of Experiment 2 ($P < 0.01$).

The fatty acids of the cholesteryl esters present an interesting pattern. Especially the different pattern between the groups fed on coconut oil or on butter oil deserves further attention. The linoleic acid content in the coconut oil is less than in the butter oil, even though only about 70% of the linoleic acid in butter oil is 18:2 ω 6.9 (unpublished observation). However the cholesteryl esters of rabbits fed on butter oil still show a much lower linoleic acid content than those of rabbits fed on coconut oil ($P < 0.01$).

The cholesteryl ester fatty acids were also determined twice during the experiment, at 30 and 39 weeks. These determinations showed exactly the same pattern. This difference was also present in the phospholipids of serum at a somewhat higher absolute level. An inverse relationship was noted between the cholesteryl oleic and linoleic acid. The difference for oleic acid between groups 2 and 3A was also significant ($P < 0.01$). Supplementation of the butter oil with soya bean oil in the proportion of 4:1 yielded an enormous increase of cholesteryl linoleate and inversely a decrease of cholesteryl oleate. The influence of the two dietary proteins, resulting in decreased serum cholesterol levels, on fatty acid composition was minimal. The PM tends to increase the linoleic acid proportion somewhat, especially in the simulated-butter oil group (6B).

The fatty acids of serum cholesteryl esters are incriminated in atherogenesis by several investigators (see: Section 3.3.2; Gottenbos, 1975). Gottenbos & Thomasson (1961) concluded from their experiments that the best relationship of atherosclerosis with any blood lipid parameter was with cholesteryl oleate. Moore and Williams (1964c) observed a drastic reduction of atherosis if the ratio linoleic: oleic acid of the plasma phospholipids increased. In a later study Moore (1969) demonstrated that at linoleic acid levels of about 40% or more in the serum cholesteryl esters of rabbits, almost no atherosclerosis occurred. Funch et al. (1962) proved that substitution of part of the butter for corn oil inhibited atherosclerosis in rabbits.

It may be calculated from the present and from Moore's (1969) data that the cholesteryl esters of the rabbits of Funch et al. (1962) should have contained about 40% linoleic acid. This might explain the absence of atherosclerosis though absolute serum cholesterol levels were not depressed by the corn oil. The extremely low serum cholesteryl linoleate levels observed in the rabbits on butter oil or simulated butter oil might provide a clue to the somewhat higher atherogenicity of butter oil compared with coconut oil, as discussed in Section 8.4. This lower linoleate and consequently higher oleate content would enhance the atherogenic properties of the cholesteryl esters more than can be expected from total cholesterol determinations alone. This hypothesis of Gottenbos (1975) remains, however, to be proven. The rabbits from the 4A group, receiving a supplement of soya bean oil rich in linoleic acid for most of this study showed a similar cholesterol pattern as the animals fed on butter oil.

Due to premature death of some hyperresponders, the terminal values are lower for 4A than 3A. Figures 12c and d are somewhat misleading in this respect because they

only present the pattern of cholesterol of the surviving animals. If the curve is presented for all animals alive at a certain bleeding point the similarity of the two groups is much more prominent. This is illustrated in Figure 14 from 12 weeks onwards. That linoleic acid supplementation to butter oil causes almost no serum cholesterol reduction in fact confirms that the hypercholesterolemia induced by butter oil is due to its cholesterol content. Cholesterol-induced hypercholesterolemia in rabbits is more difficult to reduce by linoleic acid than saturated fat-induced hypercholesterolemia.

There is a strong tendency for increased ratios free: total cholesterol with increasing serum cholesterol if the group means are compared (Table 37). The difference between Groups 2A and 3A was significant ($P < 0.05$). Also within Group 1 a positive correlation ($r = 0.51$, $P < 0.05$) was present between this ratio and total serum cholesterol. If all rabbits were taken together the correlation was even higher ($r = 0.59$, $P < 0.01$). These results are at variance with the findings reported in Experiment 1. In that experiment the ratio tended to decrease while cholesterol increased.

Several differences between these experiments are present which may explain this discrepancy. Breed differences may be present as controls in this experiment had a ratio of 20 and in Experiment 1: 43. If the data of Wigand (1959) are scrutinized it may be concluded that all his rabbits at the start of the experiment had very different ratios. This indicates great variability of this ratio at low cholesterol levels. In the experiment reported here, no correlation between serum cholesterol and the ratio free: total cholesterol could be found at cholesterol levels below 150 mg/100 ml. In fact a clear increase of the ratio only was observed in the Groups 1 and 2A with mean cholesterol levels of 285 and 255 mg/100 ml, respectively. This may partly explain the difference with Experiment 1. In Experiment 2 identical values for the ratio were found for rabbits fed on coconut oil or butter oil while the serum cholesterol levels were much higher in the former.

If the LCAT enzyme has some function in serum cholesterol esterification in rabbits, it may be hypothesized that in this breed of rabbits LCAT-activity response to increased cholesterol levels is inadequate. This would result in free-cholesterol

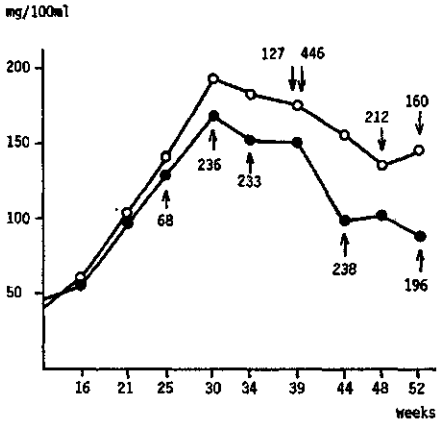

Fig. 14. Exp. 4. Mean serum cholesterol content of all living rabbits of Groups 3A and 4A, as a function of experimental time. Arrows denote death of individual rabbits. Numbers at arrows indicate serum cholesterol content at death. Group 3A: ○, butter oil; Group 4A: ●, butter oil supplemented with soya bean oil.

accumulation. By this hypothesis also the lower Hb values and increased haemolytic anaemia may be explained. Though no Hb values were determined in the first two experiments, visible anemia was never observed as was in this experiment.

10.4 Conclusion of Experiment 4

In Experiment 4 a modified protein source was used in the semi-synthetic diet to reduce the mortality rate. It was successful as hardly any animal died. A serious drawback of this mixed-protein diet was the almost complete absence of cholesterolemic response upon feeding coconut or butter oil. As the experiment was meant to investigate the different effects of butter oil, the cholesterol in butter oil and a linoleic acid supplementation to it, it was decided after 14 weeks on these diets to divide the groups of 20 into subgroups of 10. The subgroups, continuing on the protein mixture would provide an answer as to whether the cholesterol response was delayed. From the groups changed over to casein it could be determined whether this breed of rabbits reacted in the same way as the Dutch Belt rabbits, used in former experiments.

Because of the high ('normal') mortality of the casein-fed rabbits, only limited conclusions can be drawn from these groups. The course of body weight and serum cholesterol, as well as total lipids, for the rabbits fed on the mixed protein showed a consistent pattern throughout the 52 weeks of the experiment. No delayed response of serum lipids could be detected. Only rabbits fed on coconut oil showed a slight but significant increase of serum cholesterol on the mixed-protein diet. Butter oil, which in casein diets normally induces a smaller cholesterol increase, did not elevate serum cholesterol levels at all in rabbits fed on mixed protein. These data confirm the strong hypercholesterolemic action of coconut oil. At the same time they suggest that the response of rabbits to dietary fats on a mized protein diet differs only quantitatively from the results obtained with a casein diet. It seems as if the sensitivity threshold of the rabbits is increased.

Changes in blood lipids seemed to inversely parallel changes in body weight. It has been discussed, however, that forced limiting of food intake has not been reported to increase blood lipids but is without effect or, if anything, reduces serum cholesterol and atherogenicity.

A serious problem and probably the cause of many deaths in the animals fed on casein is formed by anaemia though the rations amply supplied iron. This anaemia has a strikingly similar pattern to the haemolytic anaemia produced by cholesterol feeding of guinea pigs and rabbits or the anaemia found in patients with familial lecithin: cholesterol acyltransferase (LCAT) deficiency. It is probably caused by an increased free cholesterol content of the erythrocytes. It is suggested that the rabbits used in this experiment, had a (relative) deficiency of LCAT activity resulting in depressed free cholesterol removal from peripheral tissues, including erythrocytes, to the liver. The breed of rabbits used in this experiment may be more susceptible to LCAT (relative) deficiency, as in their serum lipids the ratio of free to total cholesterol increased at increasing cholesterol levels. This was not observed in the former experiments with

Dutch Belt rabbits.

The ultimate goal of this experiment, to assess whether butter oil causes a degree of atherosclerosis predictable from the serum cholesterol levels could not be reached, because of the groups were split up, the high mortality within the casein groups and the lack of response of the mixed-protein groups. One finding however may provide a clue to this question. The rabbits fed on butter oil had considerably less linoleic acid and consequently more oleic acid in their cholesteryl esters than, for example, rabbits fed on coconut oil. The simulated butter oil induced a similar pattern. This pattern is considered as conducive to atherogenesis (Section 3.3.2).

11 Effects of feeding different proteins and coconut oil to rabbits in semi-synthetic diets (Experiment 5)

11.1 Objectives

It has been demonstrated in Experiment 4 that the source of dietary protein profoundly influences the performance of rabbits fed on semi-synthetic diets supplemented with coconut or butter oil. A comparison was made of casein and a mixed protein consisting of casein, gelatin, fish protein and Soya bean protein. The amino acid requirements of rabbits have been studied quantitatively only recently (Adamson & Fisher, 1973).

It has been discussed extensively in Section 4.3 that the tentative amino acid requirements for rabbits are somewhat peculiar. Some amino acids specifically seem to depress growth in young rabbits if present slightly above their optimum levels. These very amino acids are present in casein in large amounts. Arginine, on the other hand, was suggested to be required in greater than usual amounts by rabbits.

The concept of amino acid imbalance might explain many of the problems encountered in rabbits fed on casein. The main symptoms of an amino acid imbalance are growth depression due to reduced food intake, a decrease of the limiting amino acid in the blood and fatty degeneration of the liver (Lang, 1974, p. 219). The amino acid requirements of the rabbit resemble those of the chick rather closely. An imbalanced protein may virtually be similar to a low protein content. In chickens a low dietary protein content induces hypercholesterolemia (Yeh & Leveille, 1969, 1972, 1973) due to reduced excretion of cholesterol and bile acids. The effects on cholesterol metabolism may be explained by a reduced turnover of serum cholesterol.

In this experiment several protein mixtures of increasing complexity were compared. Group 1 got casein and served as a control. Group 2 was fed on a mixture of casein and gelatin, which reduces the surplus of lysine and normalizes the extreme ratio of lysine: arginine present in casein. The contents of the other amino acids were, if anything, decreased. To restore the S-containing amino acids partially, fishmeal concentrate was incorporated in the protein of Group 3. The mixture of the four proteins, as supplied in the previous Experiment 4 to the B groups, was given to Group 4 as another control.

The objective of this experiment was to study the effects of four different protein compositions on body weight gain, food intake, serum lipids and lipoproteins, haemoglobin content and lecithin: cholesterol acyl transferase (LCAT) activity in serum. After completion of the experimental period the rabbits were used for a cholesterol turnover study by intravenous injection of 4-^{14}C-cholesterol, and by analysis of the radioactivity die-away curve.

11.2 Experimental details

Thirty-six male White Viennese × Alaska crossbred rabbits were accustomed to the diets as described before (Sections 6.1 - 6.3). They were about six months old at the start of the experimental period. Four similar groups of nine rabbits each were formed by distributing the rabbits over the groups taking into account their serum cholesterol level and body weight. The composition of the diets is shown in Table 39. The amino acid composition is given in Table 40.

During the experimental period food consumption was determined weekly on a group basis and the rabbits were weighed every two weeks. Blood was withdrawn generally every four weeks, for cholesterol, haemoglobin and haematocrit determinations. Several times lipoprotein and serum protein determinations as well as liver function tests, e.g. SGOT, γ-GT and bilirubin were performed. After 58 weeks the experiment was terminated. At that point a full lipid analysis of serum was carried out as well as LCAT measurements.

Table 39. Ingredients and analysed nutrients of diets; Experiment 5 (weight %).

	Dietary group code			
	1 Cas	2 CaGel	3 CaGelFi	4 PM
Corn starch	36.7	36.9	36.9	36.1
Coconut oil[1]	13.8	13.8	13.8	13.8
Casein[2]	20.2	12.0	7.5	6.2
Gelatin[2]		8.0	5.0	4.3
Fish protein concentrate[2]			7.5	6.2
Soyabean protein[2]				4.1
Sawdust[2]	21.5	21.5	21.5	21.5
Vitamin premix[3]	1.2	1.2	1.2	1.2
Mineral premix[4]	1.0	1.0	1.0	1.0
KCl	1.37	1.37	1.37	1.37
NaCl	0.85	0.85	0.85	0.85
Dicalcium phosphate	2.9	2.9	2.9	2.9
$MgCO_3$	0.3	0.3	0.3	0.3
MgO	0.2	0.2	0.2	0.2
Crude protein	18.4	18.3	18.1	17.9
Calcium	0.84	0.83	1.11	1.05
Phosphorus	0.74	0.71	0.85	0.82
Iron (mg/kg)	486	473	525	512
Dry matter	89.4	89.6	89.3	89.7

1. Fatty acid composition, see Table 34.
2. Composition, see Table 33.
3. See Table 2a and b.
4. As in Table 2c; Mg separately added.

Table 40. Amino acid composition of the dietary protein as calculated from amino acid analyses of the protein sources; Experiment 5 (weight %).

	Dietary group code[1]				Tentative requirements[2]
	1 Cas	2 CaGel	3 CaGelFi	4 PM	
Methionine	0.57	0.40	0.43	0.39	
Cystine	0.10	0.06	0.10	0.12	
S-amino acids	0.67	0.46	0.53	0.51	0.60
Lysine	1.45	1.14	1.18	1.16	0.70
Arginine	0.67	1.00	0.97	1.03	1.00
Tryptophan	0.32	0.18	0.21	0.21	0.15
Leucine	2.18	1.20	1.40	1.35	0.90
Isoleucine	1.24	0.75	0.82	0.79	0.70
Histidine	0.68	0.44	0.42	0.41	0.45
Phenylalanine	1.04	0.75	0.75	0.74	0.60
Tyrosine	1.14	0.70	0.65	0.63	
Valine	1.57	1.05	0.98	0.92	0.70
Threonine	0.98	0.66	0.74	0.72	0.50

1. For explanation of codes see Table 39.
2. From Adamson & Fisher (1973).

After termination the rabbits were removed to another laboratory for a cholesterol turnover study in the animals of the Groups 1, 2 and 3 (for methods, see Section 6.6). Five animals from the same breed and stock but about six months younger were added later to serve as normal controls. These rabbits were fed on the normal laboratory pellets throughout the experiment.

11.3 Results and discussion

11.3.1 Food consumption and body weight

Food consumption figures are shown in Table 41. In the same table also body weight at the beginning and end of the experiment for the surviving animals and body weight changes are presented. Body weights of rabbits fed on different diets during the experiment are shown in Fig. 15. It is evident that food intake and body weight changes differ with the different proteins. An increased food intake and a change from body weight loss to weight gain was observed when the casein was replaced by more complex protein sources. The body weights had a normal distribution (Shapiro-Wilk test) while the standard deviations of the body weights within groups did not differ significantly (Bartlett test).

At about 28 weeks an analysis of variance was performed on body weights. A

Table 41. Food consumption (g per rabbit per day), body weight and weight changes of the surviving animals (g ± s.e.); Experiment 5. Number of animals between brackets.

	Diets[1]			
	1 Cas (6)	2 CaGel (8)	3 CaGelFi (9)	4 PM (9)
Body weight:				
at start	2784 ± 69	2853 ± 63	2851 ± 92	2849 ± 54
at end	2355 ± 158	2805 ± 156	3231 ± 143	3119 ± 82
change	−430 ± 117	−23 ± 143	381 ± 63	270 ± 63
Food intake:				
weeks 1 – 30	41.7	48.1	55.0	57.3
weeks 30 – 58	42.0	50.9	56.8	55.6

1. For diet composition see Table 39.

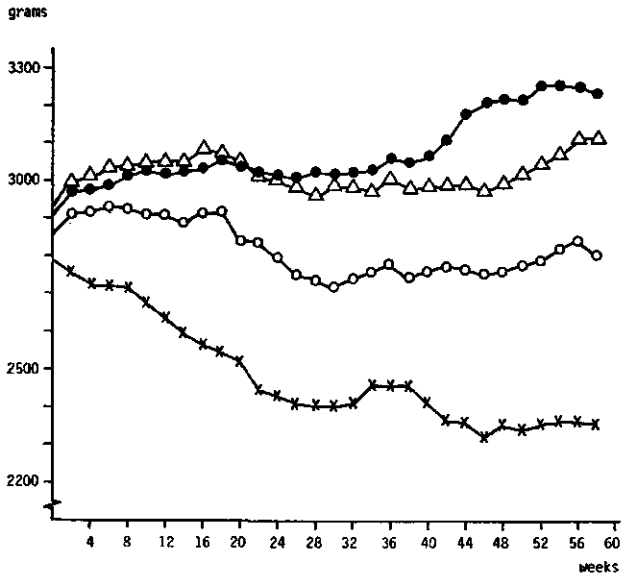

Fig. 15. Exp. 5. Mean body weight of rabbits, surviving the experiment, as a function of experimental time. All rabbits were fed on coconut oil. Group 1: x, casein; Group 2: ○, casein/gelatin; Group 3: ●, casein/gelatin/fish protein; Group 4: △, casein/gelatin/fish protein/soya bean protein.

significant group effect was present ($P < 0.01$). With the Student-Newman-Keuls sequential range test it appeared that the body weights of rabbits from Group 1 were significantly lower than from the other groups ($P < 0.05$ in all three comparisons). The other groups did not differ among themselves. At the end of the experiment again a significant group effect was noted ($P < 0.001$). The sequential range test confirmed again the differences between Group 1 and the other groups ($P < 0.05$) in all three cases. The weights of Group 2 were almost significantly different from those of Groups 3 and 4 ($0.05 < P < 0.1$).

The means of the body weight changes reflect the differences in body weight. The

rabbits fed on casein lost weight, the rabbits fed on casein/gelatin maintained weight while the more complex protein mixtures induced similar weight gains. The data for Groups 1 and 4 confirm the findings in the previous Experiment 4. The differences in body weight change between Group 1 and the other groups were significant ($P < 0.05$). Also the differences in weight change between Group 2 and the other two groups were significant ($P < 0.05$). The difference between mean weight changes of Groups 3 and 4 was not significant.

The food consumption data evidently reflect the body weight changes. There is also a close similarity between food intake during the first and second half of the experiment. A more detailed inspection of the data taught that this pattern of food intake had already been established during the first two weeks on the diets. The food-intake figure for Group 4 in this experiment is comparable with the food intake by Group 2(B) in Experiment 4, i.e. 56 g against 61 g. The same applies for the Groups 1 of this and the previous experiment, 42 against 43 g.

The data for body weight and food intake support the hypothesis that casein is not an optimal protein for rabbits. The cause of this is not yet clear. The absolute amounts of all the amino acids essential for rabbits (Adamson & Fisher, 1973) are higher in casein than in the tentative requirement pattern. An absolute deficiency of amino acids is therefore unlikely. Only arginine is present in relatively lower amounts than tentatively required. In the study of Adamson & Fisher (1973), however, a level of 0.50% reduced growth only slightly as compared with 1%.

That true deficiency of some amino acids is probably not the cause of the poor performance of the rabbits fed on casein is also suggested by the results from the rabbits fed on casein/gelatin mixture. This mixture contained all amino acids, except arginine, in smaller amounts than casein. Still, food intake and body weight are considerably improved by this protein compared with casein. Cheeke (1971) reported detrimental effects on growth if excess lysine (1.63% of the diet) was present in the diet of growing rabbits. An attempt to feed rabbits a diet supplemented with 5.0 g lysine per day failed because the animals refused to eat it (Weigensberg et al., 1964). Adamson & Fisher (1973) observed growth depression when some amino acids, especially lysine, isoleucine, phenylalanine and threonine, were present in slightly larger amounts than required for maximal growth. Because these amino acids were reduced in the casein/gelatin mixture it is suggested that casein per se does not cause an amino acid deficiency as such but rather an amino acid imbalance. The increased arginine and decreased lysine content of the casein/gelatin mixture, resulting in a decreased ratio lysine: arginine, may be responsible for the larger food intake and increased growth of rabbits of Group 2. It is suggested that a lysine-arginine antagonism also exists in rabbits as it is well known in chickens (see Section 4.3).

11.3.2 Mortality

The mortality in this experiment seems to reflect the food intake and growth pattern. Of the group fed on casein three animals died or were killed during the 58

weeks of the experiment, which figure is comparable with the mortality in the previous experiment. One animal had a broken backbone, one showed severely ulcerated feet and a haemoglobin content of 5.5 g/100 ml. These moribund rabbits were killed at 8 and 48 weeks respectively. One animal of Group 1 died spontaneously from unknown cause at 43 weeks. At autopsy severe obstipation was observed. One animal from Group 2 had to be killed because of paralysis of the back, due to a spinal fracture at 54 weeks. None of the rabbits of Groups 3 or 4 died. The low mortality rate of the groups fed on proteins other than casein alone, together with the improved food intake and increased body weights represent important improvements of this model.

11.3.3 Serum cholesterol

The pattern of the serum cholesterol content for the four groups is shown in Fig. 16. Only the data of those animals which survived the experiment are presented. The animal from Group 2 which died prematurely at 54 weeks had a cholesterol content of

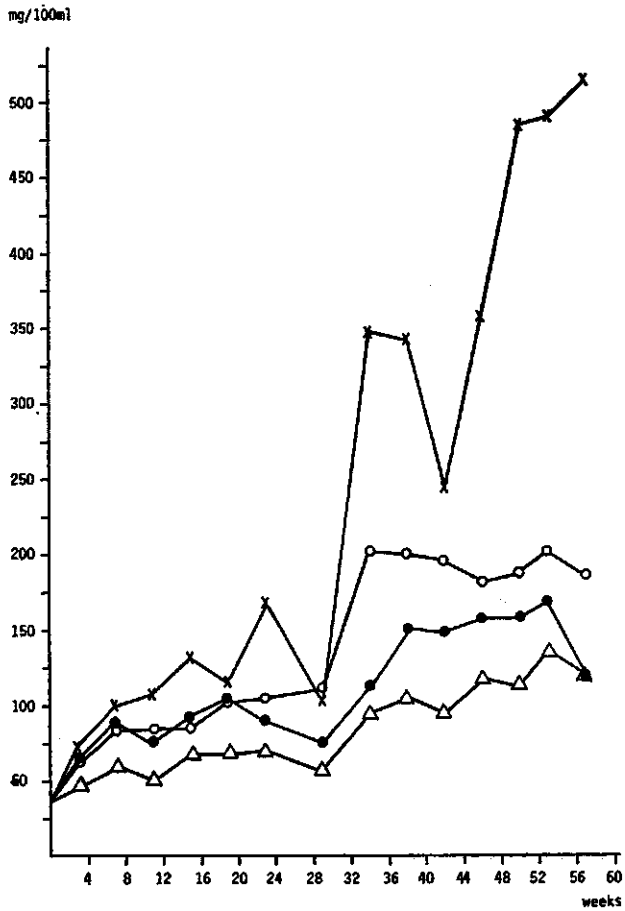

Fig. 16. Exp. 5. Mean serum cholesterol content of rabbits, surviving the experiment, as a function of experimental time. All rabbits were fed on coconut oil. Group 1: x, casein; Group 2: ○, casein/gelatin; Group 3: ●, casein/gelatin/fish protein; Group 4: △, casein/gelatin/fish protein/soya bean protein.

155 mg/100 ml. The animal from Group 1 which died at 48 weeks had a cholesterol content of 401 mg/100 ml at 46 weeks.

It may be easily seen from Fig. 16 that the casein diet induces a sharp increase of serum cholesterol while the protein mixtures stay far behind. The curves are, however, rather capricious due to the great variability between and among individuals. To indicate more precisely the trend for the different diets, more or less independent of intra-individual variations, the cumulative average serum cholesterol content has been calculated for individual rabbits at every bleeding point. From these values the group means were calculated. The results of this procedure are shown in Fig. 17. It is evident that serum cholesterol increases during the experiment from Group 4→3→2→1. The data for serum cholesterol at the start and at the end of the experiment are presented in Table 42. Neither the terminal nor the average cholesterol data have a normal distribution. After logarithmic transformation, however, the hypothesis of normality cannot be rejected (Shapiro-Wilk test).

By analysis of variance on the log-transformed values, it could be concluded that cholesterol at the start was not different between the groups. The same analysis at 58 weeks indicated that significant differences had developed between the four groups ($P < 0.001$). A sequential range test demonstrated that the log final cholesterol level of Group 1 was higher than of the other groups ($P < 0.01$ in all three cases). Analysis of variance on the log average cholesterol values at 58 weeks also yielded a significant group effect ($P < 0.001$). The mean of Group 1 was higher than the mean of the other groups ($P < 0.01$, in all three cases). The difference between Group 2 and 4 also was significant ($P < 0.05$), while that between 3 and 4 was of borderline significance ($0.05 < P < 0.1$).

In the previous experiment a significant negative correlation was noted between body weight and serum cholesterol. It was however concluded that this correlation may very well be caused by coincidence. In this experiment the non-parametric

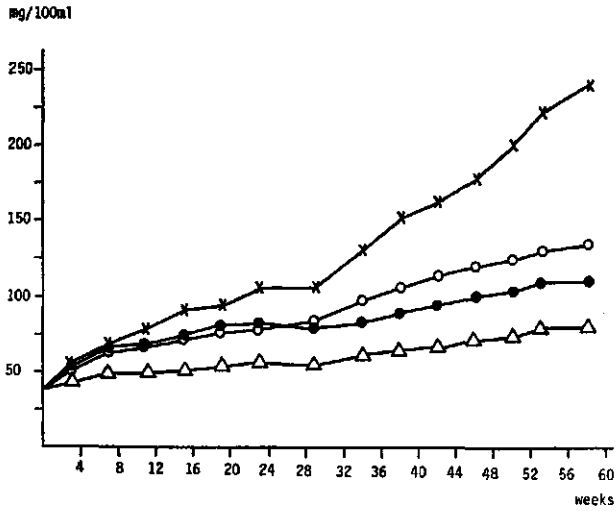

Fig. 17. Exp. 5. Means of the average serum cholesterol content of rabbits, surviving the experiment, as a function of experimental time. All rabbits were fed on coconut oil. Group 1: x, casein; Group 2: ○, casein/gelatin; Group 3: ●, casein/gelatin/fish protein; Group 4: △, casein/gelatin/fish protein/soya bean protein.

Table 42. Initial and final serum cholesterol level and cumulative average serum cholesterol level of surviving rabbits; Experiment 5 (mg/100 ml ± s.e.). Number of animals between brackets.

	Diets[1]				Significance of difference[2]
	1 Cas (6)	2 CaGel (8)	3 CaGelFi (9)	4 PM (9)	
Initial (Week 0)	36 ± 4	36 ± 3	41 ± 5	36 ± 3	1 2 3 4
Final (Week 58)	514 ± 118	186 ± 59	120 ± 11	120 ± 16	
Cumulative average	241 ± 45	135 ± 27	111 ± 8	83 ± 6	
Log final	2.6470 ± 0.1007	2.1646 ± 0.1060	2.0663 ± 0.0415	2.1584 ± 0.1526	1 2 3 4
Log average	2.4106 ± 0.0630	2.0858 ± 0.0707	1.9313 ± 0.1165	1.9107 ± 0.0339	1 2[3] 3 4[3]

1. For composition of diets see Table 39.
2. Differences are significant ($P < 0.01$), except when the diet numbers share a common underlining.
3. The difference of log average cholesterol between Groups 2 and 4 was significant at $P < 0.05$.

Spaerman rank correlation test was applied to investigate the relationship between weight loss or gain and average serum cholesterol. The results are very intricate. Within the group fed on casein a correlation coefficient of $r_s = 0.54$ ($P > 0.1$) was found. Within the group fed on casein/gelatin a significant negative correlation appeared to be present ($r_s = -0.79$; $0.02 < P < 0.05$). For the other two groups a small positive and negative correlation was observed ($r_s = 0.22$; $r_s = -0.10$). These data do not support the suggestion that there is a significant relationship between serum cholesterol and body weight changes in these rabbits.

11.3.4 Haemoglobin

In Section 10.3.4 the possible relationship between serum cholesterol and blood haemoglobin levels has been discussed extensively. On account of this supposed relationship, haemoglobin and haematocrit levels were determined regularly in this experiment from Week 7 onwards. At Week 7, however, decreased levels were already established, which remained rather constant during the rest of the experiment. At that moment serum cholesterol levels had increased only moderately. In Table 43 some data about haemoglobin and other haematologic parameters are summarized. At 58 weeks significant differences of Hb between groups existed ($P < 0.05$). However, only the difference of Hb between Groups 1 and 4 approached the 5% level. If the average Hb concentration of individual rabbits over the experimental period was used, a significant group effect was again present ($P < 0.01$). In this case the differences between Group 4 and both 1 and 2 were significant ($P < 0.05$). Also the difference between Groups 3 nd 1 was significant ($P < 0.05$).

The pattern of the Hc values resembled closely that of the Hb concentrations. This

Table 43. Haematologic findings in rabbits fed on coconut oil with different proteins; Experiment 5 (± s.e.).

	Diets[1]				
	1 Cas	2 CaGel	3 CaGelFi	4 PM	controls
Haemoglobin (g/100 ml)					
at 7 weeks	11.0 ± 0.3	11.5 ± 0.3	12.7 ± 0.3	13.0 ± 0.3	
at 58 weeks	10.3 ± 1.0	11.0 ± 0.8	12.4 ± 0.4	12.8 ± 0.4	13.5 ± 0.2
Haematocrit (%)					
at 58 weeks	34 ± 3	36 ± 2	38 ± 1	39 ± 1	43 ± 1
RBC[2] ($\times 10^6$/mm^3)	4.5 ± 0.2	.	5.9 ± 0.1	.	6.1 ± 0.1
MCHC[2] (%)	32.8 ± 2.6	.	32.0 ± 0.4	.	30.9 ± 1.3
MCV[2] (μm^3)	76.6 ± 2.0	.	67.8 ± 1.4	.	71.8 ± 1.3

1. For diet composition see Table 39.
2. RBC: red blood-cell count; MCHC: mean corpuscular haemoglobin concentration (Hb/Hc); MCV: mean corpuscular volume (Hc/RBC).

was also reflected in the almost constant MCHC ratio. The relationship between Hb and Hc values was calculated for the four dietary groups separately. The coefficients of correlation were significant within all four groups (1: $r = 0.95$; 2: $r = 0.97$; 3: $r = 0.87$; 4: $r = 0.97$; $P < 0.01$ in all cases). The calculated regression equations were hardly different.

The data confirm the suggestion made in the discussion of results of the previous experiment that the anaemia encountered in this model is almost entirely due to a reduced number of circulating erythrocytes with a normal or slightly greater size and a normal haemoglobin content. Microscopic examination of blood smears revealed again reticulocytosis, especially in the low-haemoglobin rabbits. Large numbers of reticulocytes have also been observed in cholesterol-fed anaemic rabbits (Hestorff et al., 1965; Westerman et al., 1970).

In Section 10.3.4 it has been hypothesized that the low Hb values might be related to elevated serum cholesterol levels. If all animals were taken together, a significant negative correlation between Hb and serum cholesterol could be demonstrated. In this experiment Hb measurements started seven weeks after beginning of the experiment. At that moment the group means for Hb had already reached the level which was maintained throughout the remainder of the experimental period with minor fluctuations. Serum cholesterol had increased only moderately, from 36 at the start till 100 mg/100 ml at Week 7 in Group 1 and 84 mg in Group 2. The greatest increase of serum cholesterol occurred in the second half of the experiment, see Fig. 16, but nevertheless mean Hb content remained constant. This would imply that, if serum cholesterol is involved in the development of this anaemia, especially the lower increments of this lipid class are responsible.

If the Hb and cholesterol values are plotted in a scatter diagram there is no linear relationship. As a test for correlation, therefore, the Spearman rank correlation coefficient was calculated per group between serum cholesterol and Hb. A negative correlation was found for Groups 2, 3 and 4 which was only significant for Group 4 ($r_s = -0.75$; $P < 0.05$). The correlation found for Group 1 was slightly positive due to one animal which had paired a high cholesterol level to a high Hb content throughout the experiment. If this animal was omitted, the correlation coefficient changed from $r_s = +0.26$ to -0.30 (n.s.). The same results were obtained if instead of the total cholesterol of serum the free cholesterol content was used as criterion.

The small number of animals per group in this experiment makes it difficult to draw a final conclusion about the origin of the anaemia. The very early onset of the decrease of Hb values, even before serum cholesterol had increased above 100 mg/100 ml, and the constant level of Hb after this early onset in most animals suggest that this general decrease has a similar origin as the first phase of the cholesterol-induced anaemia in rabbits. Westerman et al. (1970) demonstrated that the first phase of the anaemia in rabbits, induced by cholesterol feeding, was dilutional in origin. The plasma volume increased twofold while the red cell mass remained constant in this phase. The cholesterol content of the erythrocytes did not increase during this phase which

lasted about five weeks. During the second phase, haematocrit decreased from 30-36% till 20 – 30%, the erythrocyte cholesterol content almost doubled and the anaemia became haemolytic in origin.

It may very well be that the anaemia initially observed at seven weeks was also dilutional in origin. Because these rabbits generally had only moderately elevated serum cholesterol levels, the second phase was probably not reached except in some incidental cases. Unfortunately, blood volume was not determined in this experiment. Great individual variability was also reported by Westerman et al. (1970) as they found some animals displaying a decreased and others an increased haemolysis tendency upon cholesterol feeding. This may explain the poor correlation, mentioned above, between cholesterol and Hb in these rabbits.

11.3.5 Serum lipids at termination

After 58 weeks of experiment serum lipids were completely analyzed. The results are shown in Table 44. The mean values for Group 2 were obscured by the presence of one hyperresponding animal. If the results of this animal were omitted, the means for Groups 2, 3 and 4 would have been similar and not significantly different. The mean total lipids, total sterol, and triglycerides for Group 1 were all significantly higher than for Groups 3 and 4 ($P < 0.01$). The ratio free: total cholesterol was increased in the rabbits of Group 1 and 2 as compared with the Groups 3 and 4 ($P < 0.05$). The overall differences in ratio phospholipid: cholesterol were not significant ($P > 0.05$). Analysis of variance on the phospholipid: free cholesterol ratio yielded a small group effect ($P < 0.1$; $H = 6.57$, Kruskal-Wallis test). A significant difference was present between Groups 1 and 3 ($P < 0.05$). The finding of Huang & Kako (1970)

Table 44. Concentration of lipid classes in serum (mg/100 ml ± s.e.) and some ratios (× 100); Experiment 5. Number of animals between brackets.

	Diets[1]				
	1 Cas (6)	2 CaGel (8)	3 CaGelFi (9)	4 PM (9)	controls chow (5)
Total lipids	1458 ± 354	624 ± 247	389 ± 26	345 ± 42	199 ± 27
Triglycerides	276 ± 66	165 ± 116	47 ± 8	50 ± 10	63 ± 10
Phospholipids	388 ± 137	149 ± 38	109 ± 8	104 ± 10	58 ± 7
Total cholesterol	599 ± 152	214 ± 72	144 ± 16	120 ± 18	47 ± 7
Free cholesterol	291 ± 122	95 ± 34	50 ± 5	46 ± 8	20 ± 3
Esterified cholesterol	309 ± 45	119 ± 37	94 ± 11	74 ± 11	26 ± 5
Free/total cholesterol	43 ± 5	43 ± 2	35 ± 1	38 ± 1	44 ± 3
Phospholipids/total cholesterol	72 ± 6	80 ± 7	89 ± 11	96 ± 12	201 ± 19
Phospholipids/free cholesterol	146 ± 9	198 ± 23	239 ± 35	243 ± 32	302 ± 16
Phospholipids/esterified cholesterol	121 ± 29	145 ± 17	132 ± 16	166 ± 24	242 ± 35

1. For composition of diets see Table 39.

that triglycerides were positively correlated with free cholesterol could be confirmed with the combined data of Groups 1 and 2 ($r = 0.56$; $P < 0.05$).

11.4 Serum lipoproteins

At several points during Experiment 5 and at termination lipoproteins were analysed in detail. At 50 weeks and after termination of the experimental period, before the turnover study started, a lipid analysis of the lipoprotein fractions was made. The lipoprotein fractions were obtained by ultracentrifugation of pooled serum samples. An example of the results obtained by polyacrylamide gel electrophoresis is presented in Table 45.

The rabbits from Group 1 had significantly more β and pre-β lipoproteins than those of the other groups ($P < 0.01$). The differences between the other groups were small and not significant. The amount of α lipoproteins appears rather constant, even if total lipids increase several fold. It may be concluded from the data shown in Table 45 that the increase of serum lipids in the experimental groups is entirely limited to the (V)LDL, with primarily an increase of β lipoproteins and secondarily of pre-β lipoproteins.

It has been suggested that β lipoproteins originate from pre-β lipoproteins as a result of lipolysis and apoprotein transfer (Bilheimer et al., 1972; Eisenberg et al., 1972). We therefore compared the lipoprotein pattern in the fasting and fed state of animals with a constant serum cholesterol level. This might reveal whether also in rabbits a reciprocal relationship exists between β lipoproteins (LDL) and pre-β (VLDL) lipoproteins (Lees & Wilson, 1970). The result of this comparison is shown in Table 46. In the same table also the lipoprotein patterns of fasted animals are shown which showed over a period of eight weeks an increase, a decrease or no change in serum cholesterol.

The α lipoproteins show minor changes upon fasting. The greatest difference between the fed and fasting state is the decrease of the proportion of pre-β lipoproteins and the increase of β lipoproteins ($P < 0.01$ and $P < 0.05$, respectively).

Table 45. Lipoprotein pattern in serum from fasting rabbits, (mg/100 ml ± s.e.); Experiment 5, 58 weeks. Number of animals between brackets.

Groups[1]		Lipoproteins			Total lipids	Total cholesterol
		α	β	pre-β		
7 (Cas)	(5)	154 ± 37	588 ± 114	387 ± 87	1130 ± 161	384 ± 61
8 (CaGel)	(7)	168 ± 25	161 ± 40	55 ± 13	384 ± 69	132 ± 23
9 (CaGelFi)	(9)	219 ± 18	136 ± 23	35 ± 9	389 ± 59	120 ± 11
10 (PM)	(8)	158 ± 21	124 ± 21	29 ± 21	311 ± 28	98 ± 9
Controls	(5)	150 ± 22	36 ± 15	13 ± 4	199 ± 27	31 ± 6

1. For diet composition see Table 39.

Table 46. Distribution of serum lipoprotein fractions in rabbits (%) as determined by scanning after gel electrophoresis; Experiment 5. Influence of fasting and changes in fasting serum cholesterol concentration. Number of animals between brackets.

	Lipoproteins			Total serum cholesterol (mg/100 ml)
	α	β	pre-β	
Fasting:				
fed (10)	39	24	38	207
fasting (10)	44	39	17	202
Cholesterol constant:				
before (5)	50	41	9	129
after 8 weeks (5)	47	40	13	134
Cholesterol increase:				
before (5)	40	37	22	220
after 8 weeks (5)	18	53	30	367
Cholesterol decrease:				
before (5)	36	47	17	266
after 8 weeks (5)	52	37	10	153

If serum cholesterol content of fasting rabbits remained constant over a period of two months, the lipoprotein pattern also did not change much. An increase of serum cholesterol, however, inevitably led to a relative decrease of α and an increase of β and pre-β lipoproteins, and vice versa.

The results presented in Table 45 and 46 together are clear evidence that rabbits made hypercholesterolemic react by increasing their β and pre-β lipoproteins. Their α lipoprotein content remained constant. The findings in Table 46 support the theory that LDL are derived from VLDL. The high VLDL or pre-β lipoprotein levels found in Group-1 samples may be the consequence of ineffective removal of VLDL.

While electrophoresis was performed, it was noted that mobility of β lipoproteins was decreased as compared with α lipoproteins in some samples. As a consequence the separation between β and pre-β lipoproteins was worsened. If the distance run by the β lipoproteins is expressed as a percentage of that of the α lipoproteins a measure for relative mobility is obtained. This measure was for Group 1: 13.4%, for Group 4: 18.6%, and for the other groups in between. Human β lipoproteins from normal persons had a mobility of 18.9%. The difference between Groups 1 and 4 was highly significant ($P < 0.01$; Q-test, Wilcoxon). This different mobility suggests a divergent composition. Also in the ultracentrifuge the behaviour of the β-lipoproteins was different in the sample from Group 1. Normally the LDL remain in the infranatant together with the HDL after a run of 16 h at 100,000 *g* (ρ 1.006). The LDL of Group 1, however, were floating on top of this infranatant, indicating a lower density.

The detailed analysis of the lipoprotein fractions is presented in Table 47. The lipoproteins were obtained from fasting serum at 50 weeks by ultracentrifugation. The

Table 47. Lipid composition (% of total lipids) as determined by ultracentrifugation of serum lipoproteins of rabbits; Experiment 5 at 50 weeks.

	Chylomicrons			VLDL			LDL			HDL			Total lipids		
	1[1]	3	C	1	3	C	1	3	C	1	3	C	1	3	C
Triglycerides	17	25	40	23	18	48	7	9	37	6	4	16	11	8	25
Phospholipids	15	17	14	23	19	20	39	31	31	48	49	41	34	38	35
Total cholesterol	46	31	16	35	39	14	40	41	16	26	29	15	32	34	15
Free (non-esterified) cholesterol	15	8	10	12	11	6	21	13	5	7	8	3	17	9	4
Sterol esters, incl. fatty acids	51	38	10	39	47	14	32	46	18	31	35	20	35	42	18
Non-esterified fatty acids	2	12	26	3	5	13	1	1	8	8	4	20	3	3	18

1. 1: casein; 3: casein/gelatin/fish protein; C: controls: chow.

Table 48. Distribution of total lipids (%) over the serum lipoprotein fractions as determined by ultracentrifugation; Experiment 5 at 50 weeks.

	Diets[1]				
	1 Cas	2 CaGel	3 CaGelFi	4 PM	controls chow
Total lipids (mg/100 ml)	1537	596	526	493	194
Chylomicrons (%)	2	2	1	2	5
VLDL (%)	32	18	15	18	18
LDL (%)	53	49	44	44	10
HDL (%)	13	31	40	36	67

1. For diet composition see Table 39.

lipids were extracted, separated by thin-layer chromatography and quantitatively determined by densitometry. Only minor differences were present between Groups 2, 3 and 4. Therefore, only the results for the pooled samples of Group 1 and 3 and of the controls are presented.

The results show clearly the high content of cholesterol and the relatively low content of triglycerides in the experimental rabbits' lipoproteins as compared with controls. Also chylomicrons contained large amounts of cholesterol, especially those of Group 1. The high ratio free: total sterol in the LDL and less so in the VLDL of serum from Group 1 is remarkable.

Table 48 presents the distribution of total serum lipids over the lipoprotein fractions. These results, obtained by ultracentrifugation compare favourably with the results presented in Tables 45 and 46 obtained by electrophoresis. A high serum cholesterol content is accompanied by an increased proportion of LDL and VLDL by both techniques and a relatively low proportion of HDL. The HDL content, however, seems to remain constant. The lower electrophoretic mobility of the β lipoproteins of Group 1 was expressed by the lower density in the ultracentrifuge. This indicates a probably decreased ratio protein: lipid in this lipoprotein fraction.

From the data reported by Barr (1953), Lemmon et al. (1954) and Camejo et al. (1973), it may be deduced that α lipoproteins (HDL) are the main carriers of serum lipids in normocholesterolemic rabbits. Almost 60% of the serum cholesterol (range 30 - 70) is present in the HDL. The changes which occur in the lipoprotein pattern and composition upon cholesterol feeding have been described by several investigators. Gofman et al. (1950) demonstrated an increase of LDL (S_f 5 - 8) when serum cholesterol increased moderately. If the cholesterol content surpassed 200 - 250 mg/100 ml, 'giant molecules' developed with a S_f value of 10 - 30. These findings were confirmed by Lemmon et al. (1954), again on a cholesterol-enriched diet, and by Garlick & Courtice (1962). These last investigators reported also a constant HDL level and an increase of VLDL ($\rho < 1.019$).

Recently, Camejo et al. (1973, 1974) reported the results of their studies with cholesterol-fed rabbits. The main conclusions were: increase of VLDL ($\rho < 1.019$), increase of liver cholesterol content, incorporation of ^{14}C-leucine in liver VLDL increased. The VLDL apoproteins of hypercholesterolemic rabbits resembled more closely the LDL than VLDL of control rabbits. The VLDL of hypercholesterolemic rabbits showed β mobility on paper electrophoresis. These results suggest that VLDL of these rabbits are actually LDL with an increased ratio lipid: protein. By gel filtration they could separate the VLDL into two sub-classes VLDL-1 ($S_f > 300$) and VLDL-2 (S_f 20 – 300). Both fractions contain much esterified cholesterol and little triglycerides, 60 and 8%, respectively. The protein and phospholipid content of VLDL-2 was double that of VLDL-1.

If cholesterol was omitted from the diet, the VLDL-1 disappeared. Camejo et al. (1974) suggested that VLDL-1 are a transient form. In fact, Fraser & Courtice (1970) had concluded from their experiments that part of the VLDL of hypercholesterolemic rabbits consists of cholesterol-rich 'remnants' of chylomicrons. These remnants might be cleared from the bloodstream very rapidly by the liver parenchyme and by cells of the reticulo- endothelial system (RES). Zilversmit et al. (1967) have demonstrated that lymph chylomicrons and VLDL of cholesterol-fed rabbits contain much more cholesterol than those of dogs and rats.

The importance of how cholesterol is absorbed was stressed by Fraser et al. (1972). They showed with rhesus monkeys that the kind of fat affects cholesterol esterification in the intestinal mucosa. With corn oil feeding a larger proportion of cholesterol in the lymph was present in the esterified form as compared with coconut oil. This may be of significance as free cholesterol may exchange with other lipoproteins and remain in the blood while esterified cholesterol is rapidly cleared from the bloodstream by the liver as chylomicron remnants. Similar experiments with rabbits (Fraser, 1974) yielded that lymph cholesterol content was dependent on dietary cholesterol but not on the amount or kind of dietary fat. If, however, the amount of dietary cholesterol was kept constant and dietary fat was increased, the proportion of free cholesterol increased also. The increase of free cholesterol as compared with esterified cholesterol was greater if a saturated fat like coconut oil was given rather than corn oil.

Free cholesterol, from the surface layer of chylomicrons, is exchangeable for other smaller lipoproteins and tissues, while the steryl esters in the core of the chylomicrons are probably more rapidly removed from the blood by the liver after they have been changed to remnants rich in cholesteryl ester (Quarfordt & Goodman, 1966). These results have lead to the hypothesis (Fraser et al., 1972; Fraser, 1974) that corn oil chylomicrons, with a greater proportion of cholesteryl esters than coconut oil chylomicrons, have a less elevating effect on serum cholesterol because of a more rapid removal. Moreover, it has been shown by tissue culture experiments that free cholesterol ehters cells more readily than steryl esters (Rothblatt & Kritchevsky, 1968).

Ockner et al. (1969) had demonstrated that in rats the kind of dietary fat influences

the partition of dietary cholesterol over lymph chylomicrons and VLDL. Unsaturated fats induce incorporation of cholesterol in chylomicrons more than do saturated fats. This may be important as the eventual metabolism of ingested lipid may be influenced by particle size. It has been demonstrated for fatty acids of triglycerides (French & Morris, 1957) and of cholesteryl esters (Quarfordt & Goodman, 1966) that removal rate from the circulation varies with the size of the lipoproteins of wchich they are components. The larger the particle size, the more rapid is their rate of removal.

No detailed information is available in the literature regarding the lipoprotein partition and composition of rabbits fed on semi-synthetic diets. The results reported here show the remarkable similarity with the results reported for hypercholesterolemia induced by dietary cholesterol. In both cases the hyperlipoproteinemia is characterized by an increase of both LDL and VLDL. The VLDL contain in both conditions a higher proportion of cholesterol and a lower proportion of triglycerides than controls fed on laboratory chow. All other lipoproteins of the rabbits fed on semi-synthetic diets contained also larger amounts of cholesterol than those of the controls.

The rabbits of Group 1 fed on casein plus coconut oil, had the highest serum lipid levels. They resembled the cholesterol-fed rabbits most closely. In the chylomicrons and LDL of this group of rabbits also an increased free cholesterol content was present. If it can be confirmed that free cholesterol inhibits lipoprotein lipase activity (Huang & Kako, 1970), then disturbed lipoprotein catabolism should be the consequence. According to Hazzard et al. (1970) the blockade of lipoprotein lipolysis at the S_f 20 – 60 level entails a prolonged circulation of the remaining VLDL.

Zilversmit (1973) has recently proposed a theory that intermediary products of the catabolism of VLDL and chylomicrons, which are cholesterol enriched, might play an important role in the atherosclerotic process. This would especially pertain to patients of Type III according to the classification of Fredrickson et al. (1967). These patients are known to be predisposed to atherosclerosis. In the lipoproteins of these patients, cholesterol-rich VLDL are reported to be present (Hazzard et al., 1970; Gent, 1972). The composition of these VLDL closely resembled the VLDL composition in the rabbits (see Table 47).

Other similarities between type III lipoproteins and the rabbit lipoproteins are that on paper electrophoresis the pre-β lipoproteins had β mobility, while on polyacrylamide gel electrophoresis the β lipoproteins showed reduced mobility. The extreme variability of cholesterol and triglyceride concentrations is a diagnostic feature of Type III patients and in distinct contrast to the steady elevations in Type II (Fredrickson et al., 1967). This forms another point of agreement with the hyperlipemia observed in the rabbits.

The results discussed above and the remarkable agreement with type III lipoproteins suggest that the known sensitivity of rabbits for atherosclerosis may be related to their peculiar lipoprotein composition. The difference in lipoprotein composition between serum samples from rabbits fed on different proteins was generally much less than between samples from rabbits fed on semi-synthetic diets or on laboratory chow diet.

11.5 Serum lecithin: cholesterol acyltransferase activity

11.5.1 The LCAT reaction and its measurement

Cholesterol occurs in the living organism in the free and esterified form. The cholesteryl esters are more apolar and therefore not suited as membrane lipid. They have a tendency to form intracellular droplets and are found in the interior of lipoproteins. The free cholesterol is either part of a lipoprotein or a biomembrane (see: Norum, 1974). Three systems for cholesterol esterification are known, the acyl-CoA: cholesterol acyltransferase (ACAT; EC 2.3.1.26), the cholesteryl ester hydrolase (CE-ase; EC 3.1.1.13) and the lecithin: cholesterol acyltransferase (LCAT; EC 2.3.1.43). The functions and properties of this last reaction have been studied extensively during recent years. Several revieuws on the reaction have been publisehd (Glomset, 1972; Glomset & Norum, 1973; Gjone & Norum, 1974).

LCAT activity is mainly present in the bloodstream and lymph while ACAT and CE-ase act mainly in tissues as liver, adrenals, and intestinal mucosa. The LCAT reaction provides in many animal species an important part of cholesteryl esters in serum (Stokke, 1974; Lacko et al., 1974b). In rabbits, however, the physiological role is not yet fully understood. Rose (1972) found that in cholesterol-fed rabbits, the liver was the major source of blood cholesteryl esters. The data of Rose (1972), however, do not exclude the possibly important contribution of intestinal mucosal lipoproteins to serum cholesteryl esters (see Section 11.4).

Glomset proposed that LCAT plays a role in the transport of cholesterol from peripheral tissues (see Glomset, 1972) to the liver. Schumaker & Adams (1969), suggested that LCAT helped to maintain the ratio surface: volume of VLDL. By either mechanism LCAT activity may influence the development of atherosclerosis. No clear relationship has been demonstrated at this moment between LCAT activity and susceptibility to atherosclerosis for different animal species (Lacko et al., 1974b; Stokke, 1974).

A close relationship between serum free cholesterol content of several animal species and their LCAT activity could be observed (Stokke, 1974). Within a species, individuals with higher free cholesterol content also generally exhibit a greater LCAT activity (Lacko et al., 1974a, b). The situation for rabbits is not clear in this respect. Lacko et al. (1974b) could not find a relationship between free cholesterol content and LCAT activity in normal rabbits. Their range of free cholesterol, however, was very small and this may obscure any possible relationship.

Hashimoto & Dayton (1971) could not detect an increase of serum cholesterol esterifying activity as a result of feeding atherogenic diets to rabbits. Wells & Rongone (1969) observed an increase upon feeding cholesterol. Rose (1971) and Stefanovich (1969) reported a reduction of LCAT activity when feeding rabbits diets containing cholesterol. The results of Stefanovich (1969) were presented as percentage esterification, without taking into consideration the great increase of serum cholesterol which must have occurred upon feeding 1% cholesterol. In part differences in experimental

conditions or breed or strain of rabbits may underly these divergent results.

Recently, a method has been developed (Stokke & Norum, 1971) which allows the measurement of LCAT activity during one hour incubation of the serum sample with labelled free cholesterol added. Before this incubation the added tracer is allowed to equilibrate with the cold cholesterol from the serum while the LCAT enzyme is temporarily inactivated. Radioactivity which appears in the cholesteryl esters can be counted and esterification can be measured on a percentage or mole basis. By this method, LCAT activity is not only determined by amount or activity of enzyme but also by the amount and kind of substrate, i.e. free cholesterol and lipoproteins. To study LCAT activity per se it is suggested to add a small proportion of the serum samples to a pooled, heat-inactivated, substrate serum. Heat inactivation may however modify the physical properties of lipoproteins and such measured activities are almost always lower than in autologous serum. It was advised to use homologous pooled substrate serum (see: Lacko et al., 1974b; Stokke, 1974; Norum, 1974).

11.5.2 Results and discussion

In the sera of the rabbits from Experiment 5 and of control rabbits LCAT activity was measured with both the native lipoproteins as substrate and with the use of a heat-inactivated rabbit serum, pooled from all these animals together. The results are shown in Table 49.

The esterification rate as presented in Table 49 compares very well with the rates reported by Lacko et al. (1974b) for normal rabbits, i.e. 45 μmol and Rose (1972), i.e. 33 $\mu mol \cdot l^{-1} \cdot h^{-1}$.

The findings suggest that esterification rate in rabbit serum can be influenced. The

Table 49. Cholesterol esterifying activity in vitro in serum of rabbits fed on semi-synthetic diets with 36% (cal/cal) coconut oil and various proteins (means ± s.e.); Experiment 5.

Dietary group[1]		Free cholesterol[2] (mg/100 ml)	Cholesterol esterification[3]		
			autologous serum		pooled serum
			fractional rate ($\% \cdot h^{-1}$)	rate ($\mu mol \cdot h^{-1} \cdot l^{-1}$)	fractional rate ($\% \cdot h^{-1}$)
1 (Cas)	(6)	291 ± 122	1.6 ± 0.4	83.1 ± 11.3	0.38 ± 0.04
2 (CaGel)	(8)	95 ± 34	3.6 ± 0.5	59.8 ± 8.3	0.20 ± 0.05
3 (CaGelFi)	(9)	50 ± 5	4.0 ± 0.5	47.4 ± 3.5	0.06 ± 0.04
4 (PM)	(9)	46 ± 8	3.9 ± 0.4	43.7 ± 7.8	0.11 ± 0.07
Controls (5)		20 ± 3	9.4 ± 1.5	43.3 ± 2.1	0.15 ± 0.13

1. For diet composition see Table 39; controls fed on laboratory rabbit chow; number of animals between brackets.
2. See Table 44 for other lipid classes.
3. Fractional esterification rate: percentage of labelled free cholesterol, esterified per hour; esterification rate: μmol of cholesteryl esters formed per l serum per hour.

absolute increase of cholesteryl esters formed by feeding casein to rabbits and to a lesser extent by feeding a mixture of casein and gelatin may be related to the increased substrate concentration. The fractional esterification rate decreased from 9.4% in controls to 1.6% in cholesterol-fed rabbits, a sixfold decrease. The rate derived from the pooled serum is low, the reason for this being discussed above. Comparison of esterification rates of sera from control rabbits and sera from casein-fed rabbits in pooled substrate shows that esterification is increased at least twofold in the casein-fed rabbit sera. This twofold increase in esterification is also seen if the absolute amount of μmoles cholesteryl esters formed is considered. The increased esterification rate can therefore be considered as an increase in enzyme activity or quantity.

It is not clear whether this increased activity is a consequence of the hypercholesterelemia. LCAT activity is mainly present in the blood, associated with HDL. It has been shown that the amount of HDL or α lipoproteins (Section 11.4) does not differ very much at differing total lipid concentrations. Fielding & Fielding (1971) have demonstrated that purified LCAT reacts directly with the cholesterol and phosphatidylcholine transported by the HDL, but only indirectly with those transported by VLDL due to rapid equilibration between lipoproteins. Remnants of triglyceride-rich lipoproteins could provide substrate for LCAT by transfer of free cholesterol to HDL, which would stimulate the LCAT reaction.

The amount of HDL is probably not critical as Marcel & Vezina (1974) could detect no effect on the LCAT reaction if pure HDL were added to human plasma. Addition of chylomicrons or VLDL caused an increase of both initial and net esterification rate, the increase being proportional up to a level of about 0.5 mmol of VLDL free cholesterol. This was confirmed in vivo by a fat-loading test.

Apart from supplying substrate (free cholesterol and lecithin) for the LCAT reaction another possibility is the transfer of specific peptides between lipoproteins. After a fat loading in man the concentration of apo-C peptides doubled in the triglyceride-rich lipoproteins as a result of a transfer from the HDL_2 (Havel et al., 1970). These apo-C peptides contain the activator of lipoprotein lipase (Havel et al., 1970) and are transferred back to HDL after the clearing of the plasma (Windmueller et al., 1971). Transfer of apo-C peptides may be accompanied by transfer of other peptides such as apo-A_1 from VLDL to HDL, which activates LCAT (Eisenberg et al., 1972). During the catabolism of VLDL and chylomicrons, therefore HDL become more suited as enzyme carrier by loss of apo-C and gain of apo-A protein, resulting in increased net esterification of free cholesterol.

The enhanced flux of cholesterol and lecithin from VLDL and chylomicrons to HDL also contributes to increased esterification rate. The results appear to confirm this theory. The sera from rabbits of Group 1 contain a lot of VLDL (Table 47 and 48) which are very rich in free cholesterol, as are the chylomicrons and LDL. It may be postulated that their HDL are still in an active esterifying state. This is much less so in the sera of rabbits fed on Diet 3 or laboratory chow (Table 47 and 48). An increased activity may therefore be expected if serum of Group 1 is added to a pooled substrate. Table 49 confirms this higher esterification rate in pooled serum though the

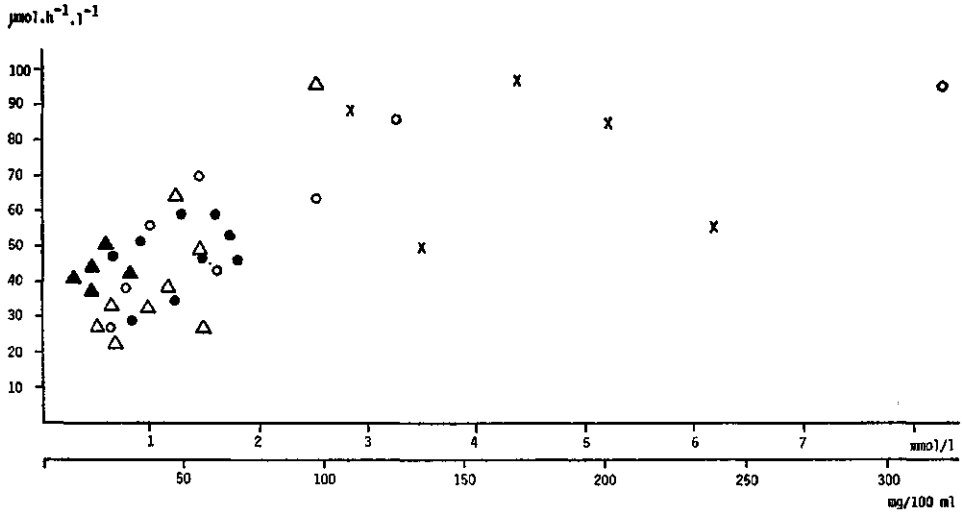

Fig. 18. Exp. 5. Relationship between the rate of cholesterol esterification (LCAT) ($\mu mol \cdot l^{-1} \cdot h^{-1}$) and the concentration of free cholesterol (mg/100 ml and mmol/l) in serum of rabbits. Group 1: x; Group 2: ○; Group 3: ●; Group 4: △; Controls (chow): ▲.

absolute values are low.

The possibility of an increased free cholesterol transfer from other lipoproteins to HDL can also not be excluded. If esterification rate is plotted against serum free cholesterol concentration, it may be seen that esterification rate increases initially but reaches a maximum of about 100 $\mu mol \cdot l^{-1} \cdot h^{-1}$ at a free cholesterol concentration of about 100 mg/100 ml (Fig. 18). Fractional esterification rate therefore declines at free cholesterol concentrations exceeding 100 mg/100 ml. It may also be concluded from Fig. 18 that in the normal range of free cholesterol concentration up till 50 mg/100 ml no correlation can be found between cholesterol concentration and esterification activity. This may explain the negative findings of Lacko et al. (1974b). The relationship between free cholesterol concentration and esterification rate is also apparent from Fig. 19. In this figure the data for Groups 1 and 2 are combined. A high correlation between [S] and [S]/V ($r = 0.87$; $P < 0.01$) was obtained.

11.5.3 Conclusion

It is concluded that in rabbits fed on semi-synthetic diets, such that serum cholesterol is changed over a wide range, absolute esterification rate increases with increments of free cholesterol in the serum above 50 mg/100 ml. It is suggested that both increased enzyme activity and substrate (free cholesterol and lecithin) availability have contributed to the increased rate of esterification. The V_{max} (91.7 $\mu mol \cdot l^{-1} \cdot h^{-1}$) is, however, relatively rapidly approached at free cholesterol concentrations of about 100 mg. At higher concentrations esterification is deficient and free cholesterol removal from

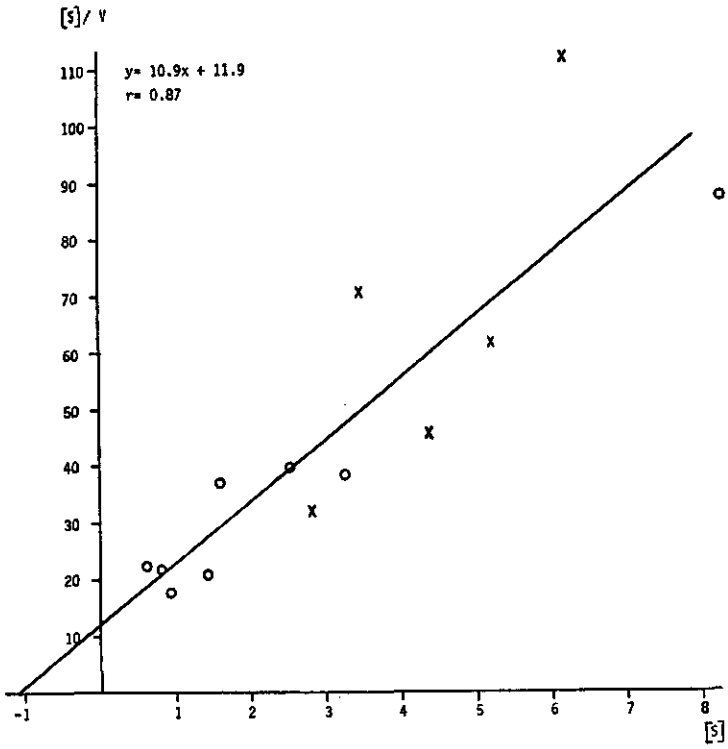

Fig. 19. Exp. 5. Plot of serum free cholesterol concentration [S] (mmol · l^{-1}) against [S]/V for the rabbits of Groups 1 and 2 in Fig. 18. V = free cholesterol esterified per 1 per h (μmol · l^{-1} ·h^{-1}). V_{max} = 1/slope of the line = 91.7 μmol · l^{-1} ·h^{-1}; K_m = intercept of the line with the ordinate = 1.09 mmol · l^{-1}.

e.g. erythrocytes may become limited. This might form a tentative explanation for cholesterol accumulation in erythrocytes of cholesterol-fed rabbits and the consequent haemolytic anaemia in these rabbits. It might also offer an explanation for the severe decrease of Hb levels in some of the rabbits. The increase in absolute esterification rate and concomitantly decreased fractional esterification rate may reconcile some opposite reports in the literature.

11.6 Cholesterol distribution and turnover in rabbits

11.6.1 The two-pool model of cholesterol turnover

The mechanism of hypercholesterolemia induced in rabbits by feeding semi-synthetic diets with casein is still largely unknown. This is due to insufficient knowledge of the factors influencing cholesterol metabolism and of the interactions between the blood cholesterol level, cholesterol synthesis and catabolism, and tissue cholesterol. In

recent years a useful kinetic approach has been developed to study cholesterol pools and turnover in man and animals (Goodman & Noble, 1968). It was reported that upon intravenous injection of labelled cholesterol the plasma cholesterol specific radioactivity-time curve could be resolved into two exponential functions. This indicated that the turnover of plasma cholesterol conformed to a simple two-pool model in man.

The presence of this two-pool system was also reported for rats (Zilversmit, 1972), monkeys (Wilson, 1970), pigeons (Bell et al., 1970) and chiks (Yeh & Leveille, 1973). From the data reported by Iritani & Wells (1966) it is deduced that this model may also be valid for rabbits, though they injected the tracer intraperitoneally. Their semilogarithmic plot of cholesterol specific activity (SA) in faeces was biphasic. The first phase lasted about 5 – 10 days. The half-life of cholesterol was 19 and 35 days during the second phase for rabbits fed sucrose and lactose, respectively. Bieberdorf & Wilson (1965) injected tracer cholesterol intravenously in rabbits. They did not study the first 8 days but found a half-life during the second phase of about 25 days. No true turnover studies with rabbits are known, in which also pool size and kinetic parameters were determined.

Because of discrepancies found in long-lasting turnover studies in man (Grundy & Ahrens, 1969) Goodman et al. (1973) have recently shown that after 30 – 40 weeks cholesterol turnover is better described by a three-pool than a two-pool model. If, however, the study is limited to the first 12 weeks after injection, then the two-pool model is the best method for the study of cholesterol turnover.

The two pools are designated Pool A and Pool B. Pool A, the readily miscible pool, comprises probably plasma, red cell and liver cholesterol, and most of the cholesterol in other viscera such as intestines, lung, spleen, and kidneys. Pool B is located in peripheral tissues, particularly muscle and also adipose tissue and probably includes also some of the cholesterol in the viscera. Cholesterol in the central nervous system is extremely difficultly miscible and belongs to the third pool (Wilson, 1970).

Turnover studies may provide a useful tool to study effects of diets, drugs etc. on cholesterol metabolism. From the analysis of the SA time curve the values of several constants are readily obtained. From these, a number of model parameters can be calculated. Fig. 20 shows a schematic presentation of the model. Parameters which can be obtained are M_A, the size of Pool A; – k_{AA}, the rate constant for total cholesterol removal from Pool A, which includes transfer into pool B (k_{AB}) and excretion from Pool A (k_A); – k_{BB}, the rate constant for total removal from Pool B; and PR_A, the production rate of cholesterol in Pool A, defined as the rate of entry of not recycled cholesterol into Pool A.

It is assumed that $k_B = 0$ because almost all steroid losses occur via tissues belonging to Pool A. With this assumption it is possible to calculate also k_{BA} and k_A. This assumption also makes it possible to determine the metabolic turnover rate in the whole body since the rate of entry into and removal from the system in a steady state will be identical with PR_A if $k_B = 0$.

The mass in Pool B can only be determined if it is assumed that either synthesis and

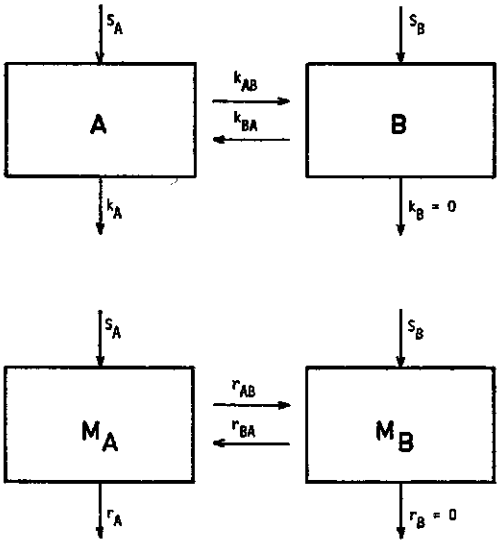

Fig. 20. Exp. 5. Two-pool model for cholesterol turnover study (Goodman & Noble, 1968).
A, B: Pools A and B; S_A and S_B: entry of cholesterol from outside the system into Pools A and B; k_A and k_B: rate constants for the removal of cholesterol outside the system from Pool A and Pool B; k_{AB} and k_{BA}: rate constants for the transfer of cholesterol from pool A to pool B and vice versa; M_A and M_B: mass of Pool A and Pool B; $r_A = k_A \cdot M_A$; $r_B = k_B \cdot M_B$ (assumed to be zero); $r_{AB} = k_{AB} \cdot M_A$; $r_{BA} = k_{BA} \cdot M_B$.

excretion in Pool A or synthesis and excretion in Pool B are both zero. None of these conditions is really existent. Nestel et al. (1969) have proposed a very useful approach to this problem. They calculated the upper limiting value for Pool B by assuming no synthesis in Pool A such that all cholesterol which enters Pool A comes from the diet or from Pool B. In this experiment the rabbits were fed no cholesterol. The dietary contribution was zero. The lower limiting value for the size of Pool B is obtained by assuming that synthesis in Pool B is nihil and therefore all cholesterol in Pool B should come from Pool A. The exact value will be somewhere in between and for normal people probably very close to the lower limit. For obese people in which cholesterol synthesis is increased in the whole body the higher value will be more realistic.

It was demonstrated by Nestel et al. (1969) that plasma cholesterol was not related to production rate, nor to the size of pool B. It was inversely related to the fractional turnover rate of cholesterol in Pool A and to the MCR (metabolic clearance rate, defined as the volume of plasma which contained the amount of cholesterol turned-over each day). It appears therefore that elevated levels of plasma cholesterol are not associated with an increase in the cholesterol content of other pools, nor in the rate of production or excretion by the body. It is suggested that high plasma cholesterol levelt are due to specific defects involving the metabolism of plasma cholesterol and not affecting the major parameters of cholesterol metabolism in the whole body (Goodman, 1970). This conclusion is consistent with the findings for patients with type II hypercholesterolemia. Miettinen (1974) reported their cholesterol production rate to be subnormal, thus concluding to defective removal of cholesterol from the blood.

Very recently, Brown & Goldstein (1974) have shown evidence for a defective removal. By incubating normal LDL and fibroblasts from type II patients and vice

versa they demonstrated that the defect was not connected with the lipoproteins but with the fibroblasts from type II patients. They concluded that type II fibroblasts were genetically resistant to LDL. This was explained by a deficiency of receptors at the fibroblast membrane for LDL. As a consequence, less LDL are bound to the membrane, less cholesterol is transferred to the cell, cellular cholesterol synthesis is less suppressed, and less proteolysis of LDL apoproteins occurs. This discovery may be a major step forwards in elucidating the regulation of cholesterol uptake at the cellular and molecular level.

Yeh & Leveille (1972; 1973) observed that chicks fed on low protein diets have higher serum cholesterol levels, but lower total body or carcass cholesterol contents (mg/g) than chicks fed high protein diets. For chicks fed on these diets they also reported differences in plasma cholesterol turnover, cholesterol synthesis rate and fecal steroid excretion. Biederdorf & Wilson (1965) could not detect in rabbits an increase of faecal steroid excretion upon serum cholesterol reduction by linoleic acid feeding. They concluded that the cholesterol removed from the circulation must have redistributed to body cholesterol pools.

Cholesterol synthetic activity is found to be decreased in vitro in liver slices from rabbits fed on semi-synthetic diets compared with laboratory chow diets. Added fats had no consistent effects (Carroll, 1971). Addition of casein to commercial diets likewise depressed cholesterol synthetic activity. Howard & Gresham (1968) also reported a 50% reduction of cholesterol synthetic activity in vitro in rabbit liver upon feeding a semi-synthetic diet as compared with a chow diet. After replacement of the casein from the semi-synthetic diet by soya bean meal a fourfold increase of faecal neutral sterol excretion was observed.

All these studies provide no answer to the question how semi-synthetic diets elevate serum lipids. It is suggested that a cholesterol distribution and turnover study may provide some insight into the difference between semi-synthetic and control diets and between semi-synthetic diets composed of different protein sources.

11.6.2 Experiment and results

An isotopic cholesterol turnover study was performed with rabbits from Experiment 5. The animals from Group 4 were excluded as their cholesterol and body weight pattern did not differ from that of Group 3. Some control rabbits, fed on laboratory chow were included to investigate the differences between a normal and a semi-synthetic diet. The parameters of cholesterol kinetics were calculated as proposed by Goodman & Noble (1968) and by Nestel et al (1969). The results are shown in Table 50 and illustrated in Fig. 21.

The differences between rabbits fed on commercial pellets and on semisynthetic diets are particularly evident for S_A, S_B and PR, relative to body weight. The control rabbits synthesize significantly more cholesterol ($P < 0.01$) in both pools but excrete also significantly more cholesterol ($P < 0.025$) than the rabbits fed on a semi-synthetic

Table 50. Parameters[1] (± s.e.) in rabbits obtained by cholesterol turnover study according to weo-pool model.

	Dietary groups[2]			
	1 Cas (n = 5)	2 CaGel (n = 5)	3 CaGelFi (n = 6)	controls chow (n = 4)
Serum cholesterol (mg/100 ml)	465 ± 87	164 ± 47	175 ± 24	15 ± 6
$t_{\frac{1}{2}}$ (A) (days)	1.29 ± 0.10	1.34 ± 0.05	1.11 ± 0.07	0.97 ± 0.10
$t_{\frac{1}{2}}$ (B) (days)	14.78 ± 1.20	14.90 ± 2.10	13.14 ± 0.80	6.68 ± 0.90
k_A (day^{-1})	0.07 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.32 ± 0.02
K_{AB} (day^{-1})	0.17 ± 0.02	0.25 ± 0.05	0.26 ± 0.02	0.26 ± 0.05
K_{BA}(day^{-1})	0.36 ± 0.03	0.25 ± 0.03	0.33 ± 0.02	0.28 ± 0.05
M_A (mg)	486 ± 54	376 ± 63	440 ± 30	206 ± 37
M_B (mg)	277 ± 30	458 ± 106	426 ± 39	391 ± 102
M_A rel. ($mg \cdot kg^{-1}$)	195 ± 14	128 ± 22	136 ± 12	83 ± 15
M_B rel. ($mg \cdot kg^{-1}$)	112 ± 12	150 ± 25	130 ± 14	156 ± 41
S_A rel. ($mg \cdot kg^{-1} \cdot d^{-1}$)	7.3 ± 0.5	6.4 ± 0.9	7.0 ± 0.4	13.0 ± 2.2
S_B rel. ($mg \cdot kg^{-1} \cdot d^{-1}$)	7.1 ± 0.5	6.3 ± 0.8	7.1 ± 0.4	13.2 ± 2.2
PR rel. ($mg \cdot kg^{-1} \cdot d^{-1}$)	14.3 ± 0.9	12.6 ± 1.7	14.0 ± 0.8	26.2 ± 4.3
MCR ($ml \cdot kg^{-1} \cdot d^{-1}$)	3.5 ± 0.6	10.2 ± 2.6	9.1 ± 1.2	166 ± 28

1. For parameters see Fig. 20. t–: half-life of cholesterol of pool A and B. PR: total synthesis (+ absorption), equals in a steady state total excretion. MCR: metabolic clearance rate = ml serum cleared from cholesterol per day (PR/cholesterol content) per kg body weight.
2. For diet composition see Table 39; number of animals between brackets.

diet. The relative mass of Pool A (M_A) is significantly smaller in those former animals ($P < 0.025$) while the mass of pool B is not different between the groups.

The results confirm the observations in vitro of Carroll (1971) and Kyd & Bouchier (1972). These investigators reported a smaller incorporation of acetate in cholesterol by liver slices from rabbits fed on semi-synthetic diets, compared with controls fed on commercial rabbit chow. Howard & Gresham (1968) noted a considerable decrease of steroid excretion in rabbits fed on casein instead of soya bean meal. These results appear to point all into the direction of a decreased cholesterol synthetic activity as well as decreased excretion rate in rabbits fed semi-synthetic diets. About the same phenomenon was recently reported for rats by Balmer & Zilversmit (1973). While comparing a chow diet with a semi-synthetic diet they observed no influence on cholesterol absorption but an enhanced cholesterol turnover and steroid excretion in chow-fed rats.

The rabbits fed on semi-synthetic diets showed a significantly lower mean rate constant for the daily cholesterol turnover in Pool A ($k_{AA} = k_A + k_{AB}$) ($P < 0.01$) and a significantly lower mean rate constant for the removal of cholesterol of Pool A from the body (k_A) ($P < 0.01$), than the chow-fed rabbits. A highly significant negative correlation was present between the relative size of Pool A (M_A rel) and the fraction of cholesterol which leaves Pool A (k_{AA}) per day per kg body weight (Fig. 22; r =

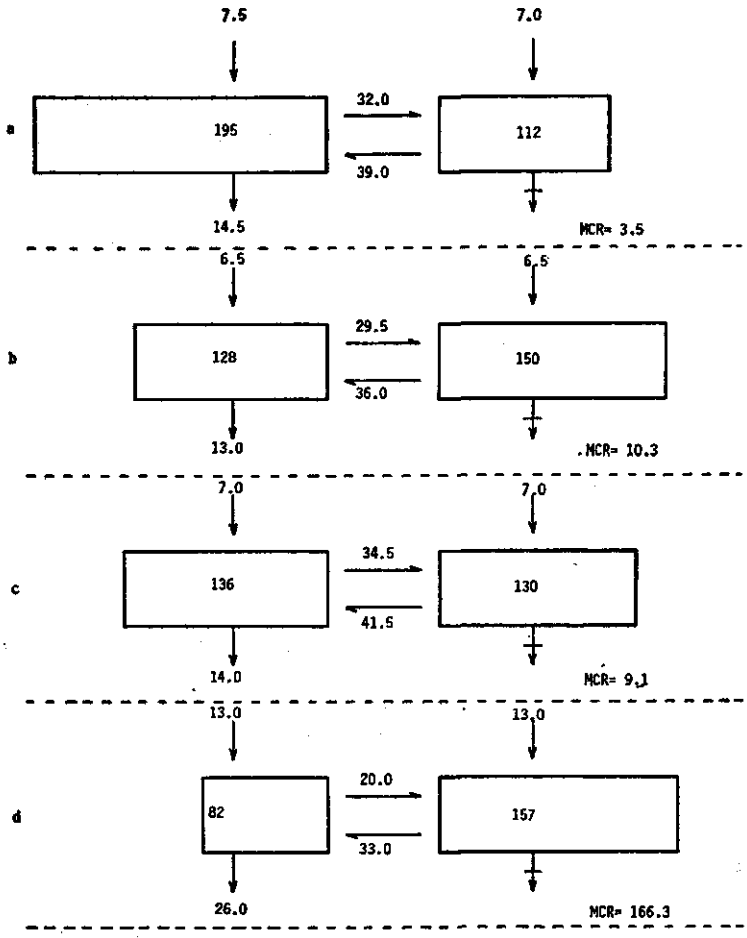

Fig. 21. Exp. 5. Models of cholesterol turnover for rabbits fed on semi-synthetic diets with different proteins and for rabbits fed on laboratory chow. a: Group 1, casein; b: Group 2, casein/gelatin; c: Group 3, casein/gelatin/fish protein; d: controls, rabbit chow. All results are presented as $mg \cdot kg^{-1} \cdot day^{-1}$. Metabolic clearance rate (MCR) as ml plasma or serum cleared from cholesterol per kg body weight per day.

-0.82; $P < 0.001$). The values of the chow-fed rabbits fall within the 95% confidence limits of this correlation. A linear correlation is assumed, which may be valid in the range of values for M_A and k_{AA} actually present. On theoretical grounds however, a hyperbolic relationship might be expected. If k_{AA} approaches zero, resulting in no removal of cholesterol from Pool A, M_A should increase infinite. If the size of Pool A (M_A) decreases and k_{AA} increases, a lower limit for M_A will be reached, below which a further decrease would not be compatible with life. The individual data, presented in Fig. 22, also indicate this trend.

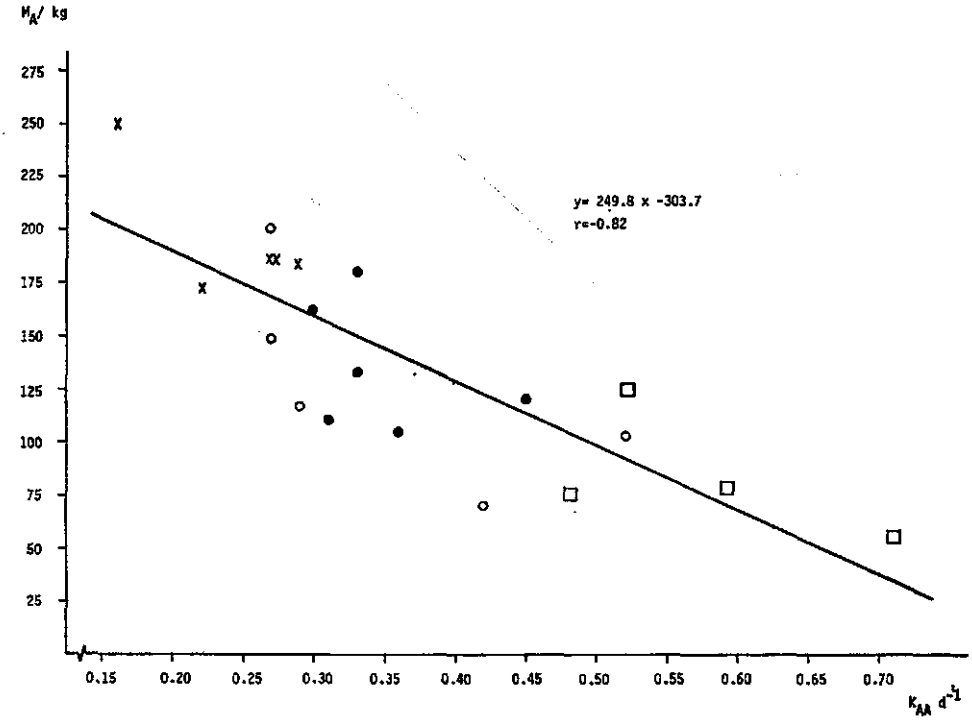

Fig. 22. Exp. 5. Correlation between the fraction of cholesterol which leaves Pool A per day ($k_{AA} = k_A + k_{AB}$) and the size of Pool A (M_A: mg $\cdot kg^{-1}$). The figure shows the regression line. Group 1: x, casein; Group 2: ○, casein/gelatin; Group 3: ●, casein/gelatin/fish protein; Controls: □, rabbit chow.

Comparison of serum cholesterol with cholesterol turnover (PRrel) revealed the existence of a strong relationship for the rabbits fed on the semi-synthetic diets. The chow-fed rabbits fell outside this relationship. With their low serum cholesterol they had too high PRs, compared with the other rabbits. This is illustrated in Fig. 23 with a conventional enzyme plot of serum cholesterol concentration [S] versus [S]/V. V is the cholesterol turnover (= PR/kg body weight) (Miettinen, 1974). From this plot a V_{max} results of 15.5 mg cholesterol $\cdot d^{-1} \cdot kg^{-1}$. This value is approached by almost all rabbits on semi-synthetic diets. The values of the chow-fed rabbits do not appear to belong to the same homogeneous population. The small number of these animals inhibits from drawing firm conclusions but it is tentatively speculated that they have a greater V_{max} (about 55 mg $\cdot kg^{-1} \cdot d^{-1}$). This may explain why chow-fed rabbits do not respond to saturated fat feeding. An increase of cholesterol influx from the diet or from endogenous synthesis should cause a greater rise in serum cholesterol level in the semi-synthetic diet-fed than in the chow-fed rabbits.

In the steady state synthesis equals per definition excretion. The smaller excretion of cholesterol by the rabbits fed on semi-synthetic diets was compensated by a similar decrease of cholesterol synthesis in Pool A and Pool B.

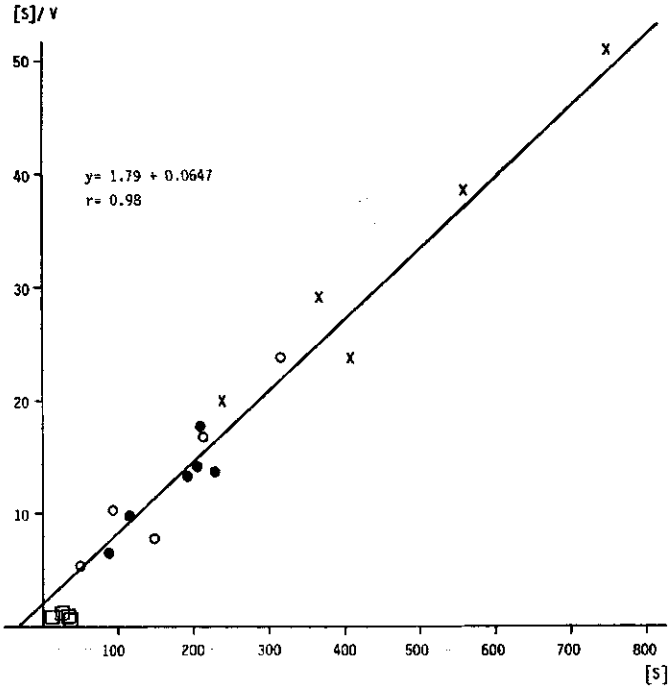

Fig. 23. Exp. 5. Plot of serum cholesterol concentration [S] (mg/100 ml) against [S]/V for the rabbits of Fig. 22. V = PR = cholesterol removal from the body ($mg \cdot kg^{-1} \cdot day^{-1}$) $\cdot V_{max}$ = 1/slope of the line = 15.5 $mg \cdot kg^{-1} \cdot day^{-1} \cdot K_m$ = intercept of the line with the ordinate = 30 mg cholesterol per 100 ml.

11.6.3 Discussion and conclusion

The differences in serum cholesterol between the groups fed the different dietary proteins are difficult to explain. The kinetic parameter k_{AB} of Group 1 is significantly lower than for the rabbits of the other groups ($P < 0.01$). This indicates the transfer of a smaller fraction of Pool A to Pool B in the former rabbits. The transfer in the opposite direction, from Pool B to Pool A, is not clearly different though a trend for a greater transfer in Group 1 seems present. These two parameters together show a tendency to an increased size of Pool A and a smaller size of Pool B in casein-fed rabbits.

The rabbits of Group 2 and particularly of Group 3 had a higher body weight (Fig. 15 and Table 41) than those of Group 1. Though it was not exactly established, the rabbits of Groups 2 and 3 had more adipose tissue, while those of Group 1 were extremely lean. It has been reported that adipose tissue, though not capable of cholesterol synthesis, may serve as a storage organ for cholesterol (see: Miettinen, 1974). The relatively greater amount of adipose tissue per kg body weight in the

animals of Groups 2 and 3 might explain the greater avidity for cholesterol of their Pools B. It may be of significance in this question that a negative correlation was found in Experiment 4 between serum cholesterol and body weight if animals from several groups were taken as a whole. Such a correlation was rarely present within groups.

In Experiment 4 a very rapid and steep increase of serum cholesterol was observed after rabbits were changed over from a mixed protein to a casein diet. It is suggested that, by the concomitant weight loss, fat and cholesterol from adipose tissue were mobilized and metabolized. As a fat like coconut oil caused only a slow increase in other rabbits, it is postulated that the rapid increase of serum cholesterol was, at least in part, caused by mobilized cholesterol, which could not be excreted.

With these data and some incidental reports from the literature it becomes possible to formulate a hypothetical model for the response of rabbits to semi-synthetic diets as related to serum cholesterol. The primary event may be a drastic reduction of faecal steroid excretion as was shown for the rat by Balmer & Zilversmit (1973). This may be the consequence of more complete re-absorption of cholesterol and bile acids, due to a prolonged transit-time of the digesta in the intestinal tract, to a deficiency of substances like crude fibre or to both. The sawdust, incorporated in our diets may be too inert to be functional as fibre. It is relevant to this question that Moore & Williams (1964a) used wheat straw as source of roughage and observed a much smaller cholesterolemic response than e.g. Funch et al. (1960), who used pure cellulose, and Wigand (1959) who used sawdust.

The decreased excretion is followed by an increase of the size of Pool A. Cholesterol synthesis will be depressed in the liver, but not in the small intestine (Kyd & Bouchier, 1972; Carroll, 1971). The fraction excreted from Pool A is reduced, while the fraction coming from Pool B remains the same or is slightly increased. The fraction transferred to Pool B remains the same but because of the increased Pool A a greater mass is transferred leading to inhibition of cholesterol synthesis in Pool B. If the rabbits have sufficient adipose tissue (or muscle) they may be able to store the excess cholesterol from Pool A and maintain low serum cholesterol values. This may be the case with the rabbits fed on mixed protein. This may also partly explain the lower serum cholesterol values and the higher carcass cholesterol content of chickens fed a high as compared with a low protein diet as shown by Yeh & Leveille (1972, 1973).

The hypothesis was made that rabbits fed on semi-synthetic diets evidently lack the ability to remove sufficient cholesterol from their body to maintain similar sizes of Pools A and B as are present in chow-fed rabbits. Diets that lower serum cholesterol will probably not only affect cholesterol turnover or excretion but also redistribute cholesterol in the body from Pool A to Pool B. The results obtained with the feeding of several proteins support this hypothesis. Also the findings of Biederdorf & Wilson (1965) that unsaturated fats lower serum cholesterol without increasing faecal steroid excretion in rabbits are in agreement with this hypothesis. As the primary defect may lie in the excretion of steroids, this model may be particularly useful in the study of the effects of dietary components on the excretion of cholesterol and bile acids.

11.7 Conclusion of Experiment 5

The objective of Experiment 5 was to study the effects on cholesterol metabolism of several diets containing different proteins or protein mixtures but with the same dietary fat: coconut oil.

An increased food intake and a change from body weight loss to weight gain was observed when the casein in the diet was replaced by more complex protein sources. The casein-fed rabbits lost weight, the animals fed on casein/gelatin (12/8) maintained weight while the other two more complex protein mixtures, containing in addition fish protein (7.5/5/7.5) or fish protein and soya bean protein (6.2/4.3/6.2/4.1) respectively, induced similar weight gain. As the casein/gelatin mixture contains all amino acids, except arginine, in smaller amounts than casein it is concluded that no absolute deficiency of amino acids is present. The poor performance of the rabbits fed on casein as sole protein, is attributed to an excess of a number of amino acids in casein as compared with the tentative requirements, e.g. lysine, isoleucine, phenylalanine, threonine. Adamson & Fisher (1973) have demonstrated that a moderate excess of these amino acids above the requirements causes depression of food intake and decreased weight gain in young growing rabbits.

The pattern of the serum cholesterol values throughout the experiment showed large fluctuations which made it difficult to draw conclusions (Fig. 16). If however the average serum cholesterol levels were considered (Fig. 17) it could be concluded that the cholesterol pattern presented the reverse of the body weight pattern. Within groups no relationship was present between individual serum cholesterol values and body weight gain or loss. The number of animals may have been too small to draw definite conclusions. Haemoglobin levels were determined from Week 7 onwards. At that time a pattern had already been established, which remained unaltered during the rest of the experiment. This pattern was inversely related to serum cholesterol levels and positively related to body weight. A close correlation was observed between haemoglobin and haematocrit, resulting in a constant mean corpuscular haemoglobin content (MCHC). The red blood cell count (RBC) decreased in correspondence with the Hb levels. It is discussed that the observed pattern resembled closely that reported by Westerman et al. (1970) for cholesterol-fed rabbits. They observed initially a decrease of Hb levels due to an increased plasma volume, thus an anaemia, dilutional in origin. In a second phase they observed a greater haemolysis tendency of erythrocytes, together with an increased cholesterol content of these cells and designated the anaemia in this phase as haemolytic in origin. It may very well be that the lowered Hb values already observed at Week 7 were also caused by blood dilution through an increased volume. The second phase characterized by haemolysis is probably not approached but by some animals, resulting in a further decrease of their Hb levels and even death.

Serum lipoproteins were investigated by electrophoresis in polyacrylamide gel and by an ultracentrifuge method. The amount of α lipoproteins appeared rather constant, despite a wide range of serum lipid values. The increase of serum lipids was entirely

limited to the β and pre-β lipoproteins. If the pattern was determined in the fasting state it could be shown that on fasting a greater part of the lipids is carried by the β lipoproteins, while in fed animals a greater part is present in the pre-β lipoproteins. The results obtained by electrophoresis were confirmed by an ultracentrifuge method. The lipoproteins from the experimental rabbits contained much more cholesterol and less triglycerides than those from the control rabbits fed on rabbit chow. The results show a remarkable resemblance with those reported for cholesterol-fed rabbits and with those of type III hyperlipoproteinemic patients. It is discussed that the extreme sensitivity of rabbits for atherosclerosis might be related to their lipoprotein distribution and composition.

It was attempted to elucidate the role of lecithin: cholesterol acyltransferase (LCAT) in cholesterol esterification in serum of rabbits with a wide range of (free) cholesterol levels. It was demonstrated that the esterification rate increased with the serum cholesterol concentration. The fractional esterification rate however decreased, indicating some relative enzyme activity deficiency. It was calculated, by the use of a conventional enzyme plot, that the maximal esterification rate of about 90 $\mu mol \cdot l^{-1} \cdot h^{-1}$ appeared at a free cholesterol concentration in the serum of about 100 mg per 100 ml. It is concluded that in these rabbits at free cholesterol concentrations in the serum above 50 mg per 100 ml esterification decreased relatively, thus reducing the removal of free cholesterol from peripheral tissues, e.g. erythrocytes. It is suggested that this may lead to accumulation of cholesterol in erythrocytes and consequently to haemolytic anaemia.

The mechanism of the hypercholesterolemia induced by semi-synthetic diets and by the several proteins in these diets as compared with rabbit chow was investigated with an isotopic cholesterol turnover study as described for a two-pool model. It was confirmed that the specific activity decay curve of serum cholesterol was conform a two-pool system. It was further demonstrated that chow-fed rabbits synthesize and excrete more cholesterol than the experimental rabbits fed on semi-synthetic diets. The experimental rabbits also showed a lower mean rate constant for removal of cholesterol from Pool A, the readily miscible pool, than the controls. A highly significant negative correlation was present between the size of Pool A and the rate constant for removal of cholesterol from Pool A (k_{AA}). All experimental groups excreted similar amounts of steroids. The size of the readily miscible Pool A was greater in the casein-fed rabbits than in the other rabbits. The reverse was the case for Pool B. This enlarged pool size may explain the similar excretion rates (r_A), even with a small rate constant (k_A) for the removal of cholesterol from Pool A in the casein-fed rabbits.

It is suggested, that the rabbits fed on the protein mixtures, having more adipose tissue per kg body weight than the casein-rabbits, may store more cholesterol in their adipose tissue. This was confirmed by the observation that the rate constants for transfer of cholesterol from Pool A to Pool B was greater in the mixed protein-fed groups (and controls) than in the casein-fed group, while the transfer in the opposite direction was greater for the casein-fed group of rabbits. It may be concluded that the size of Pool B is expanding till a maximum is reached. After this maximum is

reached, the size of Pool A will expand unless excretion is enhanced.

On feeding a semi-synthetic diet rabbits evidently cannot dispose of the excess of endogenous cholesterol. This defect in the ability to dispose of endogenous cholesterol is clearly illustrated by observations in rats fed on semi-synthetic diets (Balmer & Zilversmit, 1973). A reduced cholesterol turnover was observed in these rats, which could be restored by the addition of crude fibre or cholestyramine to the diet. It must be concluded that the sawdust used in our experiments is probably not an effective source of fibre.

It is not clear from our observations whether a reduced bile flow, an increased reabsorption of acid or neutral sterols, a delayed transit-time of the digesta through the intestinal tract or a combination of these factors is responsible for the reduced turnover of cholesterol in rabbits fed semi-synthetic diets. From experiments with pigs (Marsh et al., 1972) it may be deduced that particularly enhanced reabsorption of steroids (caused by a delayed transit time?) was responsible for hypercholesterolemia in a comparison of a milk formula and a swine mash diet.

If the above mentioned conclusion of an expanding Pool B is valid, it might be inferred that the differences in serum cholesterol, induced by the various dietary proteins, are only gradually and temporarily. This may be true as even the rabbits fed on the protein mixtures show a gradual though very slow increase of serum cholesterol if fed together with coconut oil.

12 Summary and conclusions

In Chapters 1 to 4 literature about coronary heart disease and its underlying cause atherosclerosis has been reviewed and discussed. The objectives of our investigations were presented in Chapter 1. In Chapter 2 a detailed review has been given of the risk indicators as related to atherosclerosis. In Chapter 3 several theories on the origin of atherosclerosis have been discussed which has led to the concept that atherosclerosis is the outcome of a continuous process of injury and repair, influenced by factors pertaining to the arterial wall, to blood components or to haemodynamic forces. It has been shown that at least blood pressure, tobacco smoking, probably via carbon monoxide inhalation, and serum cholesterol may be regarded as factors causally related to atherogenesis.

In Chapter 4 the application of the rabbit as experimental model for the study of atherosclerosis has been discussed. It was concluded that the rabbit, fed on a semi-synthetic diet, might provide a useful model for the study of effects of dietary factors on blood lipids and atherogenesis. The reason for the response of rabbits to fats when incorporated in semi-synthetic diets is largely unknown. From a review of the literature on nutritional requirements of rabbits, (Section 4.3) it appeared that particularly protein and amino acid requirements are poorly understood. From the scanty data it seemed as if casein, which is the nitrogen source in the semi-synthetic diet, might be responsible in part for the atherogenic properties of this kind of diet. It was therefore decided to investigate the role of dietary protein, apart from the role played by milk fat constituents in the induction of hypercholesterolemia and atherosclerosis.

From the literature no clear picture could be obtained about the effects of amount and kind of dietary protein on blood lipids or degree of atherosclerosis in man or several animal species (4.7). The role of dietary fat and cholesterol has been much more frequently studied (4.5 – 4.6). However a detailed study of the factors in milk fat which elevate serum lipids and induce atherosclerosis was lacking. It was concluded that both the cholesterol and the fatty acids as present in milk fat might be held responsible for its effect. In Chapter 5 a more detailed summary with conclusions from the literature was presented.

The results of the experiments which were performed were reported in Chapters 7 to 11. In Experiments 1 and 2 the role played by several milk fat constituents on blood lipids and atherosclerosis was studied in semi-synthetic diets containing casein as dietary protein. In Experiment 1 the effects of feeding 18% of calories as tributyrate were compared with the effects of the same amounts of coconut oil or starch. All diets contained in addition 18% (cal/cal) coconut oil. Starch and tributyrate induced

similar serum lipids, liver lipids and a similar fatty acid composition of serum lipid classes, of liver lipids and of perirenal adipose tissue. The serum lipid and liver triglyceride contents of the rabbits fed on coconut oil tended to be higher than those of the other groups. The degree of atherosclerosis was not different, probably due to the short duration of this experiment (6 months). Our results with butyric acid, together with those reported in the literature for medium-chain fatty acids (4.6.4 and 4.6.5) support the theory that fatty acids with a chain length shorter than 12 carbon atoms do not influence serum cholesterol or triglycerides or any other lipid class in serum or liver as compared with equal caloric amounts of starch. The amount of fat in the diet has evidently a strong influence on serum lipids.

In Experiment 2 the effects of 36% (cal/cal) coconut oil were compared with those induced by butter oil feeding. A third group was fed on a simulated butter oil, which was gradually adjusted to resemble the butter oil as to fatty acid composition and cholesterol content. All diets also contained casein as the source of dietary protein. Serum cholesterol declined in the following order: coconut oil > butter oil > simulated butter oil without cholesterol. If cholesterol was added to the simulated butter oil to match the complete butter oil an increase of serum cholesterol occurred in some animals. The difference in mean serum cholesterol of rabbits fed on butter oil or simulated butter oil disappeared on cholesterol supplementation. Addition of short and medium chain fatty acids to match the butter oil had not the slightest influence on serum cholesterol, thus confirming the results obtained in Experiment 1. From these data it is concluded that the main part of the hypercholesterolemic effect of butter oil in rabbits is due to its content of cholesterol. It has been discussed in Sections 4.5 and 4.6 that dietary cholesterol is also important in human nutrition as far as the establishment of serum cholesterol levels is concerned.

The question whether a relationship exists between serum cholesterol level and degree of atherosclerosis was discussed in Section 8.4. It was concluded that a strong correlation exists between serum cholesterol level and degree and extent of atherosclerosis. The correlation was better if the average serum cholesterol level over the whole experimental period was taken as the variable than with the use of the final serum cholesterol values. However, unexplained variability is great and it is almost impossible to predict individual degree of atherosclerosis from individual serum cholesterol concentrations. Mean serum cholesterol on a group basis seems a more reliable index.

Triglyceridemia, which often accompanies cholesterolemia in rabbits, does not seem to have an additive effect on extent or degree of atherosclerosis.

From the Figures 9 - 11 it may be derived that rabbits fed on butter oil with the same degree of atherosclerosis as rabbits fed on coconut oil have generally a slightly lower serum cholesterol concentration, which would indicate that butter oil-induced cholesterolemia is relatively slightly more atherogenic than coconut oil-induced cholesterolemia. It has been discussed that this effect might be caused by the cholesterol-derived origin of the cholesterolemia induced by butter oil feeding.

From the regression equations for average serum cholesterol and degree of athero-

sclerosis it could be calculated that in the conditions of this study, 90 mg/100 ml serum cholesterol is the lower value below which no aortic atherosclerosis may be expected. For cockerels a similar lower limit has been reported of 80 mg (Kakita et al., 1972) and for rodents and primates of below 145 mg (Constantinides, 1965, p. 67).

In this second experiment mortality was rather high and the general health status of the rabbits was poor in both experiments. In Experiment 3 therefore the composition of the basal semi-synthetic diet was changed to reduce mortality and to improve performance of the rabbits. The addition of 15% (w/w) coconut oil to a diet of rabbit chow did not elevate serum cholesterol and had no deleterious effect on the weight or health performance of the rabbits.

The addition of 10% (by weight) grass meal to a semi-synthetic diet was without effect as related to serum cholesterol, body weight or performance. Substitution of 20% sawdust by 20% grass meal did not affect mortality, but increased body weight and decreased serum cholesterol rather severely. It was not felt justified to include such a complex product as grass meal in a semi-synthetic diet.

To improve the acceptability of the diet various amounts of quinine were added to the diet. In a preference study, however, no clear preference was exhibited for the quinine-containing diets. In Experiment 3 the effect of substituting a mixture of proteins, resembling the amino acid composition of rabbit chow, for casein was investigated. The protein mixture (PM) consisted of six parts casein, four parts gelatin, six parts fish-protein concentrate and four parts soya bean protein. Food intake, body weight gain and general health were more satisfactory while feeding the PM diet than the casein diet. However serum cholesterol levels tended to decline with this PM diet, even though coconut oil was the dietary fat.

In Experiment 4 the influence of the protein source was further investigated in addition to effects of several dietary fats. On feeding the mixed protein, only coconut oil caused a small but consistent increase of serum cholesterol while butter oil, butter oil supplemented with soya bean oil and simulated butter oil with or without added cholesterol to match the butter oil were without effect. Even over an experimental period of more than one year not the slightest increase occurred. Body weights of the rabbits fed on coconut oil were lower than of those fed on the other fats. Almost no mortality occurred in this period with these diets.

If the rabbits were replaced from the PM to the casein diet a rapid fall in body weight and a steep increase of serum cholesterol followed. The increase of serum cholesterol was more rapid than when casein was fed from the beginning. This may be due to mobilization of depot fat and probably also of tissue cholesterol. Also mortality rate increased on these diets. It was found that many moribund rabbits had low Hb levels though the diet amply supplied iron. It was concluded from this experiment that the PM diet increased the sensitivity threshold of the rabbits for hypercholesterolemia such that coconut oil could just elevate serum cholesterol but the other fats could not.

Differences between the various fats were difficult to establish due to the high mortality rate of particularly the hyper-responding rabbits. If these prematurely dying rabbits were taken into account, no difference of serum cholesterol could be

detected between rabbits fed on butter oil and those fed on butter oil supplemented with soya bean oil. This confirms data from the literature and it was discussed that the cholesteryl esters of rabbits fed on butter oil, which contained very small amounts of linoleic acid and much oleic acid, might be more atherogenic than those of rabbits fed on butter oil supplemented with soya bean oil. Even the coconut oil fed rabbits had more linoleic acid in their serum cholesteryl esters than the butter oil fed group. It was suggested that this might partly explain the somewhat higher relative atherogenicity of the serum cholesterol of rabbits fed on butter oil compared with coconut oil (see 3.3.2).

In Experiment 5 a more detailid study was made of the effects of various protein mixtures fed in combination with coconut oil on results obtained with the semi-synthetic diet model and compared with rabbit chow. On casein feeding the rabbits lost weight as usual, on a casein/gelatin mixture the rabbits maintained weight. More complex protein mixtures, with the addition of fish protein or fish protein and soya bean protein induced similar weight gain.

It was concluded that no absolute amino acid deficiency was caused by casein, as the performance with the casein/gelatin mixture was better and as casein contains all essential amino acids in greater amounts than the tentative requirement of rabbits indicate. It is possible that casein contains an excess of some amino acids for which it has been demonstrated that even a moderate excess leads to depressed food intake and weight gain. The serum cholesterol pattern presents the reverse of the body weight pattern with only minor differences between the two most complex protein mixtures. A mixture of animal proteins may therefore be as effective in maintaining low serum cholesterol levels as vegetable proteins. This result does not agree with the results of Hamilton & Carroll (1974) for individual proteins.

Serum lipoproteins were investigated by paper and polyacrylamide gel electrophoresis and ultracentrifugation. It could be demonstrated that α lipoproteins remain constant while β lipoproteins increase with moderate increments of serum lipids followed by a rise also of pre-β lipoproteins if serum lipids increase further. The lipoproteins from all rabbits fed on a semi-synthetic diet contain large amounts of cholesterol and less triglycerides than those from control rabbits. It has been discussed that the lipoproteins resemble those of cholesterol-fed rabbits and those of type III patients from the classification of Fredrickson rather closely. It is postulated that the extreme sensitivity of rabbits for atherosclerosis might be related to their lipoprotein composition and distribution.

A relative increase of free/total cholesterol was observed in this experiment at increasing cholesterol levels. This might indicate a relatively deficient cholesterol esterification activity. As this esterification system is also suggested to be involved in the transfer of cholesterol from peripheral tissues to the liver it was hypothesized that a deficiency of activity might be implicated in cholesterol accumulation in erythrocytes, leading to haemolysis and anaemia. Determination of the esterification rate of serum (lecithin : cholesterol acyl-transferase activity: LCAT) showed that absolutely more cholesterol became esterified at increasing serum cholesterol level but that the frac-

tional esterification rate was diminished. It could be calculated that the V_{max} (maximal esterification rate) appears at a serum free cholesterol concentration of about 100 mg/100 ml which level is exceeded by the casein-fed rabbits. Our results confirm the observations reported in the literature for various species that LCAT activity increases with increasing serum cholesterol level. V_{max} however is relatively rapidly approached. Whether this is the reason for the anaemia often observed in hypercholesterolemic rabbits remains speculative.

The mechanism of the hypercholesterolemia induced by semi-synthetic diets and by the several proteins in these diets compared with rabbit chow was investigated with an isotopic cholesterol turnover study as described for a two-pool model. It was confirmed that the specific activity decay curve of serum cholesterol was in agreement with a two-pool system.

All experimental rabbits excreted the same amount of steroids but only half the amount of the chow-fed rabbits. The size of the readily miscible Pool A was greatest in the casein-fed rabbits and smallest in the chow-fed animals. The results indicate that the semi-synthetic diet causes a defect in the removal of cholesterol from the body. A maximal removal rate of 15.5 mg cholesterol per kg body weight per day was calculated for the rabbits fed on semi-synthetic diets. The chow-fed rabbits evidently did not belong to the same population. From the few data available for these animals a V_{max} was calculated of about 55 mg per kg body weight.

The chow-fed rabbits clearly can dispose of much more cholesterol than the experimental animals. The reason for this is not clear from our observations. A reduced bile flow is unlikely with these high fat diets. An enhanced re-absorption of cholesterol and bile acids is more likely. This might also explain the presence of cholesterol-rich lipoproteins in serum. Enhanced re-absorption may be due to a delayed transit time of the digesta in the intestinal tract or to the absence of specific steroid sequestering agents.

Differences between the various protein diets are less clear. The casein-fed rabbits accumulate more cholesterol in Pool A while the mixed protein fed rabbits accumulate relatively more in Pool B as do the controls. It is discussed that the casein rabbits were extremely lean while the rabbits fed the mixed protein had more adipose tissue. This adipose tissue may function as a storage organ for the excess of cholesterol from Pool A. The conclusion may be derived that also in mixed protein-fed animals the size of Pool A and serum cholesterol will increase if Pool B is approaching saturation. The very slow but consistent rise of serum cholesterol observed with these diets if coconut oil was provided is in agreement with this hypothesis.

The semi-synthetic diet model appears very useful to study factors affecting steroid absorption or re-absorption. Future investigations will be concerned with this aspect as well as with the effects of specific amino acids.

Samenvatting

Coronaire hartziekten kunnen slechts effectief worden bestreden door preventie van de eraan ten grondslag liggende veranderingen in de grotere arteriën, bekend als atherosclerose (hoofdstuk 1).

Met behulp van gegevens verkregen uit epidemiologisch en dier-experimenteel onderzoek (hoofdstuk 2), is een theorie omtrent de pathogenese van atherosclerose ontwikkeld (hoofdstuk 3). Het is mogelijk gebleken vele van de nu bekende risico-indicatoren een plaats in deze theorie toe te kennen. Het staat thans vast, dat noch een verhoogd bloedlipiden-gehalte, noch hypertensie of het roken van sigaretten voldoende of noodzakelijke voorwaarde is voor het ontstaan van atherosclerose.

Atherosclerose dient te worden beschouwd als de resultante van een continu proces van arteriële beschadiging en herstel. Dit proces kan worden beïnvloed door vele factoren. Deze kunnen functioneel worden ingedeeld in drie groepen: factoren werkend in of vanuit het bloed, de bloedbestanddelen; hemodynamische factoren; factoren werkzaam in de vaatwand zelf. De drie voornaamste risico-indicatoren, serumcholesterol, bloeddruk en sigaretten roken kunnen in dit opzicht worden beschouwd als factoren welke causaal zijn gerelateerd aan het ontstaan van atherosclerose. Deze factoren kunnen ieder afzonderlijk werkzaam zijn maar ook in combinatie.

De voeding wordt vaak in verband gebracht met atherosclerose als een der oorzaken dan wel als middel ter preventie (hoofdstuk 4). Er bestaat een nauwe relatie tussen aard en hoeveelheid vet in de voeding enerzijds en bloedlipiden, thromboseneiging en experimentele atherosclerose bij vele soorten proefdieren anderzijds. Melkvet wordt in vele Westerse landen in hoeveelheden van 30 – 40 g per hoofd per dag geconsumeerd. Gedetailleerde kennis echter omtrent de invloed van verschillende componenten uit melkvet op bloedlipiden en atherosclerose is nog fragmentarisch. Vooral de rol van het cholesterol en de vetzuren met korte ketens, aanwezig in melkvet komen in aanmerking voor nader onderzoek.

Bij proefdieren bleek ook de aard en hoeveelheid eiwit van belang. Bij de mens is de invloed van eiwit nog steeds twijfelachtig (paragraaf 4.7.3). Het gebruik van proefdieren in het atherosclerose-onderzoek is noodzakelijk omdat effecten op bloedvaten niet in vivo kunnen worden bepaald. Het konijn is een der meest gebruikte proefdieren in dit soort onderzoek (hoofdstuk 4.1). Vele bezwaren ertegen zijn verminderd sinds is aangetoond dat de arteriële veranderingen, geïnduceerd via de voeding, sterke gelijkenis vertonen met die bij de mens. Voorwaarden zijn wel dat voldoende tijd wordt gegeven voor progressie van de lesies en dat de hypercholesterolemie

slechts matig is. Een belangrijke stap vooruit werd gezet toen bleek dat konijnen die gevoederd werden met een semi-synthetisch of gezuiverd rantsoen, hypercholesterolemisch werden en atherosclerose ontwikkelden zonder cholesterol in het voer. Verzadigde vetten versterken de atherogene eigenschappen van dit diëet, terwijl polyonverzadigde vetten deze afzwakken.

Caseïne wordt meestal opgenomen in semi-synthetische rantsoenen als eiwitbron. De eiwit- of aminozuurbehoeften van het konijn zijn nog slechts ten dele bekend (hoofdstuk 4.3). Het lijkt echter alsof de samenstelling van caseïne niet geheel overeenkomt met de voorlopig vastgestelde behoefte aan aminozuren van het konijn. Naast een onderzoek naar de invloed op bloedlipiden en atherosclerose van bestanddelen uit melkvet leek het daarom van belang na te gaan in hoeverre de resultaten werden beïnvloed door de samenstelling van het semi-synthetisch basisrantsoen.

De resultaten van de uitgevoerde experimenten zijn weergegeven in hoofdstuk 7 t/m 11. In experiment 1 werden de effecten vergeleken van rantsoenen welke 18% (cal/cal) van respectievelijk tributyraat, zetmeel of cocosolie bevatten. Het basisrantsoen bevatte bovendien 18% (cal/cal) cocosolie. Zetmeel en tributyraat induceerden identieke serum- en leverlipidengehalten. Ook de vetzuurpatronen van serumlipidenfracties, van leverlipiden en van perirenaal vetweefsel waren identiek. Serumlipiden en levertriglyceridegehalten waren hoger bij voedering met cocosolie dan met de andere rantsoenen. Dit experiment duurde slechts zes maanden, hetgeen te kort bleek om verschillen in de graad van atherosclerose te induceren. De uitkomsten verkregen met boterzuur, tezamen met die vermeld in de literatuur voor middelketenvetzuren (4.6.4 en 4.6.5) vormen steun voor de opvatting dat vetzuren met een keten lengte van minder dan 12 koolstof atomen zich ten aanzien van serumlipiden gedragen als zetmeel. De hoeveelheid vet in het rantsoen had een sterke invloed op de bloedlipiden.

In experiment 2 werden de effecten van de voedering van cocosolie vergeleken met die van boterolie. Een derde groep kreeg een imitatie-boterolie gevoederd. Deze werd geleidelijk aangepast aan de samenstelling van de boterolie ten aanzien van vetzuursamenstelling en cholesterol-gehalte. Alle voeders bevatten caseïne als eiwitbron. Serumcholesterol nam af in de volgorde cocosolie, boterolie, imitatie-boterolie zonder toegevoegd cholesterol. Na toevoeging van cholesterol aan de imitatie-boterolie tot het niveau aanwezig in de echte boterolie steeg het serum-cholesterol gehalte van een aantal dieren. Het verschil in gemiddeld serumcholesterol tussen de groepen met boterolie en imitatie-boterolie gevoederde konijnen verdween na cholesterolsuppletie. De toevoeging van middel- en korte-keten-vetzuren aan de imitatie-boter had geen enkele invloed op het serumcholesterolgehalte. Deze uitkomsten hebben geleid tot de conclusie dat het grootste deel van het hypercholesterolemisch effect van boterolie bij konijnen is terug te voeren op het cholesterol aanwezig in de boterolie. De rol van het cholesterol in de menselijke voeding is bediscussiëerd in hoofdstuk 4.5 ten aanzien van de invloed op serum cholesterol.

De vraag of er een verband bestaat tussen het serumcholesterolgehalte en de graad van atherosclerose is besproken in hoofdstuk 8.4. Er bleek een sterke positieve corre-

latie tussen beide grootheden. De correlatie bleek sterker indien niet het cholesterolgehalte aan het einde van het experiment als variabele werd genomen, maar het gemiddeld cholesterolgehalte over de gehele experimentele periode. Toch bleek het moeilijk de individuele graad van atherosclerose te voorspellen uit het individuele serumcholesterolgehalte. Een verhoogd serumtriglyceridengehalte bleek geen additief effect te hebben op de mate van atherosclerose.

Hoewel het aantal dieren te klein was om definitieve uitspraken te kunnen doen bleek uit fig. 9 – 11 de tendens dat met boterolie gevoederde konijnen iets lagere serumcholesterolconcentraties bezaten dan met cocosolie gevoederde konijnen bij dezelfde graad van atherosclerose. Dit zou erop kunnen duiden dat de cholesterolemie geïnduceerd met boterolie relatief iets meer atherogen werkt dan die geïnduceerd met cocosolie. Er bestaan aanwijzingen dat de door exogeen cholesterol opgewekte cholesterolemie sterker atherogeen is dan die welke wordt opgewekt door vetten. Aangezien ook de met boterolie opgewekte cholesterolemie grotendeels is terug te voeren op exogeen cholesterol zou dit een verklaring kunnen vormen voor de waargenomen relatief sterkere atherogeniciteit van boterolie, vergeleken met cocosolie.

Uit de berekende regressievergelijkingen voor het verband tussen serumcholesterol en graad van atherosclerose kan worden afgeleid dat onder onze omstandigheden een serumcholesterol gehalte van 90 mg/100 ml de ondergrens is waarbeneden geen atherosclerose kan worden verwacht. Overeenkomstige waarden zijn gerapporteerd voor haantjes, ratten en apen.

In experiment 3 zijn een aantal kleine proefjes samengevat welke er op waren gericht door modificaties van het basisdiëet de sterfte onder de dieren te reduceren en de algemene gezondheidstoestand te verbeteren. De toevoeging van 15 gewichtsprocent cocosolie aan een handelsvoer had geen nadelige invloed op de dieren maar verhoogde evenmin het serumcholesterolgehalte. De toevoeging van 10% (w/w) grasmeel aan een semi-synthetisch rantsoen had geen invloed op serumcholesterol, lichaamsgewicht of gezondheidstoestand. De substitutie van 20% zaagsel uit het semisynthetisch voeder door 20% (w/w) grasmeel had geen invloed op sterfte, deed het lichaamsgewicht toenemen, maar deed het serumcholesterol sterk afnemen.

Tenslotte werd het caseïne uit het semi-synthetisch rantsoen vervangen door een mengsel van eiwitten (PM). Dit mengsel werd samengesteld uit zes delen caseïne, vier delen gelatine, zes delen viseiwitconcentraat en vier delen soyaeiwit zodat een aminozuursamenstelling werd verkregen gelijk aan die van commerciële konijnenpellets. Voedselopneming, gewichtstoename en algemene gezondheidstoestand waren beduidend beter met het PM-rantsoen dan met het caseïnerantsoen. Het serumcholesterolgehalte echter neigde tot dalen, hoewel het rantsoen cocosolie bevatte. Uit deze proefjes werd geconcludeerd dat vooral de aard van het eiwit verantwoordelijk zou kunnen worden gesteld voor de effecten van semi-synthetische diëten op lichaamsgewicht, serumcholesterol, mortaliteit en algemene gezondheidstoestand.

In experiment 4 werd de invloed van de aard van het eiwit verder onderzocht, naast de invloed van verscheidene vetten. Indien bovengenoemd eiwitmengsel (PM) werd gevoederd, had slechts cocosolie een geringe, zij het consistente hypercholesterol-

emische werking. Boterolie, met sojaolie gesupplementeerd boterolie of imitatie-boterolie, al dan niet met toegevoegd cholesterol, hadden deze werking niet, ook niet na een proefduur van meer dan een jaar. Met deze PM-rantsoenen trad vrijwel geen sterfte op. Overplaatsing van met een eiwitmengsel gevoederde konijnen op een caseïnediëet had een snel gewichtsverlies en een scherpe stijging van het serumcholesterol tot gevolg. Deze stijging van het serumcholesterol verliep sneller dan wanneer caseïne vanaf het begin werd gevoederd.

Vele van de met caseïne gevoederde dieren welke stierven tijdens het experiment bleken extreem lage Hb-waarden te bezitten. Het rantsoen bevatte ruimschoots ijzer en B-vitamines.

Uit dit experiment werd de conclusie getrokken dat het PM-rantsoen de gevoeligheid van konijnen voor hypercholesterolemie verlaagde, zodat nog slechts de sterk hypercholesterolemische cocosolie de serumlipiden kon verhogen. Verschillen tussen de vetten in het caseïnerantsoen waren moeilijk vast te stellen ten gevolge van tamelijk hoge sterfte onder juist de gevoelig reagerende dieren. Indien hiermede rekening werd gehouden kon geen verschil worden aangetoond tussen met boterolie gevoederde konijnen en die welke bovendien sojaolie kregen. Dit bevestigt literatuurgegevens en het is bediscussiëerd dat de cholesterolesters van met boterolie gevoederde konijnen, welke erg weinig linolzuur en veel oliezuur bevatten, relatief sterker atherogeen werkzaam zijn dan die van met sojaolie gesuppleerde dieren. Zelfs de cholesterolesters van met cocosolie gevoederde dieren bevatten meer linolzuur dan die van met boterolie gevoederde konijnen. De hypothese is opgesteld dat dit misschien de in vergelijking met cocosolie iets sterkere atherogeniteit van het serumcholesterol van met boterolie gevoederde konijnen zou kunnen verklaren.

In experiment 5 werd een meer gedetailleerde studie gemaakt van de effecten van verscheidene eiwitmengels in combinatie met cocosolie op het lipidenmetabolisme. Met caseïne verloren de dieren gewicht, met een caseïne-gelatinemengsel bleef het gewicht constant. Met meer complex samengestelde eiwitmengsels, waaraan respectievelijk viseiwit of viseiwit en soja-eiwit waren toegevoegd werd een identieke gewichtstoename bereikt. Aangezien het caseïne-gelatinemengsel betere resultaten gaf dan caseïne alleen, en caseïne alle aminozuren in grotere hoeveelheden bevat dan de voorgestelde behoefte van het konijn werd geconcludeerd dat een absolute deficiëntie aan een aminozuur niet waarschijnlijk was. De mogelijkheid bestaat dat een relatief geringe overmaat van enkele aminozuren bij het konijn leidt tot een verminderde voedselopneming. Het serumcholesterol had juist een tegengesteld verloop aan dat van de lichaamsgewichten met slechts kleine verschillen tussen de twee meest complexe eiwitmengsels. Een mengsel van dierlijke eiwitten kan derhalve even effectief het serumcholesterolgehalte laag houden als plantaardige eiwitten, hetgeen in tegenspraak is met de resultaten van Hamilton & Carroll (1974) voor afzonderlijke eiwitten.

Serumlipoproteïnen werden onderzocht met behulp van papier- en polyacrylamidegelelectroforese en met de ultracentrifuge. De α-lipoproteïnen blijven constant, terwijl bij een matige stijging van de serumlipiden de β-lipoproteïnen stijgen en bij een sterke stijging tevens de pre-β-lipoproteïnen. De lipoproteïnen van alle konijnen op

semi-synthetische rantsoenen bevatten grote hoeveelheden cholesterol en minder triglyceriden dan die van konijnen gevoederd met het handelsvoer. Het is aangetoond dat de lipoproteinen sterk lijken op die van met cholesterol gevoerde konijnen en die van patiënten van type III volgens de classificatie van Fredrickson. De hypothese is opgesteld dat de zeer grote gevoeligheid van het konijn voor atherosclerose gerelateerd zou kunnen zijn aan de onderlinge verhouding en samenstelling der lipoproteïnen.

In dit experiment werd bij hogere serum-cholesterolgehalten een toename waargenomen van de verhouding vrij/totaal cholesterol. Dit zou kunnen wijzen op een relatief tekort in de cholesterolveresteringsactiviteit. Aangezien dit veresteringssysteem ook wordt verondersteld betrokken te zijn bij de afvoer van overtollig weefselcholesterol naar de lever werd de hypothese opgesteld dat een deficiëntie in dit systeem zou kunnen leiden tot accumulatie van cholesterol in onder andere erythrocyten. Dit zou kunnen leiden tot een verhoogde hemolyse-neiging en anaemie. Bepaling van de veresteringssnelheid in serum (lecithine : cholesterol acyltransferase activiteit; LCAT) toonde aan dat in absolute zin meer cholesterol werd veresterd naarmate het serumcholesterolgehalte toenam. De fractionele veresteringssnelheid nam echter af. De maximale veresteringssnelheid (V_{max}) werd bereikt bij een concentratie van vrij cholesterol in het serum van ongeveer 100 mg/100 ml, welke concentratie in de met caseïne gevoederde konijnen werd overschreden. Deze resultaten bevestigen de gegevens in de literatuur dat in verscheidene diersoorten, waaronder de mens, de LCAT-activiteit toeneemt met de serum-cholesterolconcentratie. Bij konijnen wordt de V_{max} echter snel bereikt. Of dit als oorzaak kan worden beschouwd voor de vaak waargenomen anemie in hypercholesterolemische konijnen blijft speculatief.

Het mechanisme van de door semi-synthetische diëten geïnduceerde hypercholesterolemie en van de verschillende werking van de verschillende eiwitten in deze rantsoenen in vergelijking met handelsvoer is onderzocht door middel van een cholesterolturnoverstudie met 4-^{14}C-cholesterol. Het kon bevestigd worden dat ook het cholesterolmetabolisme van het konijn verloopt volgens een twee-'pool'-model. Alle experimentele konijnen scheidden evenveel steroïden uit, maar slechts half zoveel als de met handelsvoer gevoederde dieren. De omvang van de snel mengbare 'pool' A was het grootst bij de met caseïne gevoederde dieren en het kleinst bij de met handelsvoer gevoederde konijnen. De uitkomsten wijzen op een defect in de verwijdering van steroïden uit het lichaam indien een semi-synthetisch diëet wordt gegeven.

Met handelsvoer gevoerde konijnen kunnen aanzienlijk meer cholesterol uitscheiden. De reden voor dit verschil is niet duidelijk. Een reductie in de galproduktie en afscheiding is onwaarschijnlijk gezien het hoge vetgehalte in de semi-synthetische diëten. Een verhoogde reabsorptie van cholesterol en galzuren lijkt meer waarschijnlijk. Dit zou ook de aanwezigheid van cholesterolrijke lipoproteïnen in serum kunnen verklaren. Verhoogde re-absorptie kan het gevolg zijn van een verminderde darmpassagesnelheid van de excreta, of van de afwezigheid van steroïde-bindende stoffen in het sprijsverteringskanaal of een combinatie van beide.

Verschillen tussen de effecten opgewekt door verschillende eiwitten zijn minder duidelijk. De met caseïne gevoerde konijnen accumuleren meer cholesterol in de snel

mengbare 'pool' A terwijl voedering van gemengd eiwit leidt tot een accumulatie van cholesterol in de langzamer mengbare 'pool' B, evenals het handelsvoer. De met caseïne gevoerde konijnen waren extreem mager terwijl de met gemengd eiwit gevoederde dieren meer vetweefsel bezaten. Dit vetweefsel kan functioneren als een opslagplaats voor de overmaat aan cholesterol uit 'pool' A. Hieruit zou geconcludeerd kunnen worden dat ook in konijnen welke met gemengd eiwit zijn gevoederd, de omvang van 'pool' A zal toenemen zodra de hoeveelheid cholesterol in 'pool' B maximaal is geworden. De zeer langzame maar consistente stijging van het serumcholesterol indien cocosolie werd gevoerd in combinatie met gemengd eiwit, lijkt deze conclusie te bevestigen.

Het semi-synthetisch diëetmodel lijkt zeer bruikbaar voor het onderzoek van die factoren in de voeding welke de steroïde-resorptie beïnvloeden. Toekomstig onderzoek zal dit aspect betreffen, evenals effecten van specifieke aminozuren.

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