THE ROLE OF DIETARY PROTEINS IN ATHEROSCLEROSIS

by

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A thesis submitted to the University of Southampton for the degree of Doctor of Philosophy, Department of Nutrition, April, 1981.

TO MY DEAR PARENTS

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

NUTRITION

Doctor of Philosophy

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A series of experiments were carried out to investigate the role of dietary proteins in atherosclerosis with particular emphasis on the involvement of dietary protein in the immunological induction of atheroma and in cholesterol metabolism.

Experiments 1 and 3 failed to produce complicated arterial lesions in rabbits by the combined effects of cholesterol feeding and serum antibody production to dietary protein. Clearly, the magnitude of the immune response to dietary protein was insufficient to mimic the effect of cholesterol feeding coupled with the immunological injury induced by serum sickness or graft rejection.

The most significant observation to emerge from this part of the study was the development of immune tolerance to a given dietary protein in weanling rabbits induced by exposing their dams to the particular protein during the pre-weaning period. It was not possible to identify the nature of the tolerizing factor nor the critical stage of exposure i.e. in utero or during lactation.

Several experiments were designed to investigate the hypocholesterolaemic effect of vegetable proteins. The possible role of plant saponins as the effective agents in soya bean induced hypocholesterolaemia was investigated. The increased excretion of faecal sterols due to saponin was not associated with a reduction in serum cholesterol. Furthermore, in studies with rats and hamsters fed hypercholesterolaemic diets (1% cholesterol + 0.25% cholic acid) there was no evidence that the dietary fibre (cellulose) reduced the serum cholesterol and no evidence to suggest that the addition of saponin to the diets altered the result. It was therefore concluded that whereas saponin may reduce the absorption of cholesterol, it does not alter the effect of dietary protein or fibre on serum cholesterol. It was also found that at nitrogen intakes of near maintenance, the hypocholesterolaemic effect of soya was still evident, although somewhat reduced.

The nutritive value of methionine supplemented soya bean and cow's milk protein were compared in young rabbits. There was no evidence that nitrogen digestibility, nitrogen balance or the utilization of absorbed nitrogen differed between the two protein sources.

From the above studies it may be concluded that dietary protein may exert a significant effect on the development of atherosclerosis partly through its effect on cholesterol metabolism and partly through its possible immunological injury.

ACKNOWLEDGEMENTS

I am deeply indebted to Professor T.G. Taylor and Dr. M.J. Gibney for their invaluable advice, careful supervision and guidance during the course of this investigation and during the preparation of this thesis.

I am also grateful to Professor Taylor for provision of the excellent research facilities in this department and the Ministry of Overseas Development of the U.K. for the financial support.

I would also like to express my gratitude to Dr. P.J. Gallagher for his help and guidance in the pathological aspect of this thesis and Mrs. Glenda North for her efficient typing.

The cooperation and assistance of the staff of the animal house is also gratefully acknowledged.

Finally, I thank my parents for their continued support and encouragement throughout my formal education and my husband for his patience and support.

ABBREVIATIONS

g	-	gram
mg	-	milligram
µg	-	microgram
mmol	-	millimole
l	-	litre
ml	-	millilitre
µl	-	microlitre
i.u.	-	international unit
kg	-	kilogram
d	-	day
O.D.	-	optical density
min.	-	minute
h.	-	hour
sec.	-	seconds
^{o}C	-	degrees Centigrade
ELISA unit	-	Enzyme linked immunosorbant assay unit
%	-	percentage
NB	-	nitrogen balance
ADN intake	-	Apparent digestible nitrogen intake
N_F	-	faecal nitrogen
N_u	-	urinary nitrogen
N_I	-	Nitrogen intake
MJ	-	megajoule
m	-	metre
mm	-	millimetre

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CHAPTER 1

1. INTRODUCTION

Atherosclerosis is a pathological condition affecting the intima and inner part of the media of both large elastic arteries and certain muscular arteries. The disease is both proliferative and degenerative[1]. It takes the form of focal intimal thickenings or plaques containing lipids, complex carbohydrates and fibrous material which often encroach on the lumen of the vessel and, particularly when complicated by thrombosis and calcification may lead to impairment of the arterial circulation and ischaemia in the tissues supplied (2, 3, 4).

The most basic lesion of atherosclerosis is thought to be a small, exclusively intimal focal accumulation known as the fatty streak, consisting primarily of lipid laden macrophages known as foam cells, surrounded by lipids[5]. These lesions have been recorded in the arteries of fetuses onwards and controversy still clouds any relationship that may exist between fatty streak and raised fibro-lipid plaque. In recent years attention has been directed to the possible role of small drop like elevation of the arterial intima called the gelatinous lesion[5]. This is translucent and either colourless or pink. Histologically, they consist of oedematous separation of somewhat thickened collagen. The role of these lesions in atherosclerosis is uncertain. The fibrolipid plaque is regarded as

the archetypal lesion in atherosclerosis and its frequency in different population groups constitute a good predictor for the incidence of occlusive arterial lesions[6, 7]. The plaque is considerably elevated above the surface of the intima and consists essentially of yellow lipid laden smooth muscle cells surrounded by lipid and collagen, covered by a connective tissue cap of variable thickness. In some lesions the cap is the predominant element which imparts a pearly white appearence to the lesion. In this fibro-lipid lesion the degenerative and proliferative changes extend into the media. The lesion known as the complicated lesion appears to be a fibrous plaque that has become altered as a result of haemorrhage, calcification, cell necrosis and mural thrombosis[8].

Many hypotheses have been put forward concerning the pathogenesis of atherosclerosis.

Filtration theory

The hypothesis originally termed imbibition hypothesis[9] has undergone extensive modification right up to the present time[10, 11]. Filtration theory as it is called now is based on the concept that the metabolic needs of most of the arterial wall are served by continuous diffusion of fluid through it from the lumen towards the lymphatic channels of the adventitia, where it is carried away. Under normal circumstances most of the lipid molecules entering via the percolating fluid is carried away into the draining lymphatics. When, however, the blood is loaded with large lipid molecules, some of these

molecules may not filter through to the adventitia, but may accumulate in the wall and provoke a reaction in the tissues around them. The arterial accumulation of lipid may result from an increased rate of uptake of plasma lipid[11], from lack of an efficient system for lipid removal or from both.

Endothelium

The presence in the atherosclerotic plaque of large quantities of plasma-derived products suggest that the process of atherogenesis must be accompanied by either focal increase in the permeability of the endothelial barrier or a decline in efficiency of the mechanisms that clear plasma derived molecules from the artery wall.

A variety of animal species have been shown to have focal areas in the aortic intima that demonstrate increased permeability to various protein bound dyes[12] and labelled lipids [13]. If the endothelial permeability and intimal lipid deposition go hand in hand, the logical correlate of this proposition is to consider the range of structural alterations in the endothelium that may lead to the increased insudation of plasma derived substances and the factors that may bring about such alterations.

Endothelial injury. A number of injurious stimuli have been identified as capable of mimicing experimentally vascular cell responses similar to those presumed to occur during initial stages of atherosclerosis. They include, mechanical injury;

e.g. focal mechanical abrasion of the intima[14, 15], extensive baloon catheter denudation[16, 17, 18], desiccation[19], chemically induced injury; e.g., homocystinaemia[20, 21] or endotoxinaemia[22, 23], dietary induced hyperlipidaemia alone or in combination with mechanical injury[24]; experimentally altered haemodynamics[25, 26]; immune mediated mechanisms[27, 28].

In general most of these "injury models" share in common, varying amounts of endothelial desquamation, which is associated with basically similar histological pattern of intimal cell reaction, that is, smooth muscle cell proliferation, intra- and extra-cellular deposition of lipid and accelerated synthesis of connective tissue components, glycosaminoglycans, collagen and elastin. The disruption of the intimal lining with some of these methods is probably more abrupt and extensive than could occur naturally.

Recent experiments have shown that intimal thickening due to new tissue formation occur beneath areas of repair rather than in areas denuded of overlying endothelium[29]. Lipid accumulation in medial cells is also most marked beneath areas of re-endothelialization[30]. Thus the functional status of the endothelium appears to be more important than its physical presence or absence[31].

Thrombogenic theory

The encrustration theory proposes that atherosclerotic plaques are transformed mural thrombi[32]. This theory has

been modified extensively and is now called the thrombogenic theory. The thrombogenic theory stresses the importance of thrombus formation. It states that thrombi would become incorporated into the wall by an overgrowth of endothelial cells creeping up from the sides and growing over the thrombus [33]. The invasion of vascular cells from underneath would scavenge away the thrombic mass and replace it by scar tissue[21].

In recent years this hypothesis has been further modified with the suggestion that endothelial injury with associated platelet deposition plays a critical role in initiating intimal proliferation, a prelude to atherosclerosis.

During the formation of the thrombus, platelets adhere to the basement membrane which is exposed with de-endothelialization. The collagen component of the basement membrane induces platelet aggregation which is now known to be regulated by the synthesis of a prostaglandin, thromboxane A_2 (TXA_2) from membrane arachidonic acid[34]. The production of TXA_2 induces further platelet adhesion and further TXA_2 production. If sufficient TXA_2 is formed thrombin production is stimulated. This in turn induces the formation of fibrin which encapsulates the growing thrombus and prevents further platelets adhesion. Such fresh thrombi being rich in erythrocytes are red in colour. The thrombus may be dislodged or may be covered by endothelial cells. The breakdown of erythrocytes eventually give rise to a white mural thrombus, the basis of an atherosclerotic plaque.

It should be noted that the intact endothelium produces a prostaglandin called prostacyclin (PGI_2) which inhibits platelet

aggregation[35, 36].

The method by which fat is deposited in lesions formed in this mechanism is unclear.

Response to injury hypothesis

This hypothesis encompasses the two previous theories.

Factors such as hyperlipidaemia[24], hormone dysfunction[21], immune reactions[28], increased shear stress in hypertension[25, 26], may injure the endothelium and alter the nature of the endothelial barrier to the passage of blood contents to the arterial wall. This action alters the endothelial cell to cell, cell to connective tissue reactions and possibly detach endothelial cells from the arterial wall. Focal desquamations of endothelium caused in this way expose underlying sub-endothelial connective tissue to platelet and other elements in circulation. The platelets adhere to the sub-endothelial collagen, aggregate[37, 38, 39] and release contents of their granules[38]. The massive infiltration of platelet factors[40, 41], plasma lipoproteins and possibly other plasma constituents such as hormones at these sites of injury leads to focal proliferation of arterial smooth muscle cells, to formation of large amounts of connective tissue matrix by these cells and to deposition of lipids both within the cells and in their surrounding connective tissue matrix. According to this hypothesis[42] restoration of the endothelial barrier ultimately occurs and the lesions regress if both injury and tissue response to it are limited. However, further proliferation of smooth

muscle cells and accumulation of connective tissue and lipid occur if injury to the endothelium is continuous and repeated [31]. The effect can tip a critical balance between re-endothelialization, cell proliferation and cell destruction and removal that determine whether a lesion enlarges, remains relatively constant in size or regresses. Risk factors possibly affect this balance by causing chronic injury or by interfering with the normal tissue response to injury.

The classic picture of endothelium as a passive anatomical barrier has undergone considerable revision. The working concept of endothelial 'injury' has been similarly broadened to include endothelial dysfunction. Chemically mutagenized or virally transformed endothelial cells have been shown to be highly reactive with platelets *in vitro*. Similarly, 'altered' endothelial cells *in vivo*, conceivably could result in a localized release of platelet products into the vessel wall in the absence of frank desquamation [29, 43].

Immunological theory

There is considerable evidence to indicate that immunological injury may be important in the development of atherosclerosis[44]. Synergy of immunological arterial injury and simultaneous dietary hypercholesterolaemia produced aortic lesions in rabbits not seen in controls fed lipid supplemented diets alone[45, 46]. Most lesions of the rabbits on hyperlipidaemic diets given horse serum albumin injections were of the fatty proliferative type, strongly resembling human lesions,

in contrast to purely fatty lesions of the cholesterol fed animal without injections and the proliferative lesions of the animals given injections alone[47]. Similar observations have been made in swine[48] and in baboons[49] fed hypercholesterolaemic diets. In these experiments, injections of foreign protein produced serum sickness, because it persisted in the circulation until antibody response occurred. It was assumed that antigen then combined with antibody forming pathogenic immune complexes. After repeated injection of antigen antibody response is immediate. Serum sickness can accelerate development of arterial desease in animals[49]. Immune complex desease induced by vasectomy led to atheroscelrosis in Macaca fascicularis fed lipid rich diets[50]. Vasectomy was also associated with increased atherosclerosis in normocholesterolaemic non human primates fed lipid poor monkey chow over a long period[51].

Mechanism of immunological injury

It has been shown that permeability of blood vessels to macromolecules is increased by vasoactive amines and histamine[52] released from mast cells and platelets by the action of immune complexes[53]. In the rabbit, immune complexes formed in antibody excess cause complement dependent accumulation of activated platelets, neutrophils and mast cells in lung capillaries and induce anaphylaxis[54]. This leads to lysis of cells [55]. The discharge of vaso-active amines causes an increase in vascular permeability and contraction of smooth muscle cells.

Non-immune neutrophil mediated inflammatory reactions may also involve complement[56], and thus the involvement of complement in the pathogenesis of atherosclerosis has been out forward[57]. Complement is composed of a system of nine easily activated proteins which circulate in blood. The system can be activated partly specifically by immune complexes or unspecifically by enzymes[58], aggregates of macromolecules [59], lipopolysaccharides, lipoproteins[60, 61], viscosity and turbulence[62]. Experimentally induced infections have been shown to produce arterial damage similar to atherosclerosis[63] and evidence has been produced that activated complement can cause arterial damage[64].

Immunological injury can occur without the participation of the complement[65]. In rabbits after an injection of a protein to induce serum sickness, the binding of IgE antibodies to the surface of basophils is known to occur. These sensitized basophils on re-exposure to the antigen stimulate secretion of platelet activating factor[66] that induces platelet clumping and release of their vasoactive amines[67].

The enhanced permeability of blood vessels cause macromolecules such as immune complexes larger than 19S in the ultracentrifuge[58] to deposit in the artery wall ultimately leading to inflammatory injury. Polymorphonuclear (PMN) leucocytes have been shown to be the final agents that causes the fragmentation of the internal elastic lamina and necrosis[68, 69, 70]. It seems that the internal elastic lamina acts as a barrier to the outward spread of infection and that this is an important

substrate for PMN action.

The damage produced by immune complexes in the absence of lipid feeding results in necrotising arteritis with endothelial proliferation, sub-endothelial fibrinoid deposition, medial necrosis and polymorphonuclear leucocyte infiltration [68, 71]. In the rabbit complement and leucocytes are required for the full arterial damage to occur.

Sensitization of structural antigens of the vessel wall, produced in the cause of degenerative changes on the sites of atherosclerotic lesions, probably enhances the pathologic process[72]. Thus, an auto-immune mechanism has been put forward for the pathogenesis of atherosclerosis[73]. Arterial lesions have been produced in rabbits by immunisation with extracts of rat aorta[74] or heterologous arterial elastin and glycoprotein[75]. Formation of aortic auto-antibodies were observed in animals similarly treated[76]. Auto-antibodies against heart, kidney and spleen were found in rabbits fed cholesterol, although antibody titres were not related to the severity of the lesions[77]. In human arterial disease auto-antibodies against both normal and atheromatous aortas have been reported[78]. These occurred only in patients with advanced disease and it is possible that advanced stages of the disease might be accelerated by auto-antibodies formed as a secondary phenomenon. There is evidence that new antigens appear as a consequence of atherosclerotic change which are immunogenic[79].

Immunization of animals with foreign proteins, β-lipoprotein, γ-globulin, tetanus toxoid etc. have been observed to lead to

hypercholesterolaemia[80, 81, 74]. Attempts to inhibit atherosclerosis by injection of heterologous β-lipoprotein has been successful in some experiments[82]. Hyperlipidaemia of immune origin has been reported in multiple myeloma patients[83]. Lipoprotein auto-antibodies have been found in cases of hyperlipidaemia and atherosclerosis[84, 85, 86, 73]. Cholesterol may function in vivo as a hapten which when coupled to the protein produces a specific immune response[87].

Conditions like syphilis, rheumatoid heart disease, systemic lupus erythematosus and organ transplantation may be associated with vascular injury, arteritis or unusually severe atherosclerosis[88, 89, 90]. There is evidence to indicate that arterial injury in these conditions are immunologically mediated.

Risk factors in atherosclerosis

Various factors have been epidemiologically identified to indicate an increased risk of clinical aspects of arterial disease.

These can be broadly classified into two categories: dietary and others. Virtually all nutrients have been accorded some attention as to a possible relationship with heart disease. These include dietary fat, cholesterol, fibre, protein, carbohydrate, minerals and vitamins. The other factors include hypercholesterolaemia, hypertension, diabetes mellitus, obesity, smoking, heredity, maleness, certain bacterial and viral infections in certain individuals as well as certain personality characteristics, and lack of exercise.

PLASMA LIPIDS. Many of the above risk factors operate at least in part through a tendency to be associated with high serum cholesterol levels. Epidemiological studies have found that coronary heart disease (CHD) patients have high serum cholesterol concentrations than control patients[91, 92]. Persons living in countries where coronary heart disease is frequent have higher serum cholesterol concentrations than individuals living in countries where CHD was rare[93]. The strongest and most consistent 'risk factor' identified in the Framingham study was elevated serum cholesterol level. However, serum total cholesterol has a predictive value for the future development of CHD only in younger persons.

The distribution of cholesterol in the various lipoprotein fractions has been given considerable attention. Plasma high density lipoprotein (HDL) concentration has been found to be lower in patients with CHD than in age matched controls in epidemiological studies[94, 95, 96]. Thus high levels of HDL appear to provide protection against CHD at all levels of total serum cholesterol. However, this association of low HDL level with increased coronary risk seems to be established only within defined population groups. Also, the mechanism by which HDL could protect against CHD is not clear[97].

Low density lipoprotein (LDL) was recognised as the lipoprotein species whose elevation in plasma was most strongly correlated with atherosclerosis[98, 99]. The mechanism by which high LDL levels in plasma could lead to atherosclerosis has been explained by means of the LDL pathway[100].

LDL PATHWAY

The evidence indicates that human cells including aortic smooth muscle cells possess an elaborate biochemical pathway, the LDL pathway, that regulates the uptake, storage and synthesis of cholesterol and thus protects the cells from an overaccumulation of this sterol. When this regulatory mechanism is disrupted, as it is in patients with certain genetic disorders, a most severe form of human atherosclerosis develops.

Binding of LDL is the first step in the pathway, the cells take up LDL by endocytosis, utilize its cholesterol and suppress their own endogenous synthesis. By regulating the number of cell surface LDL receptors, and through their high affinity for LDL, cells are able to control the rate of entry of cholesterol, thereby assuring an adequate supply to the cells, and also allowing the body to maintain plasma LDL levels below the threshold range for atherosclerosis. When LDL levels are elevated in plasma due to genetic deficiency of LDL receptors as in familial hypercholesterolaemia or whether due to other genetic and/or environmental factors, cholesteryl esters deposit in the artery wall and atherosclerosis develops. It has been suggested that under these circumstances smooth muscle cells take up lipoprotein by a receptor-independent process that is not subject to feed-back regulation. Thus massive amounts of cholesteryl ester accumulate until cellular toxicity results.

DIETARY FACTORS

FAT. The amounts and the type of fat in the diet is thought to be a major risk factor in CHD through its effects on serum cholesterol concentrations[101]. Substitution of vegetable fat for animal fat in the human diet lowered serum cholesterol concentration[102, 103]. Semi-purified diets containing saturated fat were atherogenic in rabbits[104, 105, 106, 107]. This effect was repeatedly demonstrated and was traced to the high proportion of polyunsaturated fatty acids in most vegetable oils[108, 109, 110]. Some of the variable effects attributed to the degree of saturation of a fat or oil may be explained by the chain length of the fatty acid[111]. Unsaturated fatty acids cause a shift of cholesterol from plasma to the tissue pools[112,114]. The metabolic pathways of saturated and unsaturated fatty acids are different and this is thought to have different effects on serum cholesterol levels[113]. The plant sterol component of plant fats, which are structurally similar to cholesterol, decreased cholesterol absorption and may account for some of the effects of polyunsaturated fats. Increased bile and acid excretion due to high dietary polyunsaturated fats has also been reported.

DIETARY CHOLESTEROL

Feeding rabbits on diets containing cholesterol produces arterial lesions[115]. This observation together with the high cholesterol content of both human and rabbit lesions has focussed attention on dietary cholesterol as a possible causative agent

in atherosclerosis.

Despite the consistency with which dietary cholesterol produces hypercholesterolaemia and atherosclerosis in certain experimental animals, studies in humans often failed to show such a close relationship when subjects ingested large amounts of cholesterol. In a series of carefully controlled experiments a linear relationship between dietary cholesterol and serum cholesterol was shown to exist up to an intake of about 400 mg cholesterol per day[116, 117]. Above this level the relationship was much more variable. However, the association between dietary cholesterol and CHD is weak[118]. Susceptibility to cholesterol induced hypercholesterolaemia varies in both animals and man. Animals with extremely low or high responses to dietary cholesterol have been characterised as 'hypo' or 'hyper' responders[119]. Genetically determined metabolic defects in humans which lead to severe hypercholesterolaemia result in atherosclerosis and CHD even in the absence of dietary cholesterol.

CARBOHYDRATE. Ingestion of refined carbohydrates in the form of sucrose has been implicated in CHD[120], although the relationship has not been confirmed. It may be related to the low fibre content of diets rich in high refined carbohydrates. Dextrose in the diet has been found to be more atherogenic than sucrose or fructose in the rabbit[121, 122]. Fructose caused the most severe aortic sudanophilia in baboons[123] and vervet monkeys[124], fed on semi-purified diets in which carbohydrates were fructose, sucrose, glucose or starch.

FIBRE. Dietary fibre has been shown to have a protective role when included in potentially atherogenic diets[125, 126]. Fibre may exert its effect by reducing caloric intake for a given volume of food, decreasing intestinal transit time, altering the intestinal microflora and changing bile acid metabolism[127, 128, 129].

Gel forming or mucilaginous polysaccharide fibres (pectin, guar-gum) fed to animals and human subjects increases bile acid excretion and lowers plasma cholesterol concentration[130, 131, 132, 133, 134, 135]. However, non gel forming mixed dietary fibre including cereal fibre has an inconsistent effect on serum cholesterol of humans[136, 137].

PROTEIN. Dietary protein has been generally considered to be of little significance in the etiology of atherosclerosis in humans, but evidence is accumulating that it may have a significant influence,

(i) by direct effect on cholesterol metabolism

(ii) by inducing the formation of antibodies to dietary proteins which might initiate atherosclerotic process.

(i) THE EFFECT OF DIETARY PROTEIN ON CHOLESTEROL METABOLISM AND ATHEROSCLEROSIS

Many early feeding trials which investigated the use of protein to produce atherosclerosis, used proteins such as meat, milk, egg[138] and powdered beef[139] all of which contained cholesterol which had been shown to produce an arterial disease

in rabbits[140]. Subsequent research produced an arterial disease in rabbits with protein which did not contain cholesterol[141, 142, 143]. It has been consistently shown that protein derived from plant sources are hypocholesterolaemic compared to most animal proteins when fed to rabbits in low fat, cholesterol free semi-purified diets[144, 145, 146, 147], although the effect of particular protein source varied within each of the two categories[148]. The atherosclerosis associated with feeding semi-purified diets based on casein has been prevented by replacement of casein with soy protein isolate[141, 149, 150]. The hypocholesterolaemic effect of soya protein isolate was apparent even when fed in diets containing cholesterol to chicken or swine[151, 152].

High levels of protein were also shown to be more atherogenic and/or hypercholesterolaemic than low levels when fed to pigeons, squirrel monkey, baboon and the rabbit[153, 154, 155, 156] and hypocholesterolaemic when fed to chicken[157].

The effect of dietary protein source on rat serum cholesterol is uncertain. Some reports indicate hypocholesterolaemia in rats due to soya bean protein fed in low fat diets[158] as well as in hypercholesterolaemic diets[159, 160]. There are also reports where protein had no effect on serum or liver lipid levels of rats[161] given low fat diets.

Reported effects of protein source on serum cholesterol of rats is summarised in Table 1.1

Table 1.1

Reference	Protein Source	Cholesterol (%)	Bile Salts (%)	Fat (%)	Protein (%)	Serum Cholesterol (mmol/l)	Liver Cholesterol (µmol/l)
(158) Nagata et al (1980)	Soya	-	-	1	20	2.04	3.28
	Casein	-	-	1	20	2.48	4.07
(159) Katan et al (1980)	Soya	1.2	-	?	20	3.5	-
	Casein	1.2	-	?	20	10.4	-
(160) Yadev et al (1977)	Soya Flour	2	0.5	15	15	4.44	-
	Soya Isolate	2	0.5	15	15	5.36	-
	Navy bean flour	2	0.5	15	15	2.59	-
	Casein	2	0.5	15	15	9.38	-
(161) Sautier et al (1979)	Soya	-	-	7.5	25	2.39	7.09
	Casein	-	-	7.5	25	2.37	7.27

It has been shown that in humans incidence of ischaemic heart disease is equally correlated with the intake of animal protein and of fat[162, 163]. Experimental data also indicate that vegetable protein may be less cholesterolaemic than animal protein in man[164, 165, 166, 167, 168, 169], although the substitution of animal protein by vegetable protein had no significant effect on serum cholesterol levels in healthy adult males

in some short term experiments[170, 171, 172]. In contrast to healthy individuals in a series of experiments on type II A and type II B hyperlipidaemic patients serum cholesterol was lowered in the range of 10-20% by soya diets[173, 169, 167, 168]. This effect was independent of lipid composition of the diet.

Vegetable protein has been shown to have a definite hypocholesterolaemic effect in young infants[174] and it has been shown that the feeding of animal protein (milk) to infants leads to an increase in the plasma cholesterol levels within four weeks[175].

A number of experiments have attempted to elucidate the mechanism by which the protein component of semi-purified diets affect the serum cholesterol concentrations in rabbits and other animals. Rabbits given soya protein have been shown to excrete more neutral sterols[144] or more of both neutral sterols and bile acids in the faeces than casein fed rabbits. Accelerated oxidation and turnover of labelled cholesterol has also been shown in the soya fed rabbit when compared to the casein fed rabbit[176]. This mechanism cannot entirely explain the hypocholesterolaemic effect of vegetable protein. Thus two contradictory hypothesis have been put forward to explain this effect.

(a) Amino acid theory.

This theory proposes that the hypocholesterolaemic property of plant proteins, soya protein in particular, is a consequence of its amino acid composition[177, 178]. It proposes that there are cholesterol lowering and cholesterol raising amino acids.

Several experiments have been performed using soya protein isolate and casein as representative proteins. Both casein and glycinin (principal protein in soya been) support growth. The chief differences in composition of soya bean and casein are,

(1) Arginine content - 15.5% in soya protein
3.8 % in casein

(2) Glycine content - 1% soya protein
0.5% casein

(3) Valine content - 0.7% soya protein
7.9% casein

Feeding trials have been carried out with protein hydrolysates or with mixtures of amino acids in an attempt to determine whether the hypocholesterolaemic response is related to the amino acid composition[179, 180, 160, 146].

Enzymatic digests of casein and soya behaved in a manner similar to their respective intact proteins in the rabbits given low fat, cholesterol free semi-purified diets[180]. However, with amino acid mixtures equivalent to that of casein or soya protein isolate the difference is markedly reduced. The attempts to reverse the observed effect of casein or soya protein by the addition of amino acids to one protein so as to mimic the composition of the other have produced variable results[146, 180]. It appears that the intact protein has an over-riding effect in each case. In a study with rats on hypercholesterolaemic diets, it has been found that an amino acid mixture simulating soya protein, significantly reduces serum cholesterol levels compared to a casein like amino acid mixture[160].

Individual amino acids have also been given considerable attention. High dietary lysine has been reported to be hypercholesterolaemic in rabbits[181] and hypocholesterolaemic in rats[182] and hyperlipidaemic subjects[183]. However, the lysine content of soya protein and casein are similar.

Glycine supplementation reduced casein induced hypercholesterolaemia and atherosclerosis in both rabbit and rat[159]. There is a speculation that the arginine to lysine ratio of protein may affect its action on serum cholesterol and atherogenesis[177, 178]. The Arg/lys ratio of casein is 0.49 and that of soya protein is 1.13. The addition of lysine to soya protein in a quantity sufficient to lower its ratio to the level of casein resulted in a sharp increase in its atherogenicity when fed to rabbits. However, addition of arginine to casein diets had no consistent effect. Similar experiments in rats did not show any effect due to amino acid supplementation[184]. Using mixtures of soya protein and casein in rabbit diets it was possible to bring the plasma cholesterol level to an intermediate value to that obtained with either protein alone. The Arg/lys ratio of this mixture was 0.64. How such a small difference in Arg/lys ratio might affect serum cholesterol concentration is not known[179]. Results of a series of experiments done in rabbits using proteins from various sources as well as amino acid mixtures in different combinations have shown that an interaction between essential and non essential amino acids might be important in determining plasma cholesterol levels[180].

A number of human studies have demonstrated that dietary amino acid composition can influence the level of plasma cholesterol[185, 186, 187, 188]. In this instance, the source of non essential nitrogen and ratio of essential to non-essential amino acids were shown to be important with respect to plasma cholesterol levels.

(b) Saponin theory

The saponin theory suggests that the hypocholesterolaemic action of soya and other plant proteins is due to their saponin content.

Saponins are sterol or triterpene glycosides mainly of plant origin. They occur in a wide variety of plants, a few of which are used as human food e.g.: soya bean, chick pea, peanut and spinach. The amphiphilic nature of saponins dominate their physical properties in solution. They are strongly surface active, forming stable foams and acting as emulsifying agents[189]. They generally have a strong haemolytic activity and appear to form micelles in much the same way as detergents. The structure of a typical saponin from soya bean is shown in Fig. 1.1.

Although saponins are practically non-toxic to man when taken orally[190] they are known to have a number of physiological effects on animals of which most interesting is that they can lower plasma cholesterol concentrations. The cholesterol lowering action of saponin has been reported in cholesterol fed chicken[191, 192, 193], monkey[194], rat[195] and in the pig[196] on normocholesterolaemic diets. However, with diets where

exogenous cholesterol was not provided saponin did not show this effect[161] in the rat.

The mechanism by which saponin produced such effects are not completely established saponins have been shown to adsorb bile acids to dietary fibre *in vitro*[197]. This mechanism might operate *in vivo* as well as *in vitro* since increased excretion of bile acids accompanied by decreased serum cholesterol levels were observed in rats[195] and pigs[196] given saponin supplemented diets. Saponins may also act by reducing cholesterol absorption as shown in rats given hypercholesterolaemic diets[198].

It has been suggested that this effect may also occur in humans and that foods rich in saponins may reduce the risk of heart disease[199, 200, 194].

(ii) ANTIGENICITY OF FOOD PROTEINS AND THEIR RELATIONSHIP TO HEART DISEASE

It is clear that some proteins in the diet survive digestive process and cross the gastro-intestinal epithelium in immunologically reactive form[201, 202]. Absorption of whole protein from the intestinal lumen of the neonatal animal[202] as well as in the adult animal[204, 205, 205, 207] including man[208], has been shown.

The intestinal antigens stimulate the cells in the gut associated lymphoid tissue (GALT) to proliferate and differentiate into IgA bearing lymphoblasts. These cells on maturation act as IgA secreting plasma cells on the intestinal mucosa and

produce IgA in response to intestinally absorbed antigens, thus preventing further absorption of antigen[209, 210, 211]. The studies also suggest that a selective transport of IgA producing plasma cells from the GI tract to the mammary gland occur after oral immunization of the mother. Since the newborn infant is deficient in secretory antibodies ingestion of colostrum may provide passive mucosal protection of the gastrointestinal tract against penetration of intestinal organisms and antigens. Results of other studies have shown that orally induced immune response could be nearly identical to those obtained by parenteral immunization with the same antigen, i.e. mainly IgM and IgG antibodies[212]. Accordingly proteins such as horse serum albumin[210], horse raddish peroxidase[202], and bovine serum albumin[211] have been shown to produce a systemic response when given orally. Dietary soya bean protein is particularly effective this way and significant systemic immune responses have been reported in calves[213], pigs[214] and rabbits[215].

Antibodies produced against some common foods have been detected in healthy children[216, 217, 218, 219]. An allergic reaction to food protein has been displayed in large number of infants under the age of one[220, 221, 222, 223] and in small children[224]. It has been observed that the severity of allergy is maximal in children and decreases with age[225]. Allergy to soya protein is also detected in infants[226, 227], children[228], and adults[229].

Most of the abnormal arteries in infants examined at autopsy were found in cow's milk fed infants, whereas most breast fed infants had normal arteries[230]. Alternately, heart disease was found to be more prevalent in humans who had been fed cow's milk than those who were breast fed[231]. Significantly elevated levels of serum antibodies to milk and egg proteins have been reported in patients with IHD compared to controls[232]. However, subsequent studies failed to reproduce the elevated serum anti-milk antibody levels in IHD patients using the techniques originally employed, passive haemagglutination[233] and more sensitive radioimmunoassays[234] or the enzyme linked immunosorbant assay[235].

Epidemiological data indicate that highest mortality rate from CHD is found in countries where per capita intake of total milk is highest[236]. A correlation also has been found between the introduction of holder pasteurization where milk is heated for a long period of time and incidence of CHD[237, 238].

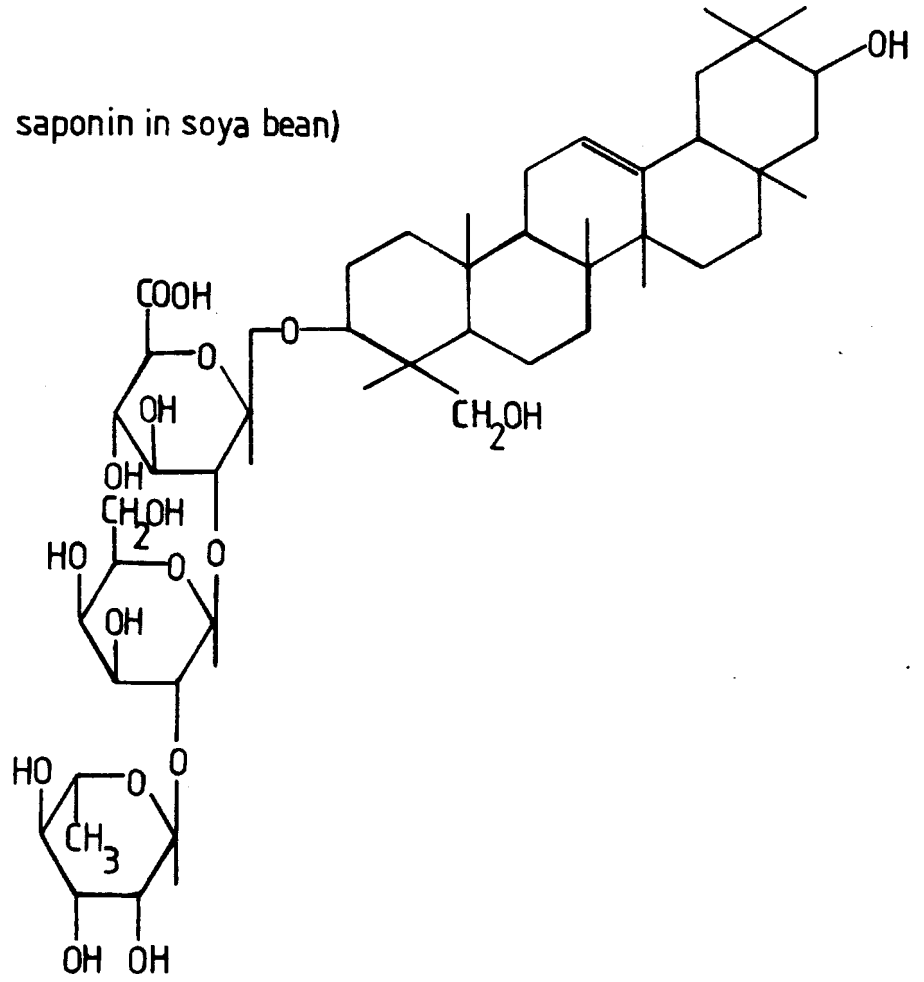
The pathogenic form of the immune response was thought to be the soluble immune complexes formed in antigen excess. Circulating immune complexes have been found to be more prevalent in bottle fed than in breast fed infants and could be important in CHD in later life[239]. Many dietary, bacterial viral or even endogenous antigens could be involved in the formation of immune complexes.

Auto-immune complexes containing β-lipoprotein as antigens and IgA, IgM, IgG as antibodies have been identified in the plasma of atherosclerotic patients[83, 86, 73], and in rabbits

fed atherogenic diets[240]. The occurrence of immune complexes were greater in patients with vascular disease than in control patients[241].

Hypersensitivity to extracts of tobacco had been described and this suggests that allergy to constitutents of tobacco may underlie the relationship between smoking and CHD and peripheral vascular disease[242, 243].

FIG. 1,1 SOYA SAPONIN 1 (major saponin in soya bean)



CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

New Zealand White rabbits both male and female from the University of Southampton colony were weaned from a commercial stock diet (Labsure animal foods, Christopher Hill Group Limited, P.O. Box 6, Castle Street, Poole) and were randomly allocated to different experimental groups. The rabbits were individually housed in stainless steel metabolism cages whenever faecal and urine collections were necessary.

Rats used in experiments were albino Wistars from the University of Southampton colony. They were selected for experiment on a weight basis and were housed in groups of 5 or 6 in each cage. PMD stock diets were fed for one week prior to weaning to the experimental diets.

Male Golden Syrian hamsters from the University of Southampton colony were weaned on to experimental diet from the PMD stock diet. Animals were selected for experiment on the basis of similar weight and housed in groups of 6 per cage.

Weekly records of food intakes and body weights were kept on all animals. Humidity was maintained at about 50% by twice daily washing of the animal room floor. Temperature of the rooms was maintained at 21^{o} and lighting followed a 12 hour light-dark cycle. All animals were fasted overnight prior to collection of blood, but otherwise were allowed free access to food

and water throughout the experiment.

2.2 Diets

The experimental diets were prepared by mixing the dry ingredients in a Hobart mixer and pelleting at 10% moisture. Pellets were 3 x 20 mm and were dried overnight at 60°C in a forced draught oven. The compositions of the diets used are given in Tables 2.1 to 2.10. The compositions of the mineral and vitamin mixtures are given in Tables 2.11 and 2.12.

Table 2.1 Stock diet RAG-(M)*

	Content g/kg diet
Maize	175
Wheat feed	322
Oat feed	100
Apple pomace	100
Soya	88
Linseed	50
Grass	100
English white fish	50
RAG Premix etc.	15

Metabolizable energy = 7.85 MJ/kg
Crude protein = 178.7 g/kg diet

*Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6, Castle Street, Poole, Dorset.

Table 2.2 Stock diet RAG*

	Content g/kg diet
Maize	175
Wheat feed	360
Oatfeed	100
Apple pomace	100
Linseed	50
Grass	100
English white fish	100
RAG Premix etc,	15

Metabolizable energy = 7.85 MJ/kg
Crude protein = 178.7 g/kg

*Labsure Animal Foods, Christopher Hill Group Ltd., P.O. Box 6 Castle Street, Poole, Dorset.

Table 2.3 Composition of basal diets (g/kg).
Experiment 1.

	Casein diet	Soya diet
Casein	303	-
Isolated soya bean protein (Promine D)	-	300
Bran	197	197
Coconut oil	177	177
Maize starch	140	140
Sucrose	98	98
DL - Methionine	1.4	5.9
*Mineral mixture 2.10.1	74	-
2.10.2	-	72
**Vitamin mixture 2.11.1	-	10
2.11.2	10	-

Metabolizable energy = 17 MJ/kg
Energy from fat = 42%
Energy from protein = 30%
Energy from carbohydrate = 28%

Cholesterol supplementation 0 or 10 g/kg

* Final dietary concentration, per MJ; Ca, 674 mg; P, 552 mg; Na, 670 mg; K, 671 mg; Mg, 266 mg; Cl, 623 mg; I, 250 µg; Fe, 13.5 mg; Mn, 9 mg; Zn, 6 mg; Cu, 1131 µg; Cr, 53 µg; Mo, 84 µg; Se, 45 µg.

** Final dietary concentration, per MJ; Vitamin A, 600 µg; Vitamin D_3, 120 µg; Vitamin E, 18 mg; Choline chloride, 103.5 mg; Nicotinic acid, 3.106 mg; Calcium pantothenate, 1.55 mg; Pyridoxine HCl; 518 µg; Riboflavin, 518 µg; Thiamin HCl, 210 µg; Biotin, 1515 µg; Folic acid, 155 µg; Vitamin B_{12}, 2.06 µg; Myo-inositol, 104 µg.

Casein - Express Dairy Foods Ltd., South Ruislip, Middlesex.
Promine D - Central Soya, Chicago Ill, USA.
Maize Starch - C.P.C. (UK) Ltd., Trafford Park, Manchester.
Bran Wheat - Christopher Hill Group Ltd., Poole, Dorset.
Coconut Oil - Keira Trading Company, West Midlands.
Cholesterol - BDH, Poole, Dorset.

Table 2.4 Composition of diets (g/kg).
Experiments 2 and 3.

	Soya Protein	Milk Protein
Promine D	302	-
Dried Skim Milk	-	486
Casein	-	126
Lactose	253	-
Bran	197	196
Methionine	5	1.4
Maize Oil	170	164
Minerals 2.10.4	64	-
2.10.3	-	19
Vitamins 2.11.1	9	-
2.11.2	-	8

Metabolizable energy	=	16.9 MJ/kg
Energy from protein	=	31%
Energy from fat	=	40%
Energy from carbohydrate	=	29%

Final dietary concentrations of minerals and vitamins per MJ are similar to experiment 4.

Dried Skim Milk - Unigate Foods Ltd., Poole, Dorset
Lactose - BDH, Poole, Dorset.
Maize oil - refined, deodorised - BOCM Silcock Ltd., Stoneferry Road, Hull.

Table 2.5 Composition of basal diets (g/kg).
Experiment 4.

	Cow's Milk Protein	Isolated Soya Protein
Promine D	-	265
Dried Skim Milk	416	-
Casein	114	-
Bran	120	120
Safflower oil	3	5
Coconut oil	5	7
Lactose	-	222
Sucrose	39	39
Starch	277	274
Methiomine	1.3	5
*Minerals 2.10.3	17	-
2.10.4	-	55
**Vitamins 2.11.1	-	7.4
2.11.2	7.4	-

Metabolizable energy	= 14.6 MJ/kg
Energy from protein	= 30%
Energy from fat	= 5%
Energy from carbohydrate	= 65%
Saponin supplementation	0 or 10 g/kg

* Final dietary concentration, per MJ; Ca, 718 mg; P, 642 mg; Na, 415 mg; K, 590 mg; Mg, 117 mg; Cl, 214 mg; I, 270 µg; Fe, 15 mg; Mn, 7 mg; Zn; 8 mg; Cu, 975 µg; Cr, 55 µg; Mo, 97 µg; Se, 55 µg.

** Final dietary concentration, per MJ; Vitamin A, 600 µg; Vitamin D_3, 120 µg; Vitamin E, 18 mg; Choline chloride, 103.53 mg; Nicotinic acid, 3.11mg; Calcium pantothenate, 1.55 mg; Pyridoxine HCl 518 µg; Menephthone, 210 µg; Riboflavin, 518 µg; Thiamin HCl, 210 µg; Biotin, 15.5 µg; Folic acid, 155 µg; Vitamin B_{12}, 2.06 µg; Myo-inositol, 104 µg.

Table 2.6 Composition of diets (g/kg)
Experiment 5.

Dietary constituents	1	2	3	4
Promine D	284.0	281.0	268.0	271.0
Methionine	4.9	4.9	4.6	4.7
Maize Oil	8.4	8.4	8.0	8.1
Coconut Oil	84.0	84.0	80.0	80.0
Starch	954.0	450.0	428.0	432.0
Sucrose	105.5	104.0	100.0	101.0
†Vitamin 2.11.3	5.3	5.3	5.0	5.1
‡Minerals 2.10.5	41.0	40.0	39.0	39.0
Saponin	-	10.0	9.0	-
Cholic acid	2.5	2.5	2.4	2.4
Cholesterol	10.0	10.0	9.0	10.0
Solka floc	-	-	47.0	48.0

Metabolizable energy	=	17 MJ/kg
Energy from protein	=	26%
Energy from fat	=	54%
Energy from Carbohydrate	=	20%

‡ To provide per MJ energy; Ca, 387 mg; P, 322 mg; Na, 323 mg; K, 322 mg; Mg, 32.3 mg; Cl, 32.3 mg; I, 12.4 µg; Fe, 3.23 mg; Mn, 3.23 mg; Zn, 3.2 mg; Cu, 320 µg; Se, 3 µg; Fe, 30 µg; Mo, 3 µg.

† To provide per MJ energy; Vitamin A, 650 µg; Vitamin D, 38.8 µg; Vitamin E, 3.9 mg; Thiamin HCl, 260 µg; Riboflavin, 320 µg; Vitamin B_6, 390 µg; Nicotinic acid 650 µg; Calcium pantothenate, 780 µg; Biotin, 17 µg; Folic acid, 50 µg; Vitamin B_{12}, 0.32 µg; Choline chloride, 60 mg; Inositol, 160 mg; Vitamin K, 100 µg.

Solka floc - Johnsen, Jorgensen & Wettre Ltd., London
Cholic acid - BDH, Poole, Dorset.
Saponin - BDH, Poole, Dorest.

Table 2.7 Composition of diets (g/kg)
Experiment 5.

Dietary constituents	1	2	3	4
Promine D	284.0	281.2	268.0	270.5
Methionine	4.9	4.9	4.6	4.7
Maize Oil	8.4	8.4	8.0	8.1
Coconut Oil	84.0	84.0	80.0	80.4
*Cod Liver Oil	3.0	3.0	2.6	2.7
Starch	454.0	450.0	428.0	432.0
Sucrose	106.0	104.0	80.0	101.0
†Vitamin mixture 2.11.4	2.4	2.4	2.4	2.4
‡Mineral mizture 2.10.6	41.0	40.0	39.0	39.0
Saponin	-	10.0	9.0	-
Cholic acid	2.5	2.5	2.4	2.4
Cholesterol	10.0	10.0	9.0	10.0
Solka floc	-	-	47.0	48.0

Metabolizable energy = 17 MJ/kg

†To provide per MJ energy; Tocopherol, 1100 µg; Menadione, 260 µg; Thiamin HCl, 260 µg; Riboflavin, 260 µg; Pyridoxine HCl, 260 µg; Calcium pantothenate, 1.76 mg; Nicotinic acid, 880 µg; Choline chloride, 40 µg; Folic acid, 90 µg; Biotin, 4.4 g; Vitamin B_{12}, 2.2 µg; Inositol, 40 µg; Para-amino benzoic acid, 10 mg.

*Provide 600 I.U. Vitamine A and 850 I.U. of Vitamin D per g.

‡To provide per MJ energy; Ca, 380 mg; P, 320 mg; K, 360 mg; Mg, 41 mg; Na, 410 mg; Cl, 243 mg; Fe, 13.9 mg; I, 1.18 mg; Mn, 2.88 mg; Zn, 294 µg; Cu, 147 µg.

Table 2.8 Composition of diets (g/kg)
Experiment 6.1

Constituent	Promine D diet	Promosoy diet
Promine D	332	-
Promosoy 100	-	426
Bran	150	150
Oil	190	190
Starch	164	61.5
Sucrose	100	100
†Minerals 2.10.4	54	54
†Vitamins 2.11.1	10	10
Methionine	6.0	4.2
KCl	2.2	-
NaCl	-	4.3

Metabolizable energy	=	18.6 MJ/kg
Energy from protein	=	31%
Energy from carbohydrates	=	27%
Energy from fat	=	42%

† Final dietary concentrations of minerals and vitamins are the same as those given for experiment 2.

Promosoy 100 - Central Soya, Chicago Ill, USA.

Table 2.9 Composition of diets (g/kg).
Experiment 6.

	Soya Protein	Cow's Milk Protein
Promine D	83.0	-
Casein	-	36
Dried Skim Milk	-	130
Methionine	1.4	0.5
Sokla floc	188	192
Safflower Oil	7	7
Coconut Oil	10	10
Sucrose	77	77
Maize Starch	510	509
*Minerals 2.10.4	51	-
2.10.3	-	32
Lactose	66	-
*Vitamins 2.11.1	7.4	-
2.11.2	-	7.0

Metabolizable energy	=	12.84 MJ/kg
Energy from protein	=	10%
Energy from carbohydrate	=	85%
Energy from fat	=	5%

*Final dietary concentrations of minerals and vitamins are the same as those given for Experiment 2.

Table 2.10 Mineral mixtures (g/kg mix)

Salt	1	2	3	4	5	6
KIO_3	0.1	0.11	0.4	0.12	0.009	-
$CuSO_4.5H_2O$	1.05	1.09	0.76	0.88	0.55	0.3
$MnSO_4.H_2O$	2.75	2.85	-	-	-	-
$MnSO_4.4H_2O$	-	-	19.23	7.79	5.684	5.0
$ZnSO_4.7H_2O$	4.78	4.95	12.61	8.88	6.152	-
$Na_2MoO_4.2H_2O$	0.05	0.06	0.2	0.05	0.003	-
$FeSO_4.7H_2O$	12.7	9.29	33.49	12.69	0.561	-
Cr $K(SO_4)_2.12H_2O$	0.12	0.12	0.46	0.13	-	-
$Na_2SeO_4.10H_2O$	0.05	0.06	0.21	0.06	0.005	-
K_2HPO_4	65.47	-	-	-	-	322.23
KH_2PO_4	-	250.31	-	152.9	-	-
KCl	245.89	15.67	-	104.76	-	-
$CaCO_3$	143.22	313.12	231.98	242.26	96.56	299.75
$Mg(OH)_2$	109.02	104.67	60.73	27.54	33.55	-
$CaHPO_4$	365.3	58.95	639.93	254.94	433.14	-
NaCl	49.5	238.75	-	-	11.64	167.36
$NaHCO_3$	-	-	-	131.6	112.29	-
K_2SO_4	-	-	-	55.2	299.84	-
NaF	-	-	-	-	0.025	-
$CaHPO_4.2H_2O$	-	-	-	-	-	74.94
Ferric citrate $.6H_2O$	-	-	-	-	-	27.48
KI	-	-	-	-	-	0.8
$ZnCl_2$	-	-	-	-	-	0.25
$MgSO_4.7H_2O$	-	-	-	-	-	101.91

Table 2.11 Vitamin mixtures(mg/kg mix)

	1	2	3	4
Biotin	26.9	10.4	56.9	40.0
Folic acid	268.6	254.2	171.7	830.0
Inositol	179.0	192.3	547190.3	414740.0
Nicotinic acid	5371.1	5096.9	2145.8	8290.0
Calcium panto-thenate	2685.5	994.9	2575.0	16590.0
Pyridoxine HCl	895.2	739.2	1287.5	2490.0
Vitamin A (Rovimix A) 500,000	1017.2	1092.7	2145.8	-
Vitamin D (Rovimix D) 500,000	203.5	219.2	128.8	-
Rovimix E_{25}	31514.7	33857.9	12875.1	10370.0
Menanthone K	358.1	384.6	321.9	2490.0
Vitamin B_{12}	3.6	-	1.1	20.0
Riboflavin	895.2	-	1072.9	2490.0
Thiamin HCl	358.1	188.0	858.3	2490.0
Maize Starch	777185.7	834988.6	214584.5	-
Choline chloride	179037.5	121981.1	214584.4	414740.0
p-amino benzoic acid	-	-	-	124420.0

2.3 Analytical Methods

2.3.1 Collection of Samples

2.3.1.1 Serum

Fasting blood samples were taken from the marginal ear vein of the rabbit and were allowed to clot in the centrifuge tube at room temperature. They were then spun at 1000 g for 35 min in a bench centrifuge (MSE).

Rats and hamsters were killed by over-exposure to anaesthetic ether. The thorax was immediately opened to expose the heart. Blood was collected in a syringe by cardiac puncture and was allowed to clot in a centrifuge tube. Serum was separated as described for rabbit serum.

Serum samples were stored at -20°C or -70°C until required for analysis.

2.3.1.2 Tissue studies

Rabbits were killed by injection of excess of nembutal. The heart and aorta were removed en bloc and preserved in phosphate buffered formalin (PBS - Section 2.3.8.1) or buffered glutaraldehyde. Samples of carotid, renal arteries and kidney removed and fixed in buffered formalin for histological examination. The livers of rabbits, rats and hamsters, were removed, blotted dry with tissue paper, weighed and were then wrapped in aluminium foil, frozen in liquid nitrogen and stored in a deep freeze at -20°C.

Samples of kidney from the rabbits in Experiment I were also stored in the deep freeze for lipid analysis.

2.3.1.3 Faeces and Urine

The faeces of animals housed in metabolism cages were collected every 24 h and stored at 4°C. The samples for 7 days were pooled, dried in a force draught oven at 60°C for 24 h, powdered and stored until required.

Urine was collected in 30 ml of 2.5% (V/V) hydrochloric acid every 24 h, the samples and the cage washings being diluted to a convenient volume and stored at 4°C until ready for analysis. A seven day pooled sample was used for analysis.

2.3.2 Separation of Serum Lipoproteins

2.3.2.1 Separation of very low density (VLDL), low density (LDL) and high density lipoprotein (HDL) fractions of serum.

Each serum sample (3 ml) was placed in a series of 10 ml ultracentrifuge tubes which were then filled with NaCl (9 g/l). The caps were replaced and the tubes centrifuged (100,000 g) in the MSE-Superspeed 65 preparative ultracentrifuge for 16 h at 2°C. The turbid upper layer containing chylomicrons and VLDL was removed using a Pasteur pipette and made up to 5 ml in volumetric flasks. The infranate solutions were also diluted to 5 ml in volumetric flasks. Both fractions were extracted in isopropanol for cholesterol and triglyceride analysis of chylomicron + VLDL and HDL + LDL.

2.3.2.2 Preparation of HDL fraction from serum or HDL + LDL fraction of serum by method of Warnick and Albers (1978).

A working heparin manganese solution was prepared by adding 0.6 ml sodium heparin solution (40,000 USP units/ml - 280 mg/ml) to 10 ml of 0.53 M $MnCl_2$ solution. To 1.0 ml of serum or LDL + HDL fraction of serum was added 0.1 ml of heparin manganese solution with thorough mixing which produced a final heparin concentration of approximately 1.4 µg/ml and a Mn^{2+} concentraion of 0.046 M. The samples were incubated at least 10 min at room temperature and centrifuged at 1500 g for 30 min. Clear supernatant solutions containing HDL were recovered for lipid analysis by pipetting. Supernatant solutions that remained turbid after centrifugation were again subjected to centrifugation at 12,000 g for 10 min and the clear subnatant solution was recovered by pipetting with a fine tipped Pasteur pipette.

2.3.2.3 Preparation of liver homogenate

A known weight of liver (approximately 1 g) was homogenised in a glass homogenising tube containing 10 ml of distilled water. Aliquots of the homogenate (100 µl) were analysed for total cholesterol and triglyceride by an automated procedure.

2.3.3 Cholesterol and Triglyceride analysis

Serum total, VLDL, LDL + HDL, and HDL cholesterol and tissue cholesterol were determined either manually or by an automated procedure. Triglycerides in all samples were determined using

the autoanalyser II. LDL cholesterol concentration was calculated as the difference between the concentration in LDL + HDL fraction and the concentration in the HDL fraction.

2.3.3.1 Manual method for cholesterol determination

The method of Zlatkis, Zak and Boyle (1953) modified by Bowman and Wolf[245] (1962) was employed for manual determination of serum cholesterol. The method estimates cholesterol colorimeterically and is claimed to be four to five times more sensitive than the original Liebermann-Burchard colour reaction.

Serum (100 µl) was delivered into the bottom of a glass stoppered centrifuge tube containing 1.9 ml absolute ethyl alcohol. The protein precipitate that forms must be as finely divided as possible. The tube was stoppered, shaken vigorously for 10 sec. and centrifuged in a bench centrifuge at 1000 g for 10 min. The colour reagent was made as follows: 2.5 g $FeCl_3.6H_2O$ was dissolved in 100 ml pure 87% orthophosphoric acid and 8 ml of this solution was diluted to 100 ml with conc. H_2SO_4 just before using. 1.5 ml of the colour reagent was added in a slow stream so that the reagent forms a layer below that of alcohol. The contents were immediately swirled for 15 seconds in a vortex shaker. The test tubes and their contents were left at room temperature for 25-30 min. The contents of the tubes were read on a Pye-Unicam SP 500 spectrophotometer at 550 nm in plastic cuvettes of 1 cm light path. A standard curve was constructed over the range 100 µg - 500 µg per ml. All readings were made against a blank containing 1 ml absolute alcohol, 0.5 ml water and 1 ml colour reagent.

2.3.3.2 Automated procedure for cholesterol and triglyceride determination

Samples for the autoanalyser II (Technicon Instruments Corp. Ltd., Tarrytown, N.Y., 1972) were prepared by pipetting 100 µl of serum or liver homogenates into 1.9 ml of 99% isopropanol and 2.0 g zeolite (Sigma Chemicals, St. Louis, Miss.) in disposable 2.5 ml plastic tubes. The tubes were stoppered, shaken in a votex mixture and spun at 1000 g for 10 min. The supernatant was decanted into stoppered polyethylene vials for cholesterol and triglyceride analysis by the automated procedure. (Technicon Clinical Methods, No. 16A, September 1972, No. 24, March 1972).

2.3.4 Faecal sterol extraction

Weighed samples of ground faeces (dried at 60°C for 24 h) were extracted in a Soxhlet extractor with 75 ml ethanol: acetone (1:1 V/V) for 14-18 h. The contents of the flask were transferred to a 100 ml volumetric flask and the final volume was made up to 100 ml with ethanol:acetone solution.

Neutral and acid sterols were separated in accordance with the method of Wells and Makita (1962)[246].

The faecal extract (20 ml) was transferred to a glass universal bottle and the ethanol:acetone solvent evaporated in a hot water bath. Ten millilitres of 1.25 M NaOH solution was added to the residue and the contents of the bottle were well mixed and autoclaved at 15 p.s.i. for 3 h. When cool the contents of the bottle were transferred to a 100 ml separating funnel

using three 5 ml washing with distilled water to complete the transfer. Two fractions were prepared.

2.3.4.1 Neutral sterol extract

The contents of the separating funnel were extracted three times with 50 ml portions of diethyl ether. The ether extracts were collected in a round bottom flask and the solvent evaporated under vacuum. The solid sterol fraction remaining after evaporation was redissolved in 1 ml dry diethyl ether (dried with 20 Å molecular sieves) with two further washings performed with 0.5 ml diethyl ether. The neutral sterol fraction was stored at 4°C in a 2.5 ml stoppered vial.

2.3.4.2 Acid sterol extract

The aqueous mixture remaining after ether extraction was acidified with 1.2 M HCl until the solution was at pH 1.0 - 1.5. The precipitate of acid sterol was extracted with three, 10 ml portions of diethyl ether using a 100 ml separating funnel and the extracts were collected in a round bottom flask. Diethyl ether was evaporated under vacuum, the dry residue redissolved in 1 ml diethyl ether and 50 µl absolute ethanol and the sterol extract transferred into a 2.5 ml glass vial using two further 0.5 ml ether washes. The vial was stoppered and stored at 4°C.

2.3.4.3 Gas-liquid chromatography (G.L.C.) of neutral sterols.

G.L.C. analyses were performed on a Pye-Unicam 104 chromatograph using a glass column, 2.17 m long and 2 mm internal

diameter. The column was packed with 3% OV-1 on a support of diatomite CQ, 100-200 mesh. Nitrogen was used as an inert carrier gas (flow rate 15 ml/min). A flame ionization detector was employed (air pressure 15 p.s.i., hydrogen 25 p.s.i). The column oven temperature was 270°C, detector temperature 300°C and injection chamber temperature 220°C. The accuracy of the temperature settings was confirmed by thermocouple readings from the respective chamber.

Application of Samples

Standards and samples were dissolved in diethyl ether. 500 µl of each sample was pipetted into 2 ml glass vials followed by 10 µl internal standard (20 µg/µl of androsten-17β-ol-3-one propionate). The contents of the vial were evaporated and redissolved in 250 µl of diethyl ether. Sample injection was performed with 10 µl glass Hamilton syringe using a double barrel needle and 2 µl of each sample was injected. Variability of the injected volume was corrected by incorporation of the internal standard.

Visual recordings of the chromatographed peaks were made and each peak was simultaneously integrated by an Infotronics autoinjection digital integrator. Individual peaks were identified using purified standards.

2.3.4.4 Gas-liquid chromatography of bile acids

The bile acids used for all analysis were methylated with a solution of diazomethane in diethyl ether.

Preparation of diazomethane

To 100 ml of ether was added 30 ml of potassium hydroxide (400 g/l) and the mixture is cooled to 5°C. To this, with continued cooling and shaking was added 10 g finely powdered nitroso-methyl-urea in small portions over a period of 1-2 min. The deep yellow ether layer was decanted readily, dried by using pellets of potassium hydroxide. No respiratory contact was made with diazomethane.

Methylation

Diazomethane solution (500 µl) was added to each sample of faecal bile acids dissolved in 2 ml of diethyl ether. The mixture was left overnight in unstoppered vials avoiding strong light. The methylated solution was filtered through charcoal and the final volume of the solution was made up to 2 ml with diethyl ether.

Sample application

Methylated bile acid sample (500 µl) was placed in 2 ml vials. Cholesterol acetate (400 µg per 500 µl sample) was used as an internal standard. The samples (2 µl) were injected into the column with a Hamilton syringe as described in section 2.

Gas liquid chromatography

The samples were run with a column temperature of 270°C, detector temperature of 300°C and an ionization chamber temperature of 220°C. The same column as for the neutral sterol

separation was used. Individual peaks were identified using purified standards.

2.3.5 Determination of Total Nitrogen

Aliquots of faeces (approx. 1 g) or urine (2 ml) were digested in a 500 ml Kjeldahl flask for 2 hours with 20 ml of concentrated H_2SO_4 and 2 catalyst tablets (one Se_2O_3 and the other Na_2SO_4 and $CuSO_4.5H_2O$) and a glass bead.

A 50 ml conical flask containing 5 ml Boric acid (20 g/l) with 3 drops of mixed indicator (1 part of methyl red, 0.1% in ethanol + 3 parts bromo cresol green, 0.1% in ethanol + 4 parts of distilled water) was placed under the receiving end of the condenser in a Kjeldahl distillation unit. An aliquot of sample (sufficient to give a 2 ml titration with $\frac{N}{100}$ HCl) was pipetted into the still followed by 8 ml of concentrated NaOH (400 g/l) and distilled for exactly 4 min. The distillate was titrated against 0.01 N HCl.

2.3.6 Tissue Lipid Analysis

Total tissue lipids were analysed by the method of Folch, Lees and Sloane-Stanley (1957)[247].

A known weight of the tissue (approximately 1 g) was homogenised in a glass homogenising tube containing 2:1 chloroform, methanol mixture (V/V). The homogenate was filtered and the final volume made up to 20 ml. 2 ml of water was added to it, mixed and the tube capped with aluminium foil and left until complete separation into two phases was obtained.

The upper phase was removed with a suction arrangement and inside wall of the tube was rinsed with 1.5 ml of solvent mixture (pure solvent upper phase ≡ 3:48:4 chloroform, methanol, water) and upper phase again removed. This rinsing of the tube wall and interphase with pure solvent upper phase was repeated twice.

Finally, the lower phase and remaining rinsing fluid were made into one phase by the addition of methanol and the resulting solution was diluted to any desired final volume by the addition of 2:1 chloroform:methanol mixture. A measured volume of this extract was evaporated to dryness in a stream of nitrogen in weighed Erlenmeyer flasks to a constant final weight. The amount of tissue lipid was expressed in mg per g of tissue.

2.3.7 Gross Energy Value

Gross energy value was determined on a dried pelleted sample (approximately 1 g) using a Gallenkamp adiabatic bomb calorimeter.

2.3.8 Antibody Assays

2.3.8.1 Passive haemagglutination assay

This assay utilises the principle that cross linking of cells by antibody directed against surface antigens results in agglutination. The cells used here are chicken cells coated with food proteins after first modifying their surface with tannic acid. A modification of an earlier haemagglutination assay was used[248].

Preparation of formalinised chicken erythrocytes

Chicken blood was drawn into 40 g/l sodium citrate (1:9 V/V with 0.1 M phosphate buffered saline (PBS) pH 7.2 containing 8.0 g/l NaCl; 0.2 g/l KCl; 1.15 g/l Na_2PO_4; 0.2 g/l KH_2PO_4). The citrated cells were then washed with PBS until the supernatant was clear and the cells were then suspended at a 7% suspension in PBS. An equal volume of 7% fermaldehyde solution in PBS was prepared and one quarter of this was added to the cell suspension. The mixture was incubated with gentle shaking for one h at 37°C and the remaining formalin solution was added. The suspension was further incubated for 24 h at 37°C with occasional shaking. The cells were then washed ten times in PBS and resuspended as a 18% solution in PBS containing 1 g/l sodium azide as an anti-bacterial agent, and stored at 4°C.

Tanning and antigen sensitisation of erythrocytes

The formalinised cells were washed and resuspended in PBS as a 3.75% solution. Equal volumes of cells and 50 mg/l tannic acid (Sigma Chemicals, St. Louis, Miss.) in PBS were mixed and incubated for 15 min. at 37°C. The cells were then washed three times in PBS and resuspended as a 3.75% solution. One volume of 3.75% tanned cells was mixed with one volume of 0.5 mg/l water soluble soya protein (see next paragraph) in PBS and incubated for 30 min. at 37°C with occasional mixing. The cells were then washed once in PBS and resuspended as a 2.5% solution in PBS containing 1% normal horse serum. The horse serum had been previously heat inactivated for 30 min. at 56°C and absorbed against tanned chicken erythrocytes.

Preparation of water soluble soya proteins

A 5 g/l soya protein (Promine D) solution in distilled water was stirred overnight at room temperature. The suspension was allowed to settle and the supernatant was then decanted and spun for ½ h at 20,000 g on a centrifuge (MSE 18). The supernatant was freeze-dried and stored at 4°C in vacuuo.

Assay

Serum samples were taken and stored at -20°C until used. Serum was thawed and heated in 0.05 ml aliquots at 56°C for 30 min to destroy complement. It was then incubated with formalinised chicken erythrocytes for 15 min at room temperature to absorb any non-specific agglutinating agents. The cells were then centrifuged for 10 min at 600 g and 0.05 ml supernatant diluted 1:2 in PBS was used for serial dilutions on V-shaped micro-haemagglutination plates (Flow Labs, Ayrshire). To these cells was added 0.05 ml sensitized antigen coated erythrocytes. Control wells were also set up containing 0.05 ml supernatant and washed non-sensitized erythrocytes or washed sensitized cells and 0.05 ml PBS. The cells were left to agglutinate for 2-3 h at room temperature and then read. The antibody titre of the serum was given as the reciprocal of highest dilution producing the carpet pattern. The cells at the bottom of control wells were button-like in appearance (Plate 2.1).

2.3.8.2 Enzyme-linked immunosorbant assay (ELISA)

The method used was essentially that of Engvall and Perlmann (1972)[249].

Polystyrene tubes coated with antigen were incubated with appropriate dilutions of antiserum. After washing, the tubes were incubated with an anti-globulin enzyme conjugate. After further washing the tubes were assayed for enzyme activity (Fig. 2.1).

Preparation of conjugate

Peroxidase labelled antibody (Goat anti-rabbit IgG) was prepared according to the method of Avrameas and Ternynck (1971) (250).

Horse radish peroxidase (10 mg) was dissolved in 0.2 ml of 0.1 M phosphate buffer (pH 6.8) containing 1.25% glutaraldehyde. This solution was allowed to stand for 18 h at room temperature and then filtered through a sephadex G.25 column (60 x 0.9 cm) equillibrated with 0.15 M NaCl. The brown coloured fraction containing the activated peroxidase was pooled and concentrated if needed to 1 ml. To this solution, 1 ml of 0.15 M NaCl containing 5 mg of antibody was added followed by the addition of 0.1 ml 1M carbonate buffer (pH 9.5). After 24 h at $4^{o}C$, 0.1 ml of 0.2 M solution of lysine was added and allowed to stand for an additional 2 h. The preparation was then dialysed at $4^{o}C$ against several changes of buffered saline. The labelled antibody preparation was precipitated at $4^{o}C$ with an equal volume of saturated neutral solution of ammonium sulphate. The pre-

cipitate was washed with half saturated ammonium sulphate solution, dissolved in a minimum volume of distilled water and exhaustively dialysed against buffered saline. The solution was centrifuged at 20,000 g for 20 min and stored at $4^{o}C$.

Assay - Method I

Stoppered disposable polystyrene tubes (LP_3) were incubated with 1 ml of 0.2 g/l soluble soya protein or 0.6 g/l dried skim milk in 50 mM carbonate buffer (pH 9.6) containing 0.02% azide for 3 h at $37^{o}C$ and were stored overnight at $4^{o}C$.

Coated tubes were washed five times with saline (9 g/l) containing (0.5 g/l) Tween 20 (Saline T) and filled with 1 ml of diluted serum in PBST (PBS, pH 7.3 containing 0.5 g/l Tween 20 and 0.2 g/l azide) and incubated at room temperature for 5 h. The tubes were then washed five times with saline T and 1 ml of 1:500 dilution of conjugate in PBST was added and incubated at room temperature overnight. The tubes were washed a further five times with saline T and then assayed for enzyme activity for exactly 1 hour.

Hydrogen peroxide (1 ml containing 300 g/l) was diluted to 100 ml with distilled water and this stock solution was stored at $4^{o}C$. To use, 1 ml of stock H_2O_2 was diluted to 100 ml with 0.01 M phosphate buffer (pH 6.0) (prepared fresh). The dye used was O-dianisidine-hydrochloride in water. The dye (0.05 ml) was added to 6.0 ml substrate and of 10 mg/ml 1 ml of this assay mixture was added to each tube. The tubes were incubated at room temperature for exactly 1 h. The tubes were then vortex

mixed and the optical density at 460 nm of the contents was measured.

The results of the antibody titres were expressed in ELISA units where 1 ELISA unit was defined as the production of 1 µg of oxidized substrate per ml of serum per h. The O.D. range used was 0 to 0.8 and samples with absorbance above this value were diluted until the absorbance fell on to the log linear range.

Assay - Method 2

Polystyrene cuvettes (Gilford Inst. Lab.) were coated with the antigen by incubation with 200 µl of soluble soya protein (10 mg/l) or dried skim milk (30 mg/l) in 50 mM carbonate buffer (pH 9.6) containing 200 mg/l azide, in a humidity chamber overnight at $4^{o}C$.

Cuvettes thus coated were washed five times with PBST, with 30 sec soaking intervals between washes. The serum (200 µl), appropriately diluted in PBST, was added to each cuvette and incubated for 2 h at room temperature in the humidity chamber. The cuvettes were then washed five times as before and 200 µl of a 1:2000 dilution of the conjugate (horse radish peroxidase x Goat anti-rabbit IgG (H + L), Nordic Immunology) was added and incubated at room temperature for 2 h in the humidity chamber. The cuvettes were washed a further five times with PBST and then assayed for enzymic activity for exactly 15 min.

The electron donor dye used in this assay was O-phenelene diamine.

The assay mixture was prepared by dissolving 40 mg of O-phenelene diamine in 100 ml of freshly prepared 0.05 M citric acid/$NaHPO_4$ buffer (pH 5.0) followed by the addition of 40 µl of stock H_2O_2 solution.

Assay mixture (200 µl) was added to each cuvette and incubated at room temperature for exactly 15 min, at the end of which time the reaction was stopped by the addition of 200 µl of 0.3 M citrate/phosphate buffer (pH 2.85). The optical density (optical density range 0 - 1.5) of the contents were read at 405 nm in the EIA manual reader (Gilford Inst. Co.). The results for antibody levels were expressed as percentage of the values obtained for rabbit injected with the same protein in an immunisation programme.

2.3.9 Immune Complex Detection

This assay utilizes the fact that immune complexes fix complement. It is called an anti-complementary assay [251]. Serum samples either fresh or after storage at -70°C were heated at 56°C for 30 min in 0.1 ml volumes. 2.5 CH_{50} units of guinea pig complement was added to each sample and incubated for 30 min at 4°C (one CH_{50} unit of complement was the amount which caused 50% lysis of the sheep red blood cells). 3% red blood cells (Oxoid Limited, Basingstoke, Hants) sensitized with haemolytic serum (Wellcome Reagents Ltd., Beckenham, Kent) were added to the mixture and incubated for 15 min at 37°C. The samples were then diluted to 1 ml with complement fixation diluent (CFD - Oxoid Ltd., Basingstoke, Hants), and after

centrifugation at 600 g for 5 min, the haemolysis in the supernatant was measured at 450 nm on a spectrophotometer (SP600). Using the standard curve constructed for known amounts of complement, the loss in haemolytic activity due to immune complexes was determined. The amount of complement consumed provides a measure of immune complexes present in the serum.

2.3.10 Atherosclerotic Scoring System

The heart and the whole aorta was removed from the rabbit and the internal surface was subdivided into five section as follows:

(1) The aortic valves and the aorta around the coronary arteries.

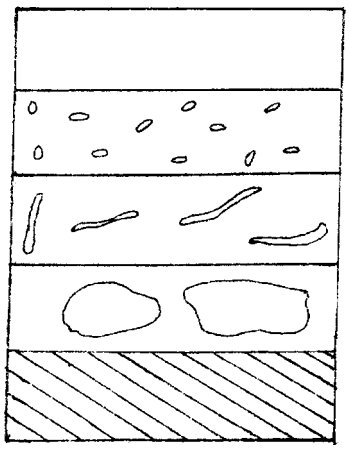
(2) The aortic arch with the orifices of the innominate and carotid arteries.

(3) The aorta around the ductus arteriosus scar.

(4) The thoracic aorta.

(5) The abdominal aorta.

Each region was then scored individually with grades 1-5 as follows[252].

Score	Condition	Macroscopic appearance of aorta
1	No Atheroma	
2	Flecks - small raised plaques	
3	Fatty streaks	
4	Larger patches of calcified atheroma	
5	Whole aorta calcified and ulcerated with atheroma	

2.3.11 Histological Methods

Samples of renal and carotid arteries and aortae from each animal were fixed in 10% buffered formalin or 6% cocodylate buffered glutaraldehyde for histological examination. The tissues were then processed into paraffin wax and haemaloxylin and eosin and elastic van-Gieson stained sections prepared.

2.3.12 Statistical Analysis[253]

The data were examined by two way analysis of variance and means compared by Tukey's test. Mean values for aorta scores were compared by a non-parametric (Mann Whitney U-test) method. Other statistical methods including regression analysis, calculation of correlation coefficients and analysis of co-variance were applied as necessary and noted in the appropriate experimental sections.

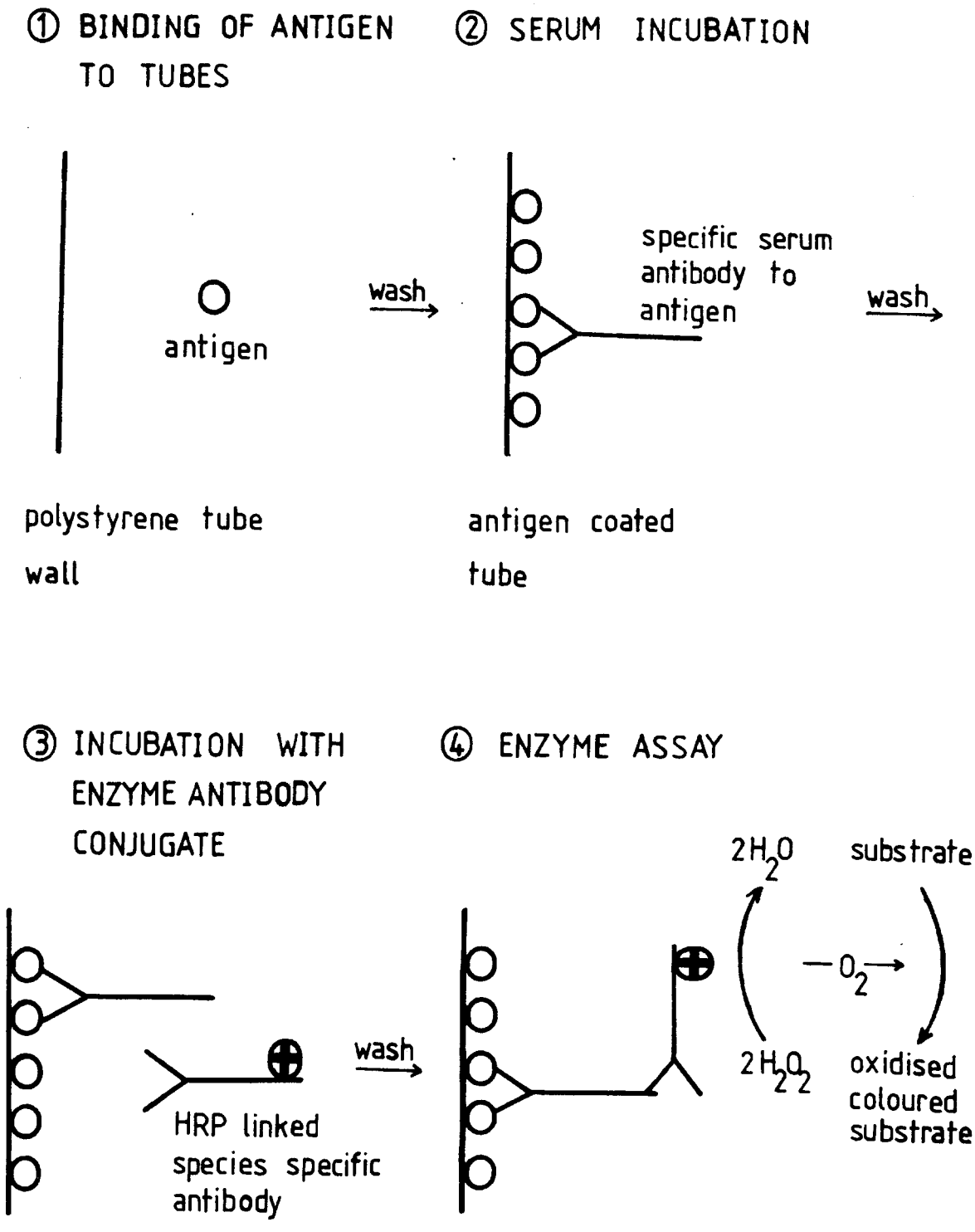


FIG. 2,1 ENZYME-LINKED IMMUNOSORBANT ASSAY

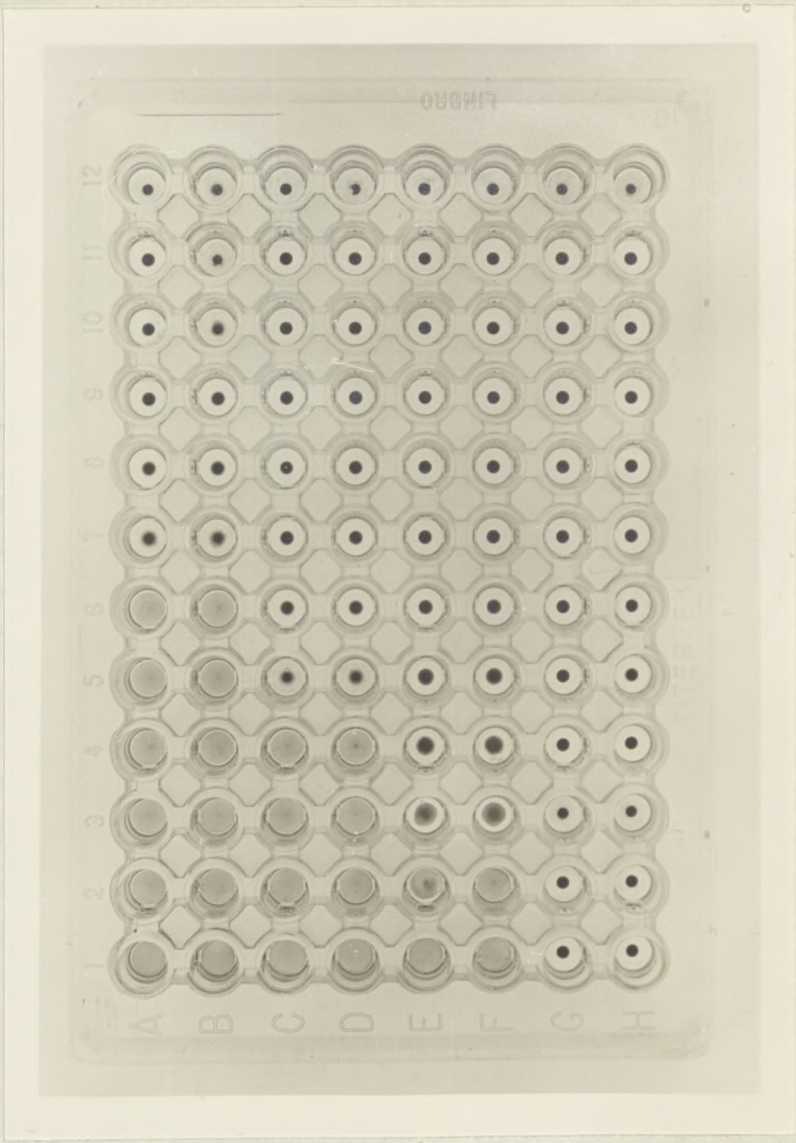


Plate 2.1 Perspex agglutination plate showing results of titration of three sera (in duplicate) carried out by the tanned cell technique employing formalin preserved chicken red blood cells. The end points are, respectively A and B - 1:320; C and D - 1:80; E and F - 1:20. The final well (12) in each row from A to F contain 1:10 dilution of the serum with uncoated cells.

Rows G and H were used to check that both kinds of cells are giving proper negative readings. G = row of coated cells in PBS alone. H = row of uncoated cells in PBS alone.

CHAPTER 3

EXPERIMENT 1

3.1 Introduction

Although the cholesterol fed rabbit is a widely used experimental model of atherosclerosis, the histological appearance of the arterial lesions bears little resemblance to the complicated atheromatous plaques responsible for human cardiovascular disease. However, the simultaneous induction of experimental serum sickness hastens the development of cholesterol atherosclerosis[254] and alters both the microscopic appearance and distribution of lesions[47].

It has also been shown that an immune response of dietary origin is also effective in producing atheromatous lesions of the human type in rabbits, by feeding an antigenic soya protein to rabbits over a period of twelve months[215]. This model had the disadvantage of requiring a relatively long period to produce significant disease. The objective of the present study was to determine whether dietary proteins could elicit sufficient antibody response to modify the histological appearance of cholesterol atherosclerosis, developed over 90 days.

3.2 Materials and Methods

Ten male and ten female New Zealand White rabbits (mean live weight 2.1 $\pm$ 0.2 kg) were weaned from a commerical stock diet (Table 2.1) and allocated in groups of five, to one of four

isonitrogenous and isoenergetic diets where dietary variables were protein source and cholesterol supplementation (Table 2.3). Protein was supplied as sodium isolates of either casein (Casumen) or soya bean protein (Promine D) and cholesterol was added to two of the diets at 10 g/kg. Food intake and live weight changes were recorded weekly. Fasting blood samples were taken by venepuncture of the marginal ear vein at the beginning of the experimental period and at monthly intervals thereafter.

3.2.1 Serum Analysis

Serum lipoproteins were separated by a combination of ultracentrifugation to segregate VLDL (d <1.019) and heparin manganous chloride precipitation of LDL from the resultant supernatant (Chapter 2.3.2). Triglyceride and cholesterol concentrations were measured by an automated procedure. A manual method was also used for cholesterol determination. Both methods are described in section 2.3.3. Antibodies to food proteins were determined by both passive haemagglutination assay and an enzyme-linked immunosorbant assay (ELISA) method 1 (section 2.3.8). Immune complex levels were determined using the complement fixation technique (section 2.3.9).

3.2.2 Tissue Studies

Animals were killed after ninety days feeding by intravenous injection of Nembutal. The heart and aorta were removed en bloc and weighed after blotting dry with tissue paper. The macroscopic extent of atherosclerosis was quantified visually by three

independent observers on a 1-5 scale (Section 2.3.10). Samples of renal and carotid arteries and aorta were processed as given in section 2.3.11 and examined histologically. The liver, left kidney, adrenal glands and the spleen were removed, blotted dry with tissue paper and weighed. Samples of liver and kidney were then removed for total lipid analysis (section 2.3.6).

3.3 Results

The effect of protein source and cholesterol supplementation on energy intake and live weight gain are given in Table 3.1. Casein fed rabbits showed significantly lower energy intake ($p < 0.05$) and correspondingly low live weight gain ($P < 0.05$). Cholesterol supplementation did not affect these parameters significantly. The poor performance of rabbits fed casein based diets has been previously reported[255, 149].

Predictably, cholesterol feeding produced substantial elevations of both total cholesterol and triglyceride in the serum. The values for total serum cholesterol measured at fortnightly intervals in the four groups of rabbits are shown in Fig. 3.1. The increase in serum cholesterol was observed in all three lipoprotein fractions, although it was less significant in the HDL fraction. Protein source did not influence the serum cholesterol or the triglyceride levels of cholesterol fed groups. In contrast, in the groups that were not receiving dietary cholesterol, soya fed rabbits had significantly lower serum cholesterol than animals given casein ($P < 0.01$) (t-test). This difference was associated with a significant reduction in LDL

cholesterol ($P < 0.01$). The cholesterol fed groups had significantly higher liver ($P < 0.01$), and adrenal ($P < 0.01$) weights. The weights of spleen, kidney and heart were also increased though not significantly. The storage lipid in the liver and kidney was significantly increased by cholesterol feeding ($P < 0.01$) but was not influenced by dietary protein source (Table 3.2).

The results for immunological assays are summarised in Table 3.3. Small amounts of circulating antibodies to dietary protein source were detected in all animals. Cholesterol feeding significantly depressed serum levels of antibody to dietary protein. This change was demonstrated by both passive haemagglutination and ELISA techniques. A good correlation between the two methods was obtained. Immune complexes, estimated by a complement fixation assay, were significantly higher ($P < 0.01$) in soya fed rabbits. This technique was unsuitable for the turbid serum of cholesterol fed rabbits.

Macroscopic atheroma scores are summarised in table 3.4. Extensive disease was present in all cholesterol fed animals and was not influenced by dietary protein source. In the non cholesterol fed groups casein fed animals showed higher aorta scores than soya fed animals. However, these values were not significantly different.

The morphological features of the plaques in the non-cholesterol fed animals were essentially similar and were not affected by the protein source. The chief morphological features

of the plaques were minimal fibrous intimal thickening and focal calcification of the superficial medial elastic fibres. Foam cells were seen occasionally (Plates 3.1 and 3.2 show typical examples of normal and lesioned arteries in non-cholestrol fed rabbits).

Histologically, the lesions in the cholesterol fed rabbits were entirely typical of cholesterol atherosclerosis. Foam cells predominated in the markedly thickened intima (Plates 3.3 & 3.4) Small amounts of fibrous tissue were seen, usually in sub-endothelial regions. In occasional animals small numbers of foam cells and focal areas of dystrophic calcification were noted in the media.

3.4 Discussion

The accumulation of lipid in the tissues as well as the increase in the weight of many organs of the body found in cholesterol fed animals are consistent with the classical picture of a 'cholesterol storage disease' described by earlier workers[256].

The histological appearance of lesions in both soya and casein sub-groups given cholesterol were entirely typical of cholesterol atherosclerosis and were not influenced by the dietary protein source. The inhibitory effect of soya bean protein on cholesterol induced atherosclerosis when compared to casein, reported by earlier workers[150] was not observed in this experiment. In fact, animals fed soya diets supplemented with cholesterol developed more disease, although the aorta

score was not significantly different from the casein fed group. One reason may be that the amount of cholesterol used in the present experiment was sufficiently large to mask any effect of the protein source. Small amounts of fibrous tissue could be identified in the intima of many rabbits, it must be accepted that some collagen is inevitable in such plaques[257, 258]. Furthermore, increases in the rate of collagen synthesis have been reported in cholesterol induced arterial disease in serveral species[259, 260, 261]. Nevertheless, by the synergy of cholesterol feeding and the induction of serum sickness, Minick et al.[47] produced atheromatous deposits which clearly contained abundant fibrous tissue. Similarly, a marked fibrous intimal thickening and mixed inflammatory cell infiltration has been observed when a significant systemic immune response followed prolonged feeding of an antigenic dietary soya protein[215]. The concept that immunological injury is associated with fibrous intimal thickening is supported by the recent observations of Rokosova and Bentley[259]: rabbits fed a cholesterol free diet and given multiple intramuscular injections of bovine serum albumin, showed significant increases in both aortic collagen and glycosaminoglycan synthesis.

The few atheromatous plaques identified in non-cholesterol fed animals showed no striking histological features. Significant intimal thickening and inner medial calcification are not uncommon[263, 264] in rabbits. Arterial lesions were significantly more common in casein fed rabbits, which has also been observed previously[141, 148, 149, 177].

In non-cholesterol fed rabbits, dietary protein had a marked influence on serum cholesterol and casein fed rabbits had significantly elevated levels of serum total and LDL cholesterol. This difference has been found in a wide ranging comparison of animal and vegetable proteins[149]. Several authors have attributed this difference to the amino acid composition of the protein sources[178, 179, 180], while others have suggested that soya bean saponins, which are present even in soya protein isolates, are responsible for lowering the circulating level of cholesterol in animals fed on soya bean protein[200]. From the data given in the later study[200], the soya diets used in the present experiment would have contained only 0.75 g/kg saponin. Thus, if the saponin theory is correct then only extremely low levels are necessary to induce hypocholesterolaemia.

Circulating antibodies to dietary protein were detected in all animals in the present study but the levels of antibody were considerably lower than had been anticipated, particularly in soya fed rabbits and were clearly inadequate to modify the histological appearance of cholesterol induced atherosclerosis. Previous studies[215] had produced considerably higher serum antibodies to dietary soya bean protein. An investigation showed trace quantities of anti-soya bean antibodies in the breeding stock and it was subsequently established that the composition of the proprietory stock diet had been altered from one based on fish meal to one based on soya bean meal (Table 2.1). Perinatal or trans-placental exposure to soya antigen is likely to

have induced a state of hyporesponsiveness, since earlier the contact with the antigen, the smaller the quantity required to induce tolerance[265]. Maternal anti soya antibodies (IgG) would probably have been carried across the placenta to the foetal rabbits[266] and further exclusion of antigen would have occurred at birth with the ingestion of colostral secretory IgA[209]. It is of interest that peripheral blood antibody levels to both soya bean protein and casein were significantly lower in cholesterol fed rabbits. Similar findings have been reported with rabbits given high fat diets and injected intravenously with heterologous erythrocytes[267].

Antibodies to heated milk protein have been implicated in the initiation of human atherosclerosis[232]. The results of the present study raise the question of hyporesponsiveness arising from both perinatal exposure to dietary antigens and continuous exposure in childhood. Studies with human volunteers have shown that the systemic immune response to dietary milk protein is least in those frequently consuming milk and greatest in those who rarely drink milk[268]. The present study indicates that hyporesponsiveness arising from perinatal exposure may considerably influence the extent of atherosclerosis in animals fed reputedly antigenic diets.

Table 3.1 Energy intake and live weight gain of rabbits on the four dietary treatments (mean ± SEM)

Dietary cholesterol (g/kg)	10		0		Analysis of Variance			
Protein source	Casein	Soya	Casein	Soya	Protein Source	Cholesterol Supplementation	Inter-action	SEM
Energy Intake (MJ/kg/d)	0.37	0.43	0.39	0.4	*	N.S.	N.S.	0.01
Live weight gain (g/kg/d)	4.29	5.42	4.76	6.12	*	N.S.	N.S.	0.46
n	5	5	5	5	10	10	10	

* Differences significant at $p < 0.05$

N.S. Differences not significant

Table 3.2 Serum and tissue lipid levels in rabbits in the four treatments (mean ± SEM).

Dietary cholesterol (g/kg)	10		0		Analysis of Variance			
Protein Source	Casein	Soya	Casein	Soya	Protein Source	Cholesterol Supplementation	Inter-action	SEM
n	5	5	5	5	10	10	10	
Serum cholesterol (mmol/l)								
Total	58	78	8.0	4.7	N.S.	**	N.S.	6.0
VLDL	32	45	1.4	0.62	N.S.	**	N.S.	8.5
LDL	25	32	5.2	2.9	N.S.	**	N.S.	2.9
HDL	1.1	1.1	1.1	0.74	N.S.	*	N.S.	0.1
Serum Triglycerides (mmol/l)	2.68	3.74	0.99	0.73	N.S.	**	N.S.	0.48
Total liver lipids (g/kg.L.wt)	5.29	5.79	3.39	2.88	N.S.	*	N.S.	0.87
Total kidney lipids (g/kg.L.wt)	0.23	0.17	0.12	0.11	N.S.	**	N.S.	0.02
Organ weights (g/kg.L.wt)								
Liver	47.3	44.24	29.55	30.19	N.S.	**	N.S.	4.0
Kidney	2.83	2.72	2.54	2.51	N.S.	N.S.	N.S.	0.14
Spleen	3.26	1.73	0.41	0.48	N.S.	N.S.	N.S.	1.1
Adrenals	0.25	0.17	0.15	0.11	**	***	N.S.	0.01
Heart and Aorta	4.53	3.02	3.14	3.41	*	N.S.	**	0.25

N.S. Differences not significant; *Differences significant at $p < 0.05$; **Differences significant at $p < 0.01$; ***Differences significant at $p < 0.001$.

Table 3.3 Antibody titres and immune complexes (mean ± SEM)

Dietary cholesterol g/kg	10		0		Analysis of variance			
Protein Source	Casein	Soya	Casein	Soya	Protein Source	Cholesterol Supplementation	Inter-action	SEM
n	5	5	5	5	10	10	10	
Antibody titres								
a) Passive haemagglutination (haemagglutination titre)	13	20	41	104	N.S.	*	-	32
b) Enzyme-linked immunosorbant assay (ELISA units)	57.3	101.5	129.5	167.2	N.S.	**	-	21.4
Immune complexes (Units of complement/ ml of serum)	-	-	0.13	0.61	**	-	-	0.15

* Differences significant at $p < 0.05$

** Differences significant at $p < 0.01$

N.S. Differences not signigicant

Table 3.4 Individual atherosclerotic scores in rabbits killed after 90 days of feeding

Dietary cholesterol g/kg	10		0	
Protein source	Casein	Soya	Casein	Soya
Scores	16	22	6	5
	21	22	9	5
	21	18	5	8
	11	21	6	5
	16	17	9	5
Mean Scores (± SEM)	17 ± 1.9	N.S. 20 ± 1.1	7 ± 0.8	N.S. 5.6 ± 0.6

N.S. No significant differences between soya and casein in both cholesterol fed and non fed groups.

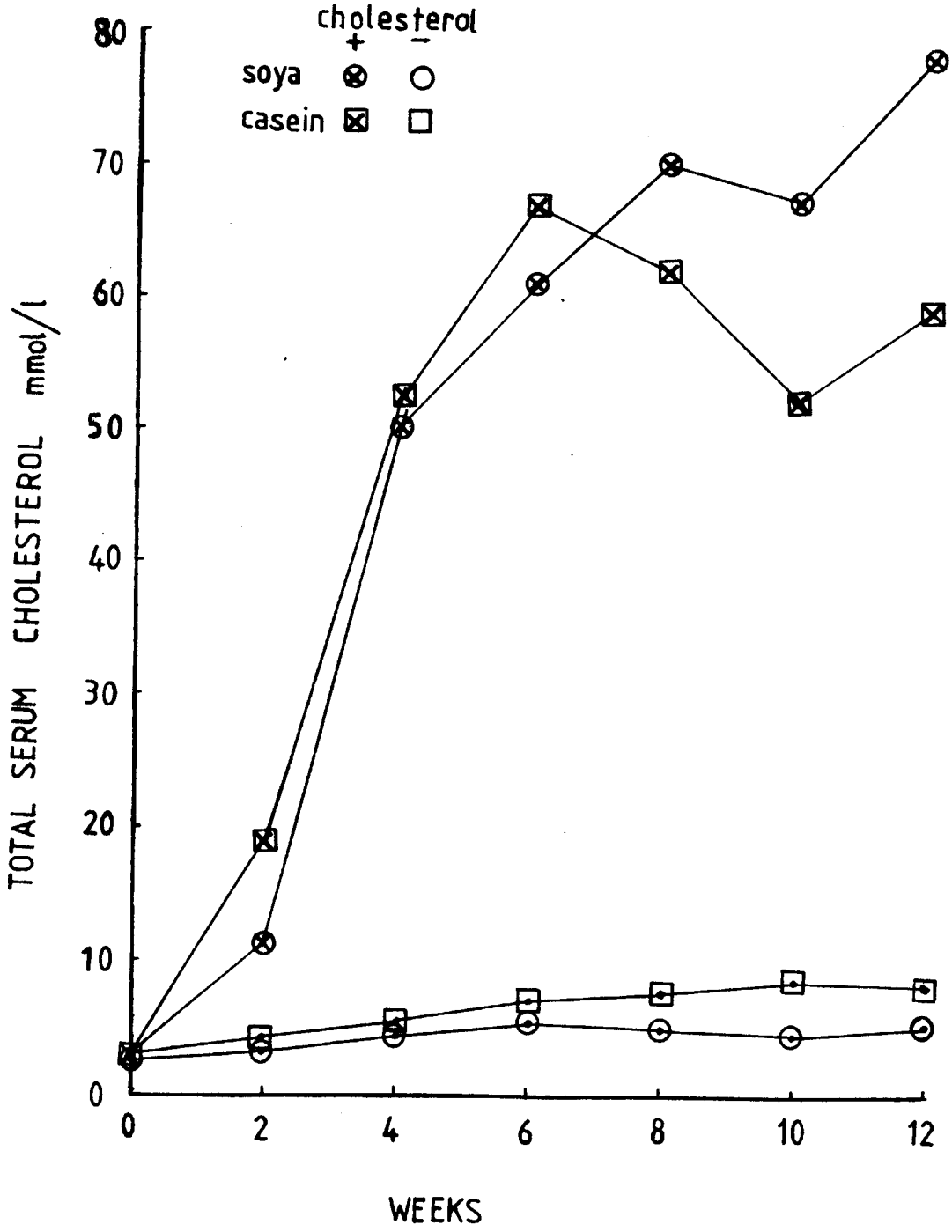


FIG. 3,1. Total serum cholesterol concentrations(mmol/l) in rabbits fed diets based on soya protein or cow's milk protein with or without cholesterol (10g/kg)

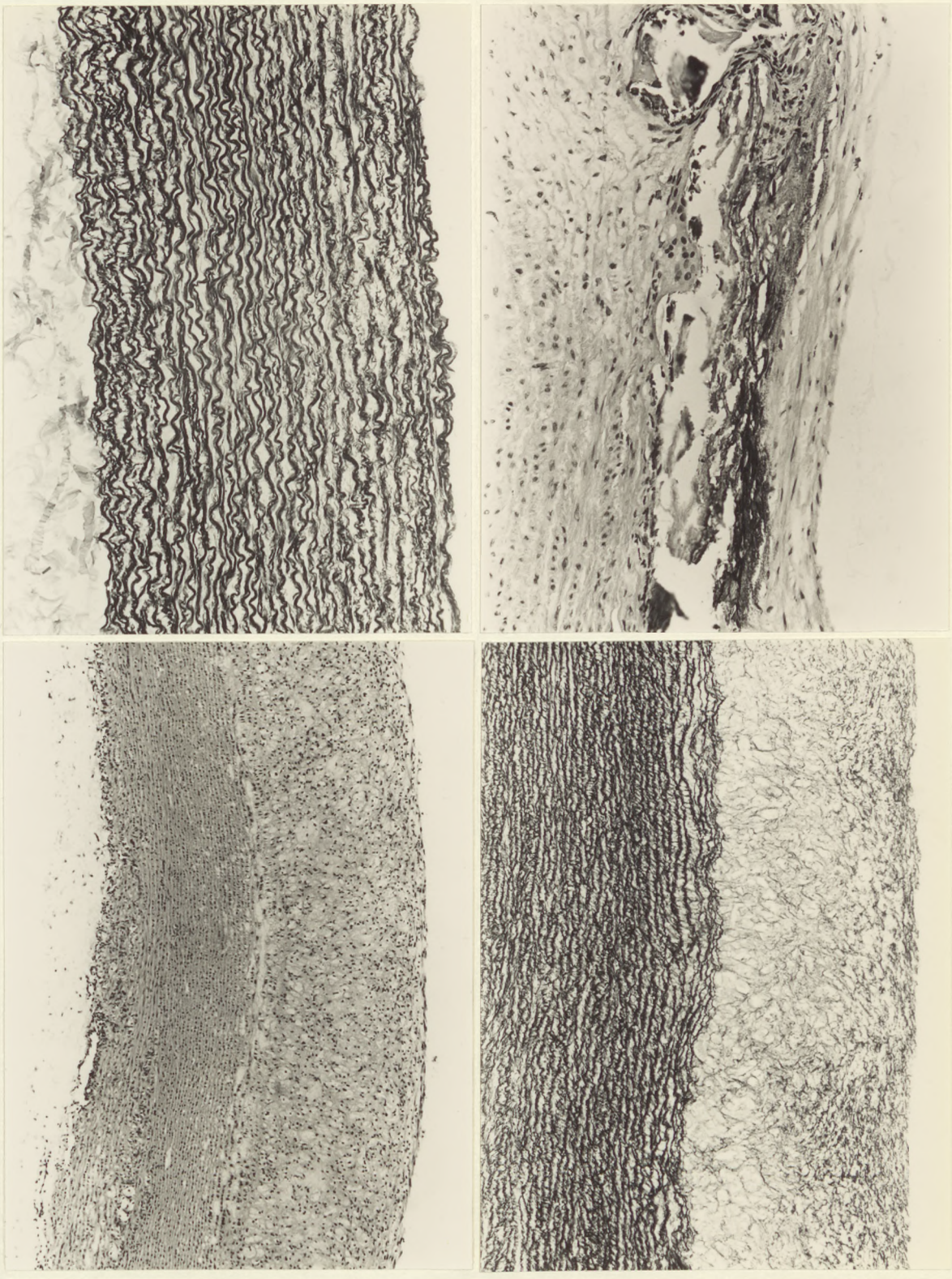


PLATE 3.1 (top left) Promine D - 0% Cholesterol Diet. Normal appearance of aorta. Note numerous elastic lamellae. No intimal thickening. Elastic - Van Gieson x 130.

PLATE 3.2 (top right) Casein - 0% Cholesterol Diet. Some fibrous intimal thickening (i) with underlying calcification and mononuclear cell infiltration. Aorta. Haematoxylin & Eosin x 195.

PLATE 3.3 (bottom left) Casein - 1% Cholesterol Diet. Marked intimal thickening (*) typical of cholesterol feeding. Aorta. Haematoxylin & Eosin x 75.

PLATE 3.4 (bottom right) Promine D - 1% Cholesterol Diet. Marked intimal thickening (*). Contrast with Plate 3.1. Aorta. Elastic Van Gieson x 105.

CHAPTER 4

EXPERIMENT 2

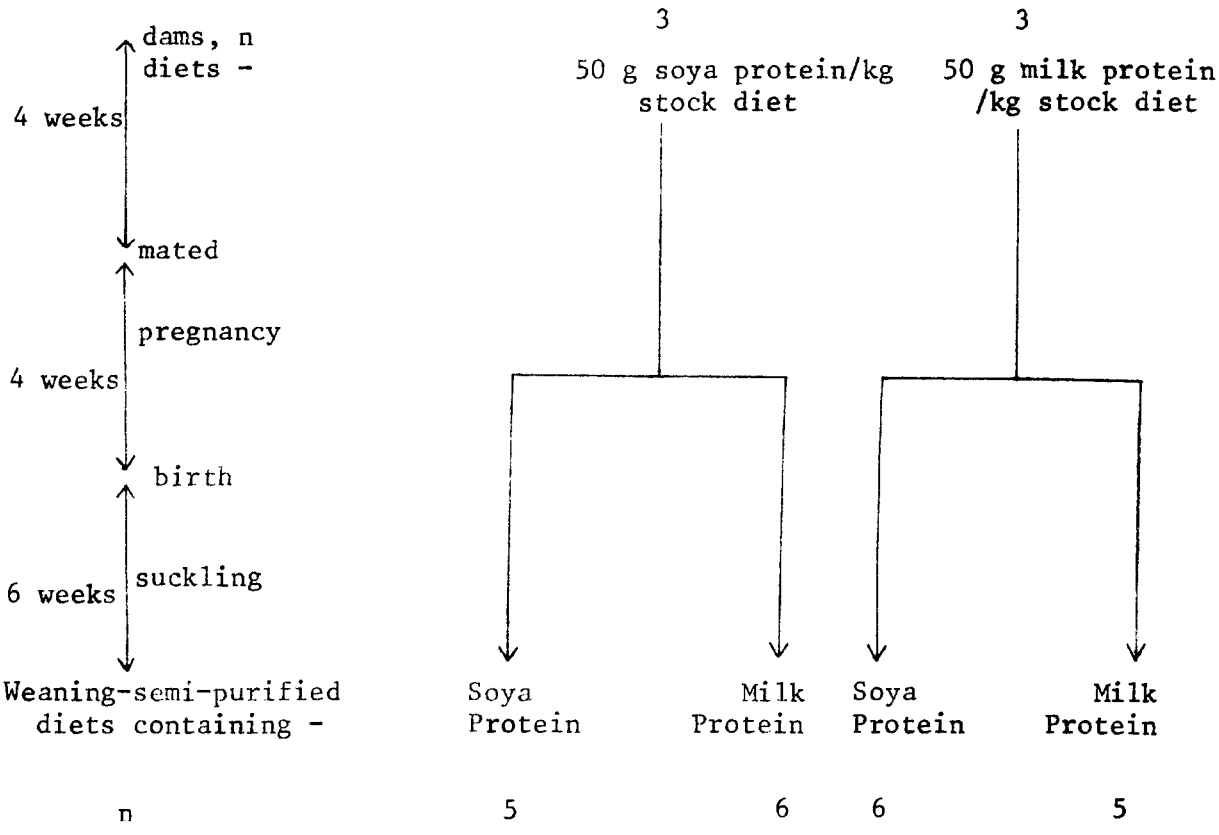
4.1 Introduction

The results of the first experiment raised the question as to the cause of reduced immune response to the dietary soya antigen as compared to levels previously observed[215]. As mentioned in the previous chapter inadvertant exposure to the soya antigen in the foetal or the neonatal period may have induced a state of immunological tolerance in the young rabbits. Although, it is well known that orally administered protein antigen can produce a local and systemic immune repsonse[210-215], induction of tolerance by oral route is less well known. The present experiment was undertaken to study the role of pre- and post-natal exposure to dietary antigens in inducing oral tolerance in rabbits.

4.2 Materials and Methods

Six female New Zealand White rabbits were fed from weaning on a stock diet (table 2.2) containing fish meal as the protein source. At three months of age the diet was supplemented with soya protein isolate (Promine D) or cow's milk protein (Dried skim milk) to provide 50 g of supplemented protein per kg of stock diet. Lactose was added to the Promine D to the same level as in the dried skim milk. Four weeks later the animals were

mated. Subsequently, four groups of 5-6 animals were made up from their offspring. At six weeks of age, two of these groups were weaned on to semi-purified diets containing the same dietary protein source as their dams. The other two groups received diets containing the alternative protein. Thus, the experimental design was as follows:



The composition of weaning diets are given in table 2.4.

Live weight changes and food intakes were recorded weekly for each animal. Blood samples were taken by venepuncture before weaning and at weekly intervals thereafter for four weeks. Serum antibody levels against food antigens were measured by the two ELISA assay methods described in section 2.3.8. Serum

total cholesterol and triglycerides were determined by auto-analyser II as described in section 2.3.3.

4.3 Results

All experimental data after 4 weeks of feeding are summarised in table 4.1. There was no significant difference in energy intakes between the different dietary treatments. However, the rabbits that came from soya fed dams showed significantly higher live weight gains than rabbits from milk fed dams, irrespective of the weaning diets. The unequal sizes of the litters and the low weight at weaning of the rabbits from soya fed dams might account for this difference. Serum total cholesterol and total triglyceride levels measured at weekly intervals are shown in table 4.2. Serum triglyceride levels did not show a consistent change whereas serum cholesterol concentrations showed a significant rise in the milk fed groups. This hypercholesterolaemic effect of milk protein started to show significance after two weeks on the diet.

Results for serum antibodies to dietary soya and milk proteins expressed in ELISA units are given in Fig. 4.1 and 4.2. After approximately three weeks, rabbits fed the same protein as their mothers had received, showed substantially lower levels of circulating antibodies than the animals given a completely novel protein. This immunosuppressive effect was more pronounced with soya protein.

4.4 Discussion

The feeding of high fat, semipurified diets containing dried skim milk as the protein source induced a significant elevation in serum cholesterol which was evident within two weeks of commencing the diets. This effect was not observed in soya fed rabbits. Although, similar effects of dietary proteins on serum cholesterl have been reported previously[145, 146, 147, 148, 149, 150], the cause of this hypercholesterolaemia is not known. Subsequent experiments will endeavour to investigate this phenomenon further.

The intestinal absorption of soya and milk protein in sufficient quantities to evoke a systemic immune response in adult rabbit is evident from the presence of specific IgG antibodies to these proteins in the rabbits. The significantly lower circulating antibody levels found in rabbits weaned onto diets containing the same protein to which their dams were exposed, support the hypothesis that exposure to a dietary antigen during pregnancy and/or suckling is associated with the development of profound tolerance to that antigen upon weaning.

At present it is only possible to speculate as to the mechanisms by which this form of tolerance is induced.

It is possible that the tolerance state is acquired by the rabbit through exposure to the antigen during the foetal or neonatal period. Macromolecules derived from dietary proteins have been shown to cross the placenta in rats[269]. In the rabbit a very small proportion of bovine γ-globulin injected into the uterine cavity was recovered intact in the fetuses[270],

although not to the same extent as the rabbit γ-globulin. Therefore, it is possible that antigen absorbed by the dam could also reach the foetus via the yolk-sac or the placenta. Furthermore, a variety of food antigens have been detected in human colostrum and milk[271, 272]. Small amounts of antigen reaching the foetal or the neonatal rabbit by either of these routes would induce a state of low-zone tolerance.

It is also possible that tolerance was acquired passively. It is well known that secretory IgA is obtained by the suckling neonate via colostrum and milk[203]. A variety of antibodies [273, 274] and living immunocompetent cells[275] directed against the antigens in the maternal diet have been detected in human colostrum and milk. The acquisition of significant amounts of food antigen specific antibodies (sIgA) by this route could inhibit the uptake of antigen[210] and therefore inhibit the production of antibodies by the young animal.

The pre-weaning rabbit is also exposed to the antigen directly while still in its mother's cage. The young animals in this experiment began to move about and to eat their dam's pelletted diets at 16-18 d of age, a time which precisely coincides with the replacement of renin by pepsin as the main gastric protease[276]. Generally, it is easier to induce a longer lasting, more complete tolerant state in younger animals when the immune system is immature[277]. Smith (1961)[278] has shown that up to 15 d of age, injection of rabbits with foreign proteins induces a state of tolerance and that after this critical stage antibody response results. Since the rabbits

in this present experiment began to nibble the maternal diet after this critical period of development of immunocompetence, this direct consumption of maternal diet by the young animals is unlikely to have produced tolerance.

Mature lymphocytes carry a record of the pattern of 'foreign' proteins[279] so that when a forein protein is introduced into the immature immune system, this causes lymphoid cell depletion[280], and if the foreign protein is reintroduced it will be treated as a 'self' protein, and no antibody production will occur. This mechanism finds apparent support in the experiments showing induction of prolonged tolerance to homografted tissue by a single injection of viable reticuloendothelial cells at birth[281], and in experiments demonstrating tolerance to tumours induced by neonatal implantation[282]. It is now suggested that this is a simplistic view of immunological tolerance[283] as fully competent lymphoid cells have been shown to remain in a state of suppression, both in artificially induced tolerance to foreign antigens and in natural tolerance towards self antigens. This suppression is dependent on T-cells and possibly macrophages. The mechanism suggested by which tolerance is induced is that antigen[284] or antigen-antibody complex activates suppressor T-cells to release factors which in turn stimulates macrophages to produce non-specific suppressor factors which act on B-cells, so suppressing antibody response. There is no single mechanism which can explain how tolerance to an antigen is brought about, but rather a complex set of interactions between T and B cells and their products and possibly macrophages[284].

Factors responsible for producing tolerance in the present studies may be the persistent tolerogen (e.g. antigen in a deaggregated form), antibody, the antigen-antibody complexes or the suppressor lymphocytes.

Experiments with mice have shown that tolerance can be induced by oral administration of antigen by a mechanism which involves both antigen-antibody complexes and suppressor lymphocytes[284, 285]. These immune complexes which were responsible for the tolerogenic effect had IgA as the antibody.

It has also been suggested that immune complexes in slight excess of antigen can be powerful tolerogens, possibly because they represent a highly multivalent form of the antigen and are particularly suited to paralyse B-cells (effector cell blockade)[287]. The theory of circulating immune complexes inducing tolerance is supported by other workers[288]. Presence of suppressor T-cells for IgG, and IgE has been demonstrated in mice orally tolerised to ovalbumin[290]. Peyer's patches have been described as the source of suppressor cells in the spleen and lymph nodes after oral administration of antigen[289]. The liver probably plays a role in deaggregating protein antigens thus converting them into a tolerogenic form[291].

Two groups[292, 293] have demonstrated that tolerance to parenterally administered antigens can be produced in weaning mice by injecting their mothers with the same antigen either before conception, during gestation or in the immediate postnatal period. Furthermore, experiments with foster mothering [293] indicate that suckling was necessary for the transmission

of this type of tolerance. In this context, it is relevant that commonest of food allergies, to cow's milk protein is more frequent in bottle than in breast fed infants[225].

Therefore the systemic tolerance induced in this way could explain how an organism can live despite the continuous and massive antigenic aggression from the gut contents, and also be a possible technique of artificial tolerance induction to harmful antigens or allergens.

Table 4.1 Results of experiment 2. The effects of the four experimental treatments of live weight gain, energy intake, serum lipids and serum antibodies to food proteins. (Mean ± SEM)

Maternal Diet	Soya		Milk		± SEM	Mean for all Rabbits		Soya	Milk	± SEM
Weaning Diet	Soya	Milk	Soya	Milk		Soya	Milk	Mean for all rabbits		
n	5	6	6	5	-	11	11	11	11	-
Weight gain (g/kg/d)	18.8	17.2	13.9	14.3	1.06	16.4	15.8	18.0	14.1	0.75
Energy Intake (MJ/kg/d)	0.65	0.65	0.66	0.61	0.06	0.66	0.63	0.65	0.64	0.04
Serum Total Cholesterol (mmol/l)	2.9	4.4	2.7	4.5	0.6	2.8	4.41*	3.65	3.6	0.43
Serum Total Triglyceride (mmol/l)	0.62	0.75	0.82	0.72	0.06	0.73	0.74	0.75	0.7	0.04
Anti Soya antibody titre (Elisa Units)	265^{b}	39.1^{c}	970.4^{a}	26.9^{c}	125	617.7	33**	152	499**	88.5
Anti Milk antibody titre (Elisa Units)	54^{c}	632^{a}	89^{c}	202^{a}	119	71	417**	343	146	84
Antibody levels (% of injected serum)										
Anti soya	0.09	0.03	1.25	0.03	0.32	-	-	-	-	-
Anti milk	0.07	4.1	0.32	3.19	0.9	-	-	-	-	-

* Differences significant at $p < 0.05$, ** Differences significant at $p < 0.01$

^{abc}Mean values for all 4 dietary treatments without a common superscript are significantly different ($p < 0.05$)

Table 4.2 Effect of feeding semi-purified diets based on soya or cow's milk protein on the serum total cholesterol and triglyceride levels of rabbits during the first 4 weeks after weaning. (Mean ± SEM)

Weeks post-weaning	0	1	2	3	4
Serum total cholesterol (mmol/l)					
Milk protein diet	3.1	3.54	3.83	4.42	4.45
Soya protein diet	3.1	2.61	2.5 *	2.5 *	2.7 *
SEM	0.3	0.4	0.6	0.6	0.5
Serum total triglyceride (mmol/l)					
Milk protein diet	0.88	0.84	0.82	0.65	0.74
Soya protein diet	0.89	0.95	0.90	0.71	0.73
SEM	0.06	0.08	0.05	0.05	0.04

*Differences significant at $p < 0.05$

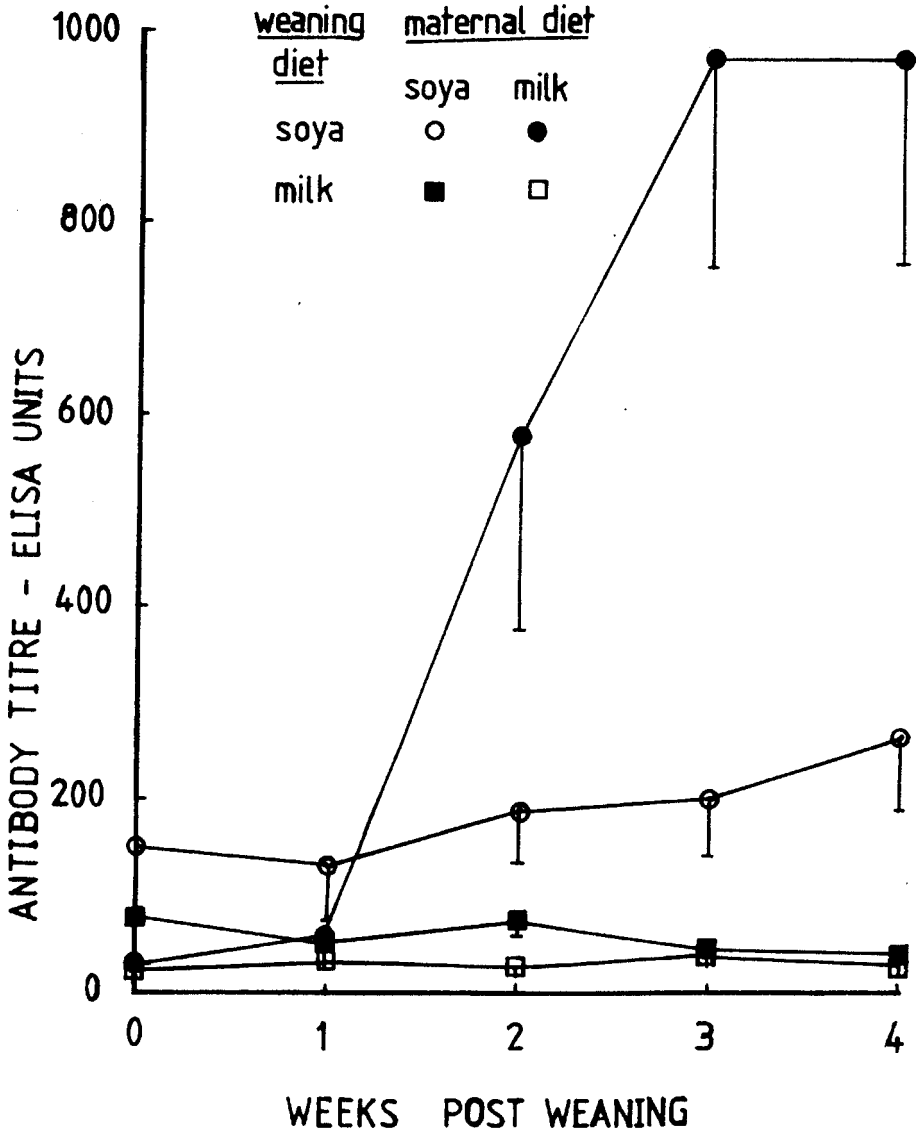


Fig. 4.1 Antisoya antibody levels (mean ± SEM) in rabbits fed diets based on soya protein or cow's milk protein at weaning.

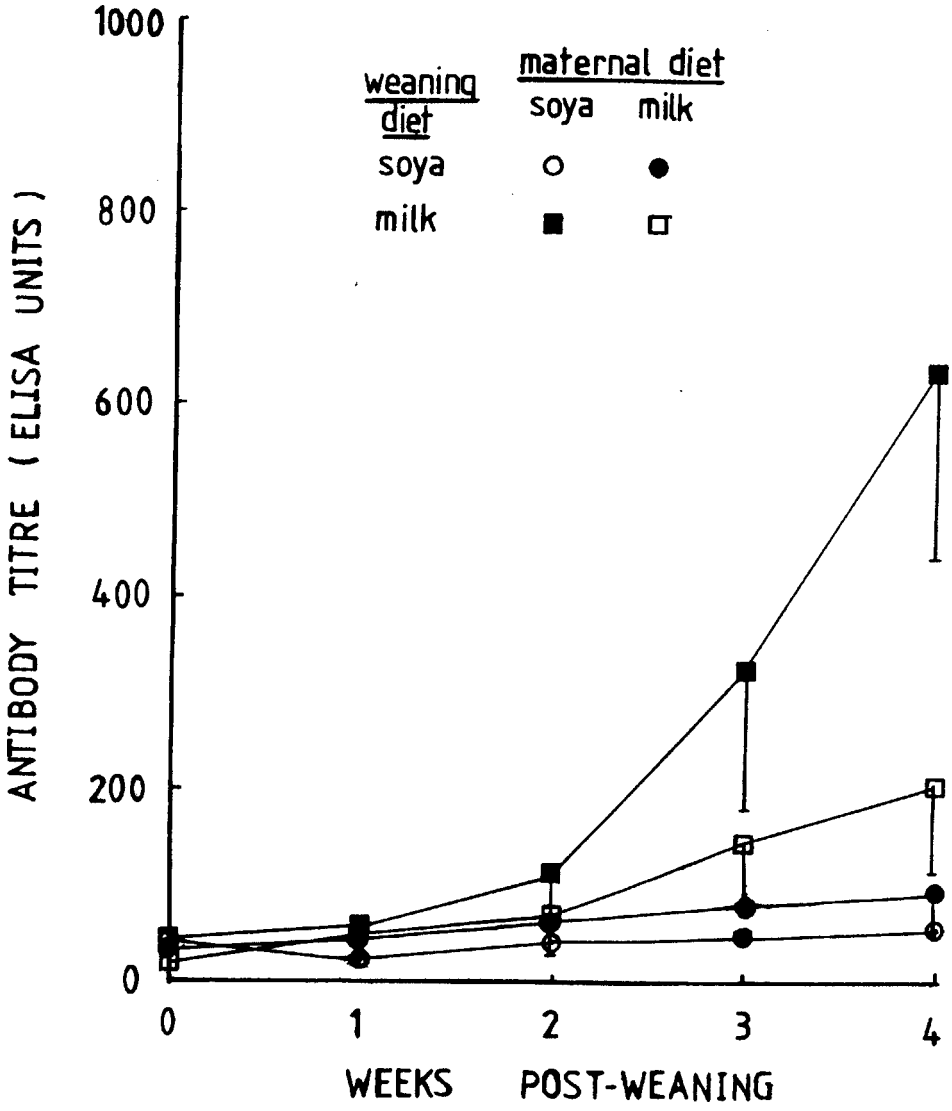


Fig. 4.2 Anti milk antibody levels (mean ± SEM) in rabbits fed diets based on soya protein or cow's milk protein at weaning.

CHAPTER 5

EXPERIMENT 3

5.1 Introduction

The low levels of circulating anti soya antibodies produced in experiment 1 did not constitute of sufficiently strong immunological challenge to alter the nature of cholesterol induced atherosclerosis. In experiment 2 it was confirmed that a state of immunological tolerance had been induced in the animals due to the inadvertent exposure to this antigen during the pre-weaning period. Accordingly, the present experiment was undertaken to investigate whether the level of antibodies in non-tolerised animals might be sufficient to modify the histological appearance of lesions produced by cholesterol feeding. This was prompted by the finding which showed that the appearance of lesions in cholesterol fed rabbits was dramatically altered by simultaneous immunological injury via serum sickness[47].

5.2 Materials and Methods

Twenty one New Zealand White rabbits (used in experiment 2) were continued on the same basal diets (table 2.4) after supplementation with cholesterol at 7.5 g/kg diet. Weight changes and food intake data were recorded weekly. Fasting blood samples were taken at the end of three months, and the animals killed by intravenous injection of nembutal. The heart

and aorta were removed en bloc. The microscopic extent of atheroma was quantified visually (section 2.3.10). The histological studies were done as described in section 2.3.11. Antibodies to food proteins were determined by the enzyme linked immunosorbant assay described in section 2.3.8.2 (method 2). Serum lipoproteins were separated as described in section 2.3.2. Serum triglyceride and total cholesterol concentrations were determined with the autoanalyser (section 2.3.3.2) and cholesterol concentration in various lipoprotein fractions were determined using the manual method (section 2.3.3.1).

5.3 Results

Results are summarised in table 5.1. There were no significant differences in the energy intake or the live weight gain of rabbits fed soya or milk protein diets fed in combination with cholesterol.

Both serum cholesterol and triglyceride levels were markedly elevated and no significant effect due to protein source was evident in the whole serum or its lipoprotein fractions, except in the HDL fraction where significantly lower ($p < 0.05$) cholesterol concentration was found in soya fed animals. These differences however were very small.

Significantly higher levels of anti soya antibodies were produced in rabbits that were introduced to soya protein after weaning. A similar pattern was observed with rabbits weaned on to milk protein, but the differences were not significant. The suppression of antibody response has been described in the

previous chapter. Anti milk antibodies appeared to be produced in higher concentrations than anti soya antibodies when expressed as a percentage of the antibody produced by parenteral challege. However, it is not possible to compare the antibodies produced against completely different antigens, in view of the possible differences in the properties of antibodies produced. Thus, differences may exist between antigen-antibody building capacity (avidity) and further differences may exist in the adhesion of antigen to the solid phase during ELISA.

The aorta scores as analysed by a non-parametric statistical method (section 2.3.12) did not show any significant difference due to protein source, irrespective of the time at which the rabbits were first exposed to the protein (table 5.2). Microscopically, atheromatous plaques characterised by intimal accumulation of lipid filled foam cells with no apparent underlying intimal alterations were seen in all the rabbits. Seven rabbits, all from the dams fed milk protein based diets, but weaned to soya based (3 rabbits) or milk based diets (4 rabbits) showed occasional lipid-fibrous plaques involving the inner media. Calcium deposition and mono-nuclear cell infiltration were seen at the base of these plaques (Plates 5.1, 5.2).

5.4 Discussion

In constrast to experiment 1, no reduction in energy intake or live weight gain was observed in the present experiment due to the diets based on milk proteins. This may have been due to the use of a mixture of casein and cow's milk protein which led

to improved palatibility of the diet.

The reduction in the extent of macroscopic atheromatous degeneration was probably due to the reduction of cholesterol concentration in the diets from 10 g/kg to 7.5 g/kg and the substitution of maize oil for coconut oil.

Although, lesions of both types were found in experiment 1, the fibro-lipid plaques were much more frequent in the present experiment. In contrast to experiment 1, where a state of tolerance to the food antigen existed, in the present experiment two groups of rabbits, either tolerised or not tolerised to the food antigen tested, were used. However, no correlation was observed between the occurrence of these lesions showing signs of an inflammatory reaction and the antibody titres. The fact that these lesions occurred only in the rabbits bred from the dams fed on diets containing milk protein indicate that the diets fed before weaning may have been responsible for the initiation of these lesions.

Spontaneously occurring medial lesions of similar histological appearance have been described by many workers, not only in the rabbit[264], but also in the horse[294], sheep[295], cat[296] and various other species. Although, the occurrence of mono-nuclear and giant cell infiltrate associated with the internal elastic lamellae indicate an active inflammatory process, the pathogenesis of this lesion is not known. Previous experiments have shown that the synergy of immunological injury induced by serum sickness or graft rejection can lead to atherosclerosis in rabbits[27, 47]. The postulated mechanism is through the

deposition of immune complexes and the ensuing necrosis of the arterial wall and accompanying inflammation result from changes in endothelial permeability triggered by the release of vaso-active amines from platelets and leucocytes[64, 65, 68, 78]. However, from the results of this experiment it appears that the antibody levels achieved by feeding of an antigenic protein to rabbits is not of sufficient magnitude to alter the nature of cholesterol atherosclerosis produced over 12 weeks.

Table 5.1 Effect of feeding cholesterol in semi-purified diets based on soya or milk protein to rabbits reared from soya or milk fed colonies for 90 d (Mean ± SEM).

Maternal Diet	Soya		Milk			Analysis of Variance	
Weaning Diet	Soya	Milk	Soya	Milk	SEM	Maternal Diet	Weaning Diet
n	5	6	5	5		11/10	10/11
Energy Intake MJ/Kg/d	0.51	0.47	0.45	0.41	0.02	N.S.	N.S.
Live Weight Gain g/Kg/d	6.91	7.17	6.38	6.34	0.35	N.S.	N.S.
Serum Cholesterol mmol/l							
Total	63	64	68	61	7.4	N.S.	N.S.
VLDL	32.6	34.7	40.3	41.2	6.3	N.S.	N.S.
LDL	29.6	27.8	26.2	19.6	3.2	N.S.	N.S.
HDL	0.7	1.1	0.77	0.92	0.12	N.S.	*
Total triglyceride mmol/l	1.69	1.77	1.73	1.52	0.13	N.S.	N.S.
Antibody levels (% injected serum)							
Anti milk antibodies	0.25	16.55	0.13	9.46	3.6	N.S.	*
Anti soya antibodies	0.16	0.04	1.18	0.02	0.2	N.S.	N.S.

* Differences significant at $p < 0.05$ N.S. No significant difference

Table 5.2 Individual aorta scores of rabbits bred from soya or milk protein fed colonies weaned on to cholesterol containing semi-purified diets based on soya or milk protein.

Maternal Diet	Milk		Soya		
Weaning Diet	Milk	Soya	Milk	Soya	
	12	10	14	13	
	20	10	14	11	
	10	11	12	15	
	12	11	14	9	
	16	14	11	11	
			14		
Mean ± SEM	14 ± 0.8	11.2 ± 0.7	13.2 ± 0.6	11.8 ± 1.0	N.S.

N.S. No significant differences were observed between protein sources and the time of introduction to the protein

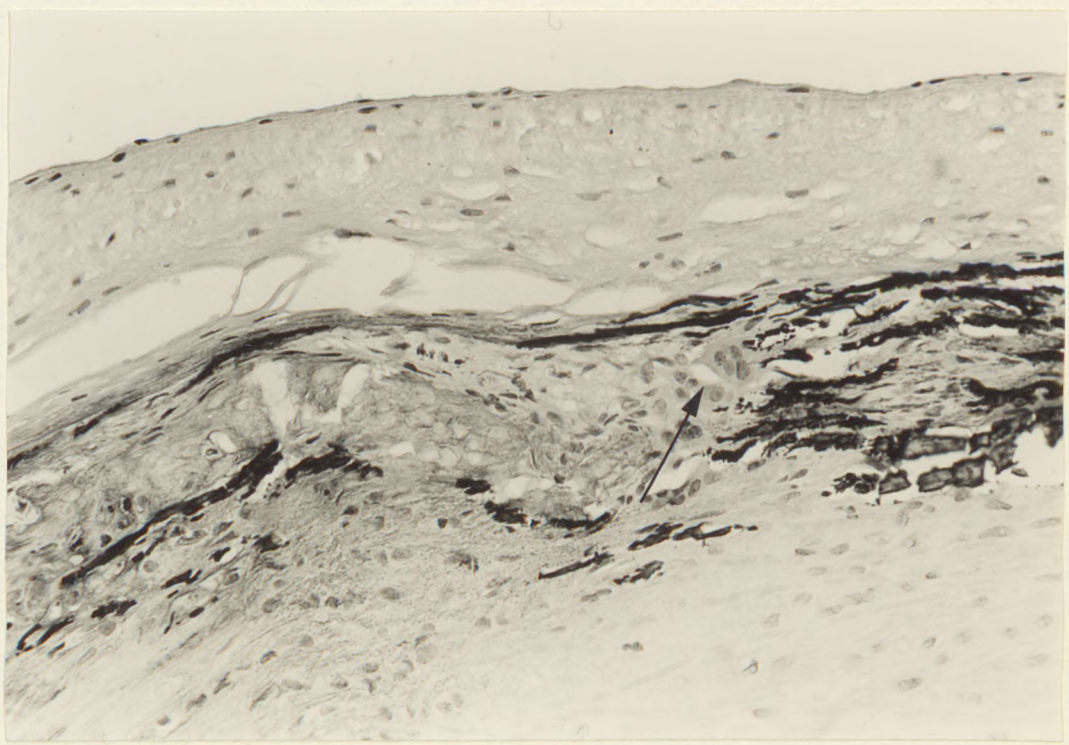


PLATE 5.1 (top) Maternal diet milk. Post weaning diet milk. Cholesterol induced intimal thickening (*) with areas of intimal calcification and mononuclear cell infiltration (arrows). Aorta. Haematoxylin & Eosin x 195.

PLATE 5.2 (bottom) Maternal diet milk. Post weaning diet soya. Some intimal thickening (*) but marked calcification, mononuclear and giant cell (arrow) infiltration. Aorta. Haematoxylin & Eosin x 215.

CHAPTER 6

EXPERIMENT 4

6.1 Introduction

The effect of dietary protein on the cholesterol metabolism of the rabbit has been known for sometime[150] and was again demonstrated in experiments 1 and 2. However, little progress has been made in elucidating the mechanisms involved. Two main hypothesis have been proposed to explain the relative hypocholesterolaemic effect of plant compared with animal proteins. One theory suggests that hypocholesterolaemic effect of plant proteins, soya proteins in particular is a fortuitous result arising from the presence of saponins[200] which have the capacity *in vitro* to adsorb bile acids to deitary fibre[197]. A second hypothesis relates the hypocholesterolaemic effect of vegetable proteins to their amino acid composition *per se*[178]. The purpose of the present study was to test the hypothesis that the hypocholesterolaemia associated with soya based diets is a function of the saponin fraction and to see whether supplementation of cow's milk protein with saponin would reduce its hypocholesterolaemic action.

6.2 Materials and Methods

Four groups of six New Zealand White rabbits were used. Each group comprised of four male and two female rabbits. The animals were housed individually in metabolism cages. Each

group was offered one of four diets for a period of 28 d. The composition of basal diets are given in table 2.5. Half the animals receiving each of the basal diets were given a dietary supplement of commercial white saponin (10 g/kg - Messrs. BDH Ltd., Poole, Dorset). The rabbits were bled from the marginal ear vein on day 28 and serum separated. Faeces were collected during the last seven days of the experiment, dried at 60°C in a force draught oven for at least 24 h. Food intake and live weight gain were recorded weekly.

High density lipoprotein fraction of serum was separated according to the method given in section 2.3.2.2 The cholesterol and triglycerides of the whole serum and that of the HDL fraction of the serum were determined either manually or on the auto-analyser II (section 2.3.3). Faecal sterols were extracted, separated into neutral and acid sterol fractions and the individual neutral and acid sterols determined, using gas-liquid chromatography (section 2.3.4). A typical gas-liquid chromatogram of faecal neutral sterols is shown in Fig. 6.1, and that of acid sterols is shown in Fig. 6.2.

6.3 Results

The mean daily energy intake was significantly increased ($p < 0.05$) by saponin supplemnetation but was not reflected by changes in live weight gain. The rabbits fed on diets based on cow's milk protein showed significantly higher total serum cholesterol and triglycerides and significantly more cholesterol in the VLDL + LDL fraction ($p < 0.005$) than the soya fed rabbits.

Dietary protein source did not influence HDL cholesterol and serum lipids were uninfluenced by the inclusion of saponin in the diets. Furthermore, there was no evidence of an interactive effect of dietary protein source and protein supplementation on serum lipids (table 6.1).

The dietary effects on the faecal excretion of total and individual neutral and acid sterols are given in table 6.2 and 6.3 respectively. Animals given soya protein excreted significantly greater amounts of both total acid and neutral sterols ($p < 0.05$) than those given milk protein. Although saponin increased the excretion of both fractions, this effect was not statistically significant. Furthermore, although there was no statistical evidence of an interaction between saponin supplementation and dietary protein source on sterol excretion, saponin appeared to increase faecal sterols only when soya protein was used. Thus the mean value for the excretion of total sterols increased from 15.9 to 24.4 mg/kg/d when saponin was added to the soya based diets, whereas corresponding change with the milk fed rabbits was from 9.6 to 10.6 mg/kg/d.

There was no significant treatment effect on excretion of deoxycholic acid or lithocholic acid which were the major bile acids found in these rabbit faeces. A similar result was observed for cholic acid which was present in small quantities in the samples. Four bile acids appearing on the gas chromatograph were not identified but probably were keto or keto hydroxy derivatives of deoxycholic acid[297]. Three of these unidentified bile acids (peaks 10, 11, 12) were excreted in significantly

greater quantities by soya fed rabbits ($p < 0.05$). In the case of the bile acid with the longest retention time (peak 12) saponin caused significant increase in its excretion but only with the soya fed rabbits ($p < 0.05$).

Although, total neutral sterol excretion was significantly increased by soya feeding, there was no significant effect due to protein source on cholesterol or coprostanol excretion. Three other peaks were tentatively identified by reference to the data of Miettinen et al.(1965)[298] as coprostanone and corresponding 3-keto homologues of the plant sterols, campesterol and stigmasterol. Significantly higher concentrations of plant sterols (campesterol + stigmasterol + sitosterol) were excreted by soya fed rabbits which probably reflects the amounts present in the diets. Two peaks with long relative retention times were observed in considerable quantities in the chromatograms of all soya fed rabbits, but were absent or present in only trace quantities in those of cow's milk protein fed rabbits. The gas-liquid chromatotgraphic data of Hellström et al. (1962)[297] suggest that these sterols may have been keto derivatives of cholesterol.

6.4 Discussion

In an investigation of the hypocholesterolaemic action of dietary fibre, Oakenfull and Fenwick (1978)[197] observed that the capacity of fibre sources to bind bild acids *in vitro* was dependent on the saponin content of the fibre source. Thus cereal fibres lacked the capacity to adsorb bile acids *in vitro*

but acquired this capacity when supplemented with saponin-rich ethanol-water extract of lucerne. The authors proposed that sources of dietary fibre rich in saponin would be more effective in reducing serum cholesterol by increasing faecal bile acid excretion. Whole soya beans contain 5-6% dry weight as saponins and as such are extremely rich sources of these compounds. On that basis, it has been proposed that the hypocholesterolaemia associated with soya bean feeding in man[167] and rabbit[145, 146, 147, 148, 149] was a function of the saponin in the soya sources. This proposal was supported by the many observations that dietary saponins have the capacity to lower serum cholesterol in chickens[191, 192, 193], rats[195] and pigs[196] (table 6.4).

The results of the present study do not uphold the saponin theory. The explanation may be in the method used to induce hypercholesterolaemia. In the present study, it was achieved by feeding a cholesterol free, low fat diet based on animal protein and as such it was a direct consequence of the balance of hepatic synthesis, catabolism and excretion of cholesterol. However, in all previous studies of the hypocholesterolaemic effect of plant saponins, the elevated levels of serum cholesterol were achieved by supplementing the diet with cholesterol thus inducing the exogenously based hypercholesterolaemia whereas that reported in the present study is endogenously based (table 6.4). It could be argued, therefore, that the hypocholesterolaemia previously observed for saponins is due to inhibition of cholesterol absorption[299].

Several authors have reported increased excretion of acid and neutral sterols when saponin is added to the diets of rats [195, 161], and pigs[196]. However, in the present study, saponins appeared to be effective only when soya was used as the protein source. Since soya feeding itself increased the intestinal pool and excretion of acid and neutral sterols, it is not surprising that when saponin is added to the diet there is a further increase in the excretion of sterols, perhaps by inhibiting their re-absorption. This does not happen with milk protein diets and there is no satisfactory explanation for this.

Figure 6.3 shows the excretion of acid and neutral sterols in relation to serum total cholesterol concentration and it can be seen that the variation in serum cholesterol occurs over a narrow range of acid and neutral sterols. The converse is also true. Therefore, milk protein fed rabbits may lack the capacity to maintain normal levels of serum cholesterol through impaired cholesterol oxidation. Data presented by Roberts et al. (1979) [300] showed an increased turnover of plasma LDL cholesterol in soya fed rabbits. Alternatively, in milk fed rabbits a normal biliary flow of bile acids and cholesterol may be associated with an enhanced capacity for re-absorption of endogenous sterols. Enhanced cholesterol absorption has been observed in casein fed pigs[152] and rabbits[176]. However, in rabbits cholesterol absorption does not increase hepatic oxidation to bile acids of de novo synthesised cholesterol[301]. The increased absorption may be due to a greater bile acid pool size in rabbits fed semi-purified casein based diets relative to those on commercial

feed[297]. Dietschy and Wilson (1970)[302] reported that an increase in bile acid pool size decreases intestinal synthesis of cholesterol and increases cholesterol absorption.

The data on the excretion of individual neutral and acid sterols do not provide a basis for explaining the different effects of soya and milk proteins on serum cholesterol. The substantial micro-flora of the rabbit caecum may have had an effect on the molar proportions of individual bile acids and neutral sterols and thus it would not be possible to constuct a hypothesis like the one recently put forward for the hypo-cholesterolaemic effect of dietary fibre in rats[303].

There is growing evidence to suggest that the hypo-cholesterolaemic effect of casein and other purified animal proteins is due to protein per se. Glycine supplementation[159] of casein reduced serum cholesterol in rabbits and cholesterol fed rats and was associated with reduced aortic atherosclerosis. Supplementation of soya bean proteins with lysine so as to increase its lysine-arginine ratio to that in casein (from 0.9 to 2.0) resulted in a sharp increase in atherogenicity and serum cholesterol concentration[178]. However, these authors have been unable to explain the mode of action of amino acid supplementation on cholesterol metabolism. The possible involvement of insulin and glucagon in the effect of protein on cholesterol metabolism has been suggested[173] and this possibility merits serious consideration.

The influence of dietary protein on cholesterol metabolism has important implications for man, both in the treatment of

hyperlipidaemia and in the interpretation of epidemiological data relating diet to the incidence of coronary heart disease. However, the data on the effect of protein source on plasma cholesterol of man is contradictory. Although some experiments reported significant reduction in serum cholesterol of normal volunteers consuming high soya diets[166], other experiments failed to show any alteration of serum cholesterol of normo-lipaemic volunteers[170, 171, 172]. In contrast to these studies on normal healthy individuals, a series of studies on type IIa and type IIb hyperlipidaemic patients showed significant reductions in serum cholesterol concentrations in the order of 10-20%[167, 168, 169, 174]. Thus, the effect of dietary protein on serum cholesterol may be similar in normo-lipaemic man and rat where as the situation in the hyperlipaemic man may be more similar to that in rabbits.

Clearly, dietary protein can play an important role in cholesterol metabolism in many species including man and its mode of action warrants further study.

Table 6.1 Mean values (± SEM) for the effect of four dietary treatments on energy intake, live weight gain and serum lipid levels.

Dietary saponin (g/kg)	0		10		±
Portein Source	Soya	Milk	Soya	Milk	SEM
Energy intake (MJ/kg/d)	0.35[a]	0.41[a]	0.47[b]	0.47[b]	0.04
Live weight gain (g/kg/d)	9.5	7.8	8.8	9.9	0.8
Serum cholesterol (mmol/l)					
Total	2.55[a]	6.22[b]	1.75[a]	5.82[b]	0.5
HDL	0.38	0.38	0.39	0.4	0.03
VLDL + LDL	2.17[a]	5.84[b]	1.36[a]	5.42[b]	0.4
Serum triglycerides (mmol/l)					
Total	0.75[a]	0.91[b]	0.74[a]	0.89[b]	0.05
HDL	0.25	0.21	0.22	0.24	0.01

[a,b] mean values without a common superscript are significantly different ($p < 0.05$).

Table 6.2 The effect of the individual dietary treatments and separate effects of protein source and saponin supplementation on the excretion of total and individual bile acids (mg/kg/d) mean values ± SEM.

	Relative retention time	Peak No.	0% saponin		10% saponin		SEM	Protein effect		Saponin Effect		SEM	Interaction
			Soya	Milk	Soya	Milk		Soya	Milk	1%	0%	±	
Number of animals	-	-	6	6	6	6	-	12	12	12	12	-	-
Total acid sterols	-	-	4.75[a]	2.46[b]	7.63[c]	2.36[b]	0.8	6.19	2.42**	5.0	3.61	0.6	N.S.
Lithocholic acid	0.77	1	1.14	0.86	1.23	1.06	0.3	1.18	0.96	1.14	1.0	0.21	N.S.
Deoxycholic acid	1.26	5	1.09	0.88	1.71	0.87	0.26	1.4	0.88	1.29	0.99	0.18	N.S.
Unknown	1.6	7	0.81	0.14	0.43	0.03	0.26	0.61	0.09	0.23	0.48	0.18	N.S.
Cholic acid	1.96	9	0.06	0.05	0.29	0.15	0.09	0.17	0.1	0.22	0.05	0.07	N.S.
Unknown	2.25	10	0.37	0.05	0.4	0.05	0.16	0.39	0.05*	0.23	0.21	0.11	N.S.
Unknown	2.57	11	0.56	0.31	1.07	0.03	0.25	0.81	0.17*	0.55	0.44	0.18	N.S.
Unknown	2.82	12	0.72[b]	0.18[b]	2.5[a]	0.16[b]	0.37	1.61	0.17**	1.33	0.45*	0.26	*

[a,b,c] Mean values for four dietary treatments without a common superscript are significantly different ($p < 0.05$). Statistical differences between the separate mean values for protein source and saponin supplementation and for interaction are indicated thus: * ($p < 0.05$); ** ($p < 0.01$).
N.S. No significant difference.

Table 6.3 The effect of individual dietary treatments and the separate effects of protein source and saponin supplementation on the excretion of total and individual neutral sterols (mg/kg/d) mean values ± SEM.

	0% saponin		1% saponin		SEM	Protein Effect		Saponin Effect		SEM	Interaction
	Soya	Milk	Soya	Milk		Soya	Milk	1%	0%		
Number of animals	6	6	6	6	6	12	12	12	12	12	
Total neutral sterols	11.13^{ab}	7.14^{b}	16.97^{a}	8.22^{b}	1.92	14.05	7.68**	12.6	9.14	1.35	N.S.
Coprostanol	2.4	0.89	2.88	2.01	0.68	2.64	1.45	2.45	1.64	0.48	N.S.
Cholesterol	2.39	3.64	2.66	3.45	0.64	2.52	3.54	3.05	3.02	0.46	N.S.
3 keto homologues of the three plant sterols	3.44	2.47	6.41	2.57	0.8	4.93	2.52**	4.49	2.95	0.5	N.S.
2 unidentified peaks (no's 8 & 9 - see text)	2.08^{a}	0.00^{b}	4.41^{a}	0.0^{b}	-	3.25	0.00**	2.21	1.0	-	-
Dry matter excretion (g/kg/d)	2.79	2.49	4.04	3.98	0.71	3.42	3.24	4.01	2.64	0.5	N.S.

^{a,b} Mean values for the four dietary treatments without a common superscript are significantly different ($p < 0.05$). Statistical differences between separate mean values for protein source and saponin supplementation and for interaction are indicated thus: * ($p < 0.05$); ** ($p < 0.01$); N.S. No significant difference.

Table 6.4 Summary of data from the literature on the effects of saponins on serum cholesterol

Reference	Species	Cholesterol supplementation g/kg diet	Saponin supplementation g/kg diet	Basal diet	Basal diet + Cholesterol	Basal diet + Saponin	Basal diet + Cholesterol + Saponin
Newman et al (1957)	Chicken	5	9	4.3	5.8	4.0	4.4
Griminger et al (1958)	Chicken	3	6	6.5	9.0	5.7	7.6
Morgan et al (1972)	Chicken	5	2.5	4.9	8.4	4.5	6.4
Morgan et al (1972)	Chicken	5	2.5	4.5	8.2	4.2	5.7
Oakenful et al (1980)	Rat	10	10	1.5	2.6	1.6	2.1
Sautier et al (1979)	Rat	0	10	2.4	-	2.1	-
	Rat	0	10	2.4	-	2.3	-
Topping et al (1980)	Pig	0	10	3.0	-	2.8	-

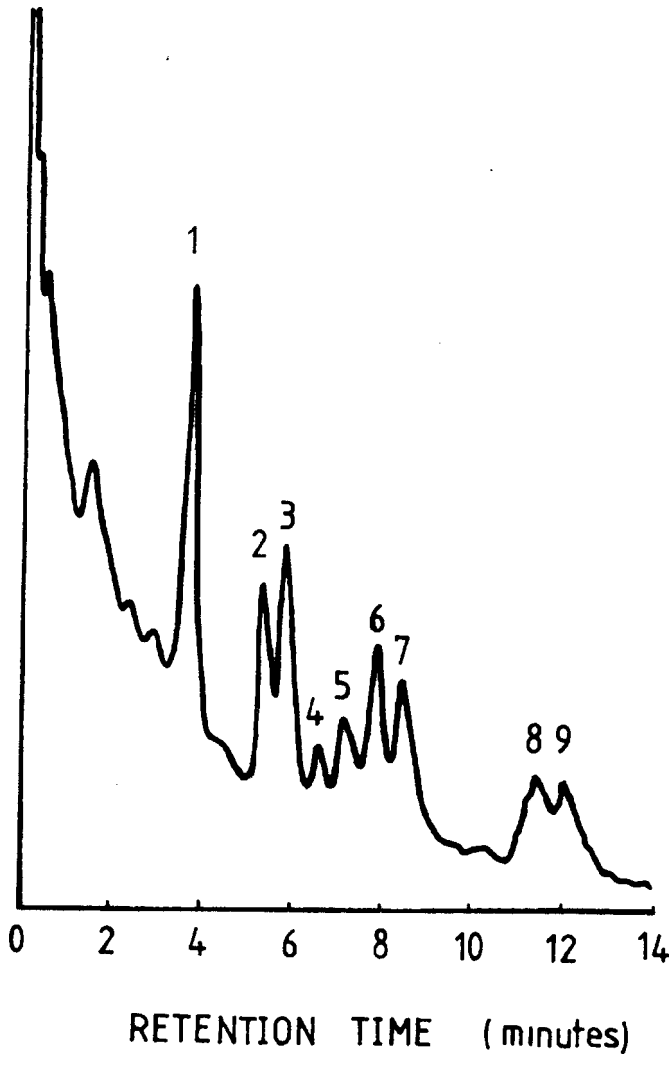


Fig. 6.1 Gas-liquid chromatogram of faecal neutral steroids, 8 peaks were resolved. 1. Internal standard. 2. Coprostanol. 3. Cholesterol. 4. Coprostanone*. 5, 6, 7, 3-keto homologues of plant sterols, compesterol*, stigmasterol* and sitosterol (* identified by comparison to the analysis of Miettinen et al. (1965))

Peaks 8, 9, were not identified, (probably keto derivatives of cholesterol).

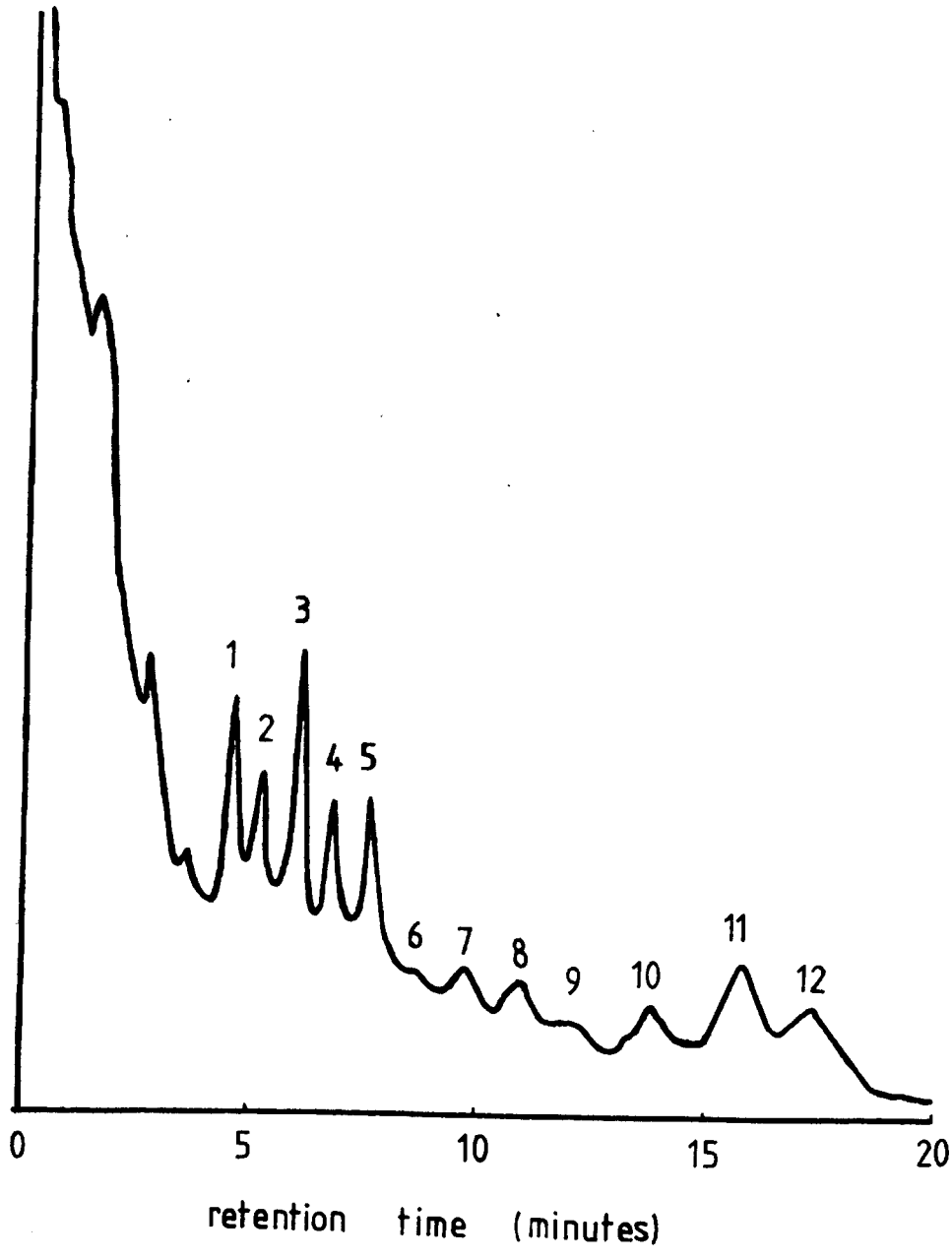


Fig. 6.2 Gas-liquid chromatogram of methylated faecal bile acids. 11 peaks were resolved. 1. Lithocholic acid. 2. Not a bile acid. 3. Internal standard. 4. Not a bile acid. 5. Deoxycholic acid. 6. Not a bile acid. 7. Probably 3β hydroxy 12 keto cholanic acid. 8. Not a bile acid. 9. Cholic acid. 10. 12, Keto litho cholic acid. 11. Unknown. 12. 3,12 diketo cholanic acid.

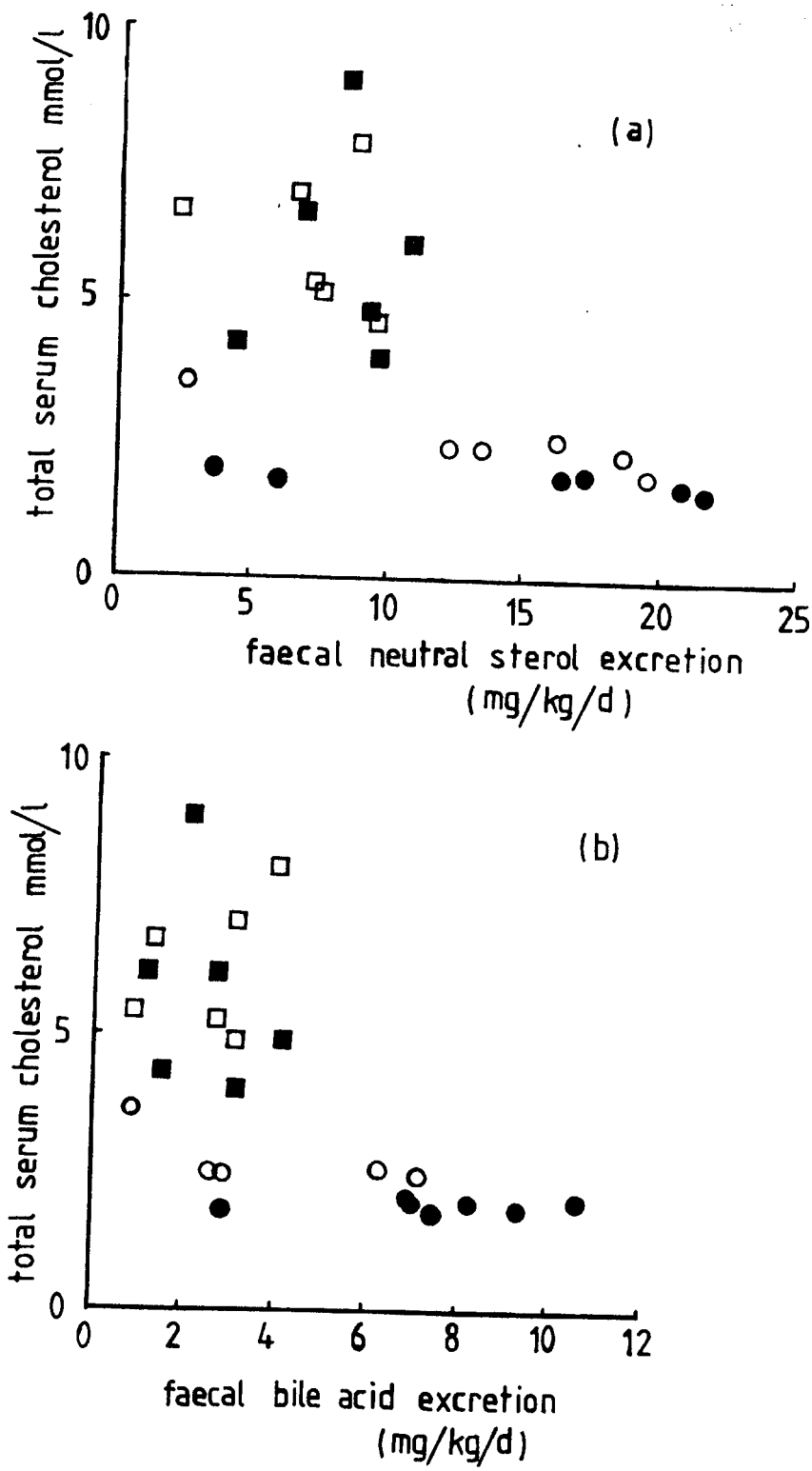


Fig. 6.3 The relationship between total serum cholesterol (mmol/l) and the excretion of (a) neutral sterols, (b) bile acids (mg/kg/d). Milk diet without saponin □ Milk diet with 10 g/kg saponin ■ ; Soya diet without saponin ○ ; Soya diet with saponin ● .

CHAPTER 7

EXPERIMENT 5

7.1 Introduction

On the basis of epidemiological studies, dietary fibre has been claimed to have a protective role against the development of coronary heart disease[125, 126]. However, the effects of different fibres on serum cholesterol or bile acid excretion vary when fed to animals or humans[304]. It is proposed that the hypocholesterolaemic effect of the fibre is due to its adsorption of bile acids in the intestine, thus interfering with the enterohepatic cycle of bile acids. The increased excretion of sterols is offset by hepatic synthesis of bile acids leading to decreased release of cholesterol into plasma from liver[126].

Data from *in vitro* studies revealed that various plant fibre fractions including lignin and wheat straw had the capacity to bind bile acids[127, 128, 129]. Although binding capacities varied with the type of fibre, all the natural fibres had great capacity to bind bile salts than did the synthetic fibres. Oakenfull and Fenwick (1979)[197] repeated these *in vitro* binding studies and found that some of the fibre preparations used in previous experiments did not in fact bind bile salts. Although, this discrepancy in results was not explained they proposed that only fibre preparations containing saponins adsorb bile acids to a significant extent.

It was demonstrated that the ability of the fibre to adsorb bile acids decreased when a saponin was extracted from lucerne. Wheat bran which did not have the ability to adsorb bile acids did so when lucerne saponin was added to it. Evidence also has been presented that dietary saponins lower plasma cholesterol levels in rats fed hypercholesterolaemic diets by inducing adsorption of bile salts to fibre in the gut[195].

However, the results of the previous experiments on the rabbit failed to support the idea that hypocholesterolaemic action of soya protein is a consequence of its saponin content. Since it was considered that saponins could act in the presence of exogenous cholesterol an experiment was designed to determine the effect of saponin on rat and hamster serum and liver lipids when fed in hypercholesterolaemic diets with and without dietary fibre. Thus the separate effects of fibre and saponin as well as their interaction could be determined.

7.2 Materials and Methods

Twenty one male weaning Wistar rats were fed ad libitum on one of four isonitrogenous isoenergetic diets for four weeks. The composition of diets are given in table 2.6. Two of the diets were supplemented with solkafloc (purified wood cellulose) as a fibre source at 50 g/kg level. Commercial white saponin was added to one of the fibre supplemented diets and one fibre free diet at 10 g/kg concentration. Thus, the four dietary treatments

were as follows: + Fibre + Saponin, + Fibre - Saponin, - Fibre + Saponin, - Fibre - Saponin. All diets contained cholesterol 10 g/kg diet, and cholic acid 2.5 g/kg diet.

In the 2nd experiment, twenty four male weaning Golden Syrian hamsters were used. The composition of diets are given in table 2.7. The experimental design is essentially similar to the above experiment.

Animals were fed in groups of 5-6 and their combined food intake recorded each week. The weight changes were recorded for each animal weekly. At the end of the experimental period of four weeks the animals were killed by over-exposure to anaesthetic diethyl ether and samples of liver and serum obtained as described in section 2.3.1. Serum and liver cholesterol and triglycerides were determined using the automated procedures described in section 2.3.3.2.

7.3 Results

Results of the rat and hamster experiments are summarised in tables 7.1 and 7.2.

In the experiment with rats, live weight gain was significantly decreased by the addition of fibre to the diet, and serum triglyceride levels were significantly increased by the same treatment. Other parameters did not show significant changes.

In the experiment with hamsters live weight gain was reduced significantly by fibre supplementation and increased significantly by saponin supplementation. None of the other parameters were affected by dietary treatments.

7.4 Discussion

The reduction in live weight gain shown by both rats and hamsters due to supplementation with dietary fibre is probably a reflection of slightly lowered energy intake in these treatments. This could be expected with the increase in volume of food when the fibre is present. However, the increase in weight gain due to saponin supplementation of the diet cannot be readily explained. This contrasts with previous work where significant reduction in weight of chicks due to saponin was observed[191, 192].

The rat has a very efficient cholesterol homeostatic mechanism and excess dietary cholesterol is rapidly converted to bile acids in the liver, and serum cholesterol is difficult to raise. Expanding the bile acid pool by bile acid feeding enhances cholesterol absorption and inhibits conversion of cholestrol to bile acids, thus leading to accumulation of cholesterol in the liver[302]. Therefore supplementing diets with cholesterol and cholic acid leads to increased retention of absorbed exogenous cholesterol and large increase in both plasma and liver cholesterol concentrations result[305]. The hamster lacks the ability to increase its bile acid synthesis-excretion rate in response to dietary cholesterol which distinguishes it from the rat and therefore the response to dietary cholesterol + cholic acid is much greater in this species[305, 306]. In the present study, both species responded well to cholesterol + cholic acid feeding resulting in massive hypercholesterolaemia, specially the hamster. In the case of the rat, cholesterol and cholic acid

feeding increased serum cholesterol three fold when compared to non-cholesterol fed rats.

In spite of the raised serum cholesterol levels, no effect due to saponin supplementation was observed either alone or in the presence of fibre. This contrasts with the results obtained by Oakenfull and Fenwick (1979)[197]. In their study the increase in serum cholesterol in the cholesterol fed animal was only moderate, e.g. 60 and 100 mg cholesterol per 100 ml serum in control and cholesterol fed animals, respectively. This is further evidence of the value of a dietary cholic acid supplement in increasing blood cholesterol of cholesterol fed rats. However, in their study Oakenfull and Fenwick found that saponin lowered serum cholesterol in cholesterol fed rats, but this was not significant. It is possible that had these authors used cholic acid to raise blood cholesterol to a maximum they would not have observed the hypocholesterolaemic effect due to saponin. If it is assumed that saponin does indeed adsorb bile acids to fibre, then it is possible that addition of 0.25% cholic acid to the diet in the present study was more than could be adsorbed to fibre by 1% saponin, leaving sufficient additional cholic acid for absorption and subsequent suppression of hepatic cholesterol oxidation. A recent observation also shows that bile acid excretion is proportional to the saponin content of the diet[307].

The purified wood cellulose (solkafloc) alone has been shown to lack any consistent hypocholesterolaemic activity in the rat [308]. Indeed, slight increases in plasma and liver cholesterol of rats have been observed with supplementation of hyper-

cholesterolaemic diets with cellulose[133]. In the present study this inconsistent effect was noted where the hamster showed no effect due to fibre supplementation while, in the rat, serum triglyceride levels were significantly increased with the supplementation.

Increased excretion of faecal neutral sterols and/or bile acids have been observed when diets were enriched with saponaria [195, 196, 161], alfalfa[299] or soya saponins[307, 309]. Decreased absorption of [^{14}C] cholesterol in the rat due to alfalfa saponin and formation of non-absorbable complexes of saponin with sterols in the gastro-intestinal tract have been described [299]. In some of these experiments plasma or liver cholesterol concentrations were not affected[161, 309]. It is therefore possible that alterations in sterol metabolism could occur to some extent without changes in plasma or liver cholesterol.

In man and experimental animals fibre fractions that do not contain saponins such as cellulose[310, 311], hemi-cellulose and pectin[131, 132, 135] have been shown to increase bile acid output as well as lowering plasma cholesterol levels in some instances.

There is as yet no general agreement as to the role of dietary fibre in bile acid excretion[312]. However, the data suggest that certain fibre fractions adsorb bile acids while others form gels with water which could then trap bile acids and increase their excretion.

Table 7.1 Mean values (± SEM) for live weight, live weight gain, energy intake, serum and liver lipids of rats fed on the four experimental diets for four weeks.

% Fibre	% Saponin	No of Animals	Final Live weight (g)	Live weight gain (g/rat/d)	Energy Intake (MJ/rat/d)	Serum total cholesterol (mmol/l)	Serum total Triglyceride (mmol/l)	Liver weight (g)	Liver Lipids (mmol/g) Chol-esterol	Triglyc-eride
0	0	5	282	5.66	0.442	5.51	0.9	16.74	0.18	0.036
0	1	5	289	6.04	0.41	5.0	0.92	17.34	0.2	0.036
5	0	6	288	5.71	0.352	5.76	1.14	17.5	0.2	0.036
5	1	5	262	5.34	0.393	5.98	1.24	16.5	0.22	0.036
± SEM	-	5/6	13.8	0.09	-	0.68	0.08	1.08	0.017	0.123
+ Fibre	-	12	275	5.54	0.372	5.87	1.19	17.01	0.21	0.036
- Fibre	1	12	286	5.85*	0.425	5.23	0.91**	17.04	0.19	0.036
-	+ Saponin	12	275	5.69	0.402	5.46	1.08	16.93	0.2	0.036
-	- Saponin	12	285	5.68	0.397	5.64	1.02	17.12	0.2	0.036
± SEM			9.3	0.06	-	0.46	0.05	0.72	0.01	0.08

*Differences significant at $p < 0.05$

**Differences significant at $p < 0.01$

Table 7.2 Mean values (± SEM) for live weight, live weight gain, energy intake, serum and liver lipids in hamsters fed on the four experimental diets for four weeks (mean ± SEM).

% Fibre	% Saponin	No of Animals	Final Live Weight (g)	Live Weight gain (g/ hamster/d)	Energy Intake (MJ/hamster/ d)	Serum Total Cholesterol (mmol/l)	Serum Total Triglyceride (mmol/l)	Live weight (g)	Liver Lipids (mmol/g) Cholesterol	Triglyceride
0	0	6	107.8	1.9	0.113	7.55	3.4	7.1	0.13	0.09
0	1	6	103.1	2.0	0.103	8.3	3.3	6.4	0.11	0.09
5	0	6	102.5	1.5	0.103	7.3	3.1	6.8	0.11	0.1
5	1	6	96.8	1.8	0.107	7.31	2.9	6.7	0.12	0.1
SEM	-	-	3.9	0.07	-	0.3	0.16	0.3	0.009	0.0006
+ Fibre	-	12	99.65	1.6	0.105	7.31	3.0	6.7	0.12	0.1
- Fibre	-	12	105.4	2.0**	0.108	7.93	3.3	6.8	0.11	0.09
-	+ Saponin	12	105.1	1.7	0.108	7.43	3.2	6.9	0.11	0.1
-	- Saponin	12	99.15	1.9**	0.105	7.81	3.1	6.6	0.11	0.09
			2.74	0.05	-	0.22	0.12	0.22	0.006	0.0004

**Differences significant at $p < 0.01$

CHAPTER 8

8.1 Introduction

Experiments reported in this thesis (Chapters 3, 4 and 6) and elsewhere have shown that the nature of the dietary protein may influence the type and severity of atherosclerotic lesions in rabbits. Most of the interest in this field has been in the immunogenic or the hypo- or hyper-cholesterolaemic effects of proteins, and to date there has not been any comparative study of the nutritional value of proteins exhibiting contrasting properties in these respects. The experiments reported in this chapter set out to consider this aspect.

Experiment 6.1

In a previous study carried out in this department on the immunogenic effect of dietary proteins on the development of atherosicerotic lesions in rabbits[215], high fat diets (maize and coconut oil) based on two soya protein preparations were used. The protein preparations were promine D (a soya protein isolate used in the previous experiments in this thesis) and promosoy 100, a soya flour, the latter believed to be less antigenic[214]. Antibodies against soya protein antigens were detected in all the rabbits and the incidence of complicated atherosclerotic lesions appeared to be greater in promine D fed animals. A significant correlation was also found between the severity of atherosclerosis (aorta scores) and the final live weights of the rabbits. These data suggested that the nutritive value of the proteins may have been different. This experiment sets out to examine this possibility.

Experiment 6.2

The different effects of soya protein and cow's milk protein on the cholesterol levels of the rabbit has been demonstrated in previous chapters (Chapters 3, 4 and 6). Since some of these differences could be attributed to the differences in the nutritive value of the proteins, experiment 6.2 was undertaken to evaluate the nutritive value of soya and cow's milk protein using nitrogen balance studies in rabbits.

Experiment 6.3

The recommended value for the crude protein to metabolizable energy ratio (CP:ME) required for the optimal growth of the young rabbit is 14.3 g/MJ[313]. In the previous studies where hypercholesterolaemia due to cow's milk protein was observed (Chapters 3, 4 and 6), the CP:ME ratio was about 18 g/MJ. It was therefore considered possible that the different surplus amino acids that were available from these two high protein diets may have had different effects on the cholesterol metabolism of the rabbits. Therefore, experiment 6.3 was undertaken to evaluate soya and cow's milk protein in diets at CP:ME ratio of 6 g/MJ.

8.2.1 Animals and Materials

Experiment 6.1

Four male New Zealand White rabbits were offered one of four, isonitrogenous, isoenergetic diets in a 4 X 4 Latin square design. The diets differed in their soya protein source (Promine D or Promosoy 100) and fat source (maize or coconut oil). The

composition of these high fat and high protein (30% protein energy) diets are given in table 2.8.

Experiment 6.2

Twenty two New Zealand White rabbits were divided into two groups of seven males and four females and were offered one of two high protein (30% protein energy), low fat (5% fat energy) diets. The compositions of the diets based on soya bean protein (Promine D) and cow's milk protein (Casumen + dried skim milk) are given in table 2.5.

Experiment 6.3

Twelve, New Zealand White rabbits were divided into two groups of each of three males and three females and were offered one of two, isonitrogenous, isoenergetic diets based on soya bean protein and cow's milk protein. The composition of the diets which were low in protein (10% protein energy) and fat (5% fat energy) are given in table 2.9.

All diets in experiments 6.1, 6.2 and 6.3 were supplemented with methionine to provide a final concentration of methionine + cystine to 3.9 g/16 g nitrogen.

8.2.2 Methods

In all three experiments the animals were housed in metabolism cages and food and fresh water were available ad libitum. After an initial equillibration period of seven days, faeces and urine were collected during a 7 day experimental period, as described in section 2.3.1.3.

Food intake and weight gain were recorded daily during the experimental period.

In the experiment 6.1 the gross energy of diets and faeces were determined by an adiabatic bomb calorimeter (section 2.3.7) and total fat in the diets and faeces were determined by chloroform-methanol extraction (section 2.3.6). Percentage dry matter content of food and faeces were obtained by drying to a constant weight in an oven at 105°C for 48 h.

The aliquots of food, faeces and urine of each 7 d collection were analysed for total nitrogen (section 2.3.5).

8.3 Results and Calculations

Using the notations, Nitrogen Intake = N_I
Faecal nitrogen = N_F
Urinary nitrogen = N_u

following parameters were calculated,

$$\text{Apparent nitrogen digestibility (ADN)} = \frac{(N_I - N_F)}{N_I} \times 100$$

$$\text{Nitrogen Balance (NB)} = N_I - (N_F + N_u)$$

$$\text{Utilization of apparently digested nitrogen} = \left[\frac{N_I - (N_F + N_u)}{N_I - N_F}\right] 100$$

The results of experiment 6.1 are summarised in table 8.1 and those of experiments 6.2 and 6.3 in table 8.2.

No significant differences were found between treatments in the live weight gain as estimated by regression analysis of daily live weights in any of the experiments.

In experiment 6.1 the apparent digestibility of energy, dry matter and nitrogen was found to be significantly reduced in rabbits given soya flour as opposed to soya protein isolate ($p < 0.01$). However, no significant interaction was found between protein and fat sources. In experiments 6.2 and 6.3 no significant differences were found in the apparent digestibility values between the protein sources, isolated soya protein and cow's milk protein.

The apparent digestible nitrogen intake and nitrogen balance in experiments 6.1 and 6.2 were not significantly different. Furthermore, covariance analysis did not show any significant differences in nitrogen balance between rabbits fed soya isolate, soya flour or the cow's milk protein in these two experiments. The relationship between ADN intake and nitrogen balance in experiments 6.1 and 6.2 are shown in Fig. 8.1 and 8.2 respectively. No significant differences were observed in the slopes of the regression lines indicating equal rates of utilization of nitrogen between protein sources.

In experiment 6.3 the ADN intake and nitrogen balance were significantly lowered in the rabbits fed soya protein based diets as compared to those fed cow's milk protein based diets. However, the covariance analysis did not indicate any significant difference in nitrogen balance or the utilization of apparently digested nitrogen between the two diets.

8.4 Discussion

The better performance of the animals in experiment 8.1 is probably due to the higher palatibility of the diets, that led to increased energy intake. The reduction in apparent digestibility of dry matter, energy, and nitrogen in the diet based on soya flour is probably a consequence of the method of industrial preparation of this product. Equally the complex carbohydrate fraction associated with the protein might be interfering with the digestion as promosoy 100 has only 60% protein as opposed to 90% protein in promine D. Lowered growth performance of preruminant calves fed soya flour based diets have been reported[213, 314]. However, in the present experiment, the reduction in the digestibility of the diets was not reflected in the performance of the animals, probably because an adequate amount of protein was still available to the animals. The data do not indicate major differences in nutritive value of the two proteins and therefore the correlation of atherosclerosis with the final live weight in the previous experiment[215] may have arisen through a reduction in food intake as arterial disease developed.

It is generally accepted that lowered protein to energy ratio in a diet leads to higher biological value[315]. This fact is evident from the results of the three epxeriments. In experiment 6.3 where CP:ME ratio is low, higher value for the utilization of apparently digestible nitrogen was observed, with the cow's milk protein based diets. However, in comparison to the apparent biological values observed in other species[315]

this value is still low. This may have been due to the fact that no correction was made for the endogenous nitrogen losses. The rabbit has a large caecum and bacterial activity in the caecum and the colon can increase the amount of protein in the digesta. Since no attempt was made to prevent coprophagy in the present set of experiments the ADN intake may have been underestimated. Furthermore, it is possible that bacterial degradation of the undigested protein and peptide could take place in the caecum with the production of ammonia[317]. Subsequent absorption of ammonia from the gut and its conversion to urea could result in higher excretion of nitrogen in the urine. Coprophagy has been shown to increase digestibility of nitrogen, nitrogen balance and weight gain of rabbits, but the apparent biological value was increased significantly only with low quality protein diets, e.g. gelatin, where as no significant increase was observed with casein based diets[318]. Another possibility for the lowered ADN retention, in experiment 6.3 may have been partly due to the lowered energy intake. The diets used in this experiment were hard and inpalatable to some of the animals. However, other workers also recorded low ADN retention values in rabbits[316, 318] with high or low quality proteins. There is no satisfactory explanation for this poor utilization of protein in rabbits.

The nutritive value of isolated soya protein and cow's milk protein has been compared in young animals of various species. In the pig[320] and the lamb[319], isolated soya protein has been found to be inferior to cow's milk protein whereas in the calf[321]

no difference was found in the utilization of nitrogen between these two protein sources. Although this comparison has not been reported in the rabbit lowered weight gain due to casein based semi-purified diets have been reported[322, 255]. Experiment 6.3 which provided only a marginal level of protein for the maintenance of the rabbits, should have indicated a difference in the utilization of nitrogen from the two proteins if the protein quality was truly different. The results however indicated that the weanling rabbit is able to utilize methionine supplemented soya or cow's milk proteins equally well from semi-purified diets. The diets used in experiment 6.2 contained a higher CP:ME ratio than that recommended for the rabbit and therefore it is not surprising that significant effects due to protein source were not observed in either weight gain or energy utilization. The live weight changes associated with casein based diets in earlier experiments[255, 322] may have been due to reduction in voluntary food intake.

Hypercholesterolaemia due to milk protein was evident even at the low level of protein (10% energy from protein) in the diet (experiment 6.3), although the levels of cholesterol were lower than that observed for the high protein cow's milk protein based diets in experiment 6.2. This result is in aggreement with the work on the rabbits using casein at different levels in the diet [156] and also in pigeon[153] and squirrel monkey[154] where high levels of protein were more atherogenic and/or hypercholesterolaemic than lower levels. The effect of dietary protein level on the serum cholesterol of the chicken were however different and

low levels of protein were much more hypercholesterolaemic than high levels[157, 323, 324]. The sensitivity of the different species to different amino acids might be the cause for this difference. The fact that the level of soya protein in the diet did not alter the serum cholesterol of the rabbit, whereas the effect due to milk protein was dose related indicate that the hypercholesterolaemia due to milk protein may be due to an imbalance of amino acids.

The saponin content of soya protein based diet in experiments 6.2 and 6.3 were 0.009% and 0.003% respectively according to Oakenfull et al.[200], and is extremely low to have any effect on cholesterol metabolism.

The results thus indicate that methionine supplemented soya bean protein and cow's milk protein are nutritionally similar in rabbit and the hypocholesterolaemia associated with soya protein is not due to an amino acid deficiency, but the hypercholesterolaemia associated with milk protein in the rabbit may be partly due to an imbalance of amino acids.

Table 8.1 The mean values (± SEM) for the weight gain, energy intake, apparent digestibilities and nitrogen balance studies of rabbits in experiment 6.1.

	Protein Source			Fat Source			SEM	Interaction
	Promine D		Promosoy	Coconut		Maize		
n	8		8	8		8		
Energy Intake MJ/kg/d	0.55	N.S.	0.59	0.54	N.S.	0.6	0.05	N.S.
Live weight gain g/kg/d	12.7	N.S.	11.0	11.0	N.S.	13.0	2.7	N.S.
Apparent digestibility (%)								
Dry matter	85.6	**	82.9	84.8	N.S.	83.7	1.9	N.S.
Energy	88.5	**	86.8	88.4	N.S.	86.9	1.2	N.S.
Fat	94.8	N.S.	95.4	95.8	N.S.	94.4	0.27	N.S.
Nitrogen	90.8	**	88.7	88.7	N.S.	86.96	0.02	N.S.
Apparent digestible nitrogen (ADN) intake g/kg/d	1.53	N.S.	1.37	1.4	N.S.	1.5	0.01	-
Nitrogen Balance g/kg/d	0.49	N.S.	0.33	0.38	N.S.	0.44	0.008	N.S.
Adjusted nitrogen balance g/kg/d	0.44	N.S.	0.38	0.42	N.S.	0.40	-	N.S.
ADN retention (%)	34.1	**	25.0	28.0	N.S.	31.0	1.5	-
Adjusted ADN retention (%)	30.3	N.S.	26.2	28.9	N.S.	27.5	-	-

** Differences significant at $p < 0.01$
N.S. No significant difference

Table 8.2 The mean values (± SEM) for the weight gain, energy intake, serum lipid levels, digestibility and nitrogen balance studies in rabbits in experiments 6.2 and 6.3

	Experiment 8.2			Experiment 8.3		
	Promine D	Cow's milk Protein	SEM	Promine D	Cow's Milk Protein	SEM
No. of animals	11	11		6	6	
Mean live weight (kg)	2.73	2.45	0.11	2.26	2.16	0.1
Live weight gain (g/kg/d)	9.14	8.83	0.5	5.6	9.0	2.3
Energy intake (MJ/kg/d)	0.43	0.44	0.03	0.33	0.34	0.02
ADN intake (g/kg/d)	1.09	1.1	0.06	0.27	0.32**	0.01
Nitrogen Balance (g/kg/d)	0.39	0.33	0.04	0.03	0.16**	0.02
Adjusted nitrogen balance (g/kg/d)	0.39	0.34	-	0.07	0.12	-
ADN retention (%)	33.8	28.6	3.5	10.6	48.1**	7.5
Adjusted ADN retention (%)	36.0	30.8	-	23.6	40.6	-
% apparent digestibility						
Dry matter	87.9	88.7	1.2	79.7	82.5	2.5
Nitrogen	88.3	89.3	1.6	81.6	84.3	3.1
Serum total cholesterol mmol/l	2.55	6.22**	0.5	2.28	3.19*	0.3
Serum total triglycerides mmol/l	0.75	0.91**	0.05	0.73	0.82	0.07

* Differences significant at $p < 0.05$ ** Differences significant at $p < 0.01$

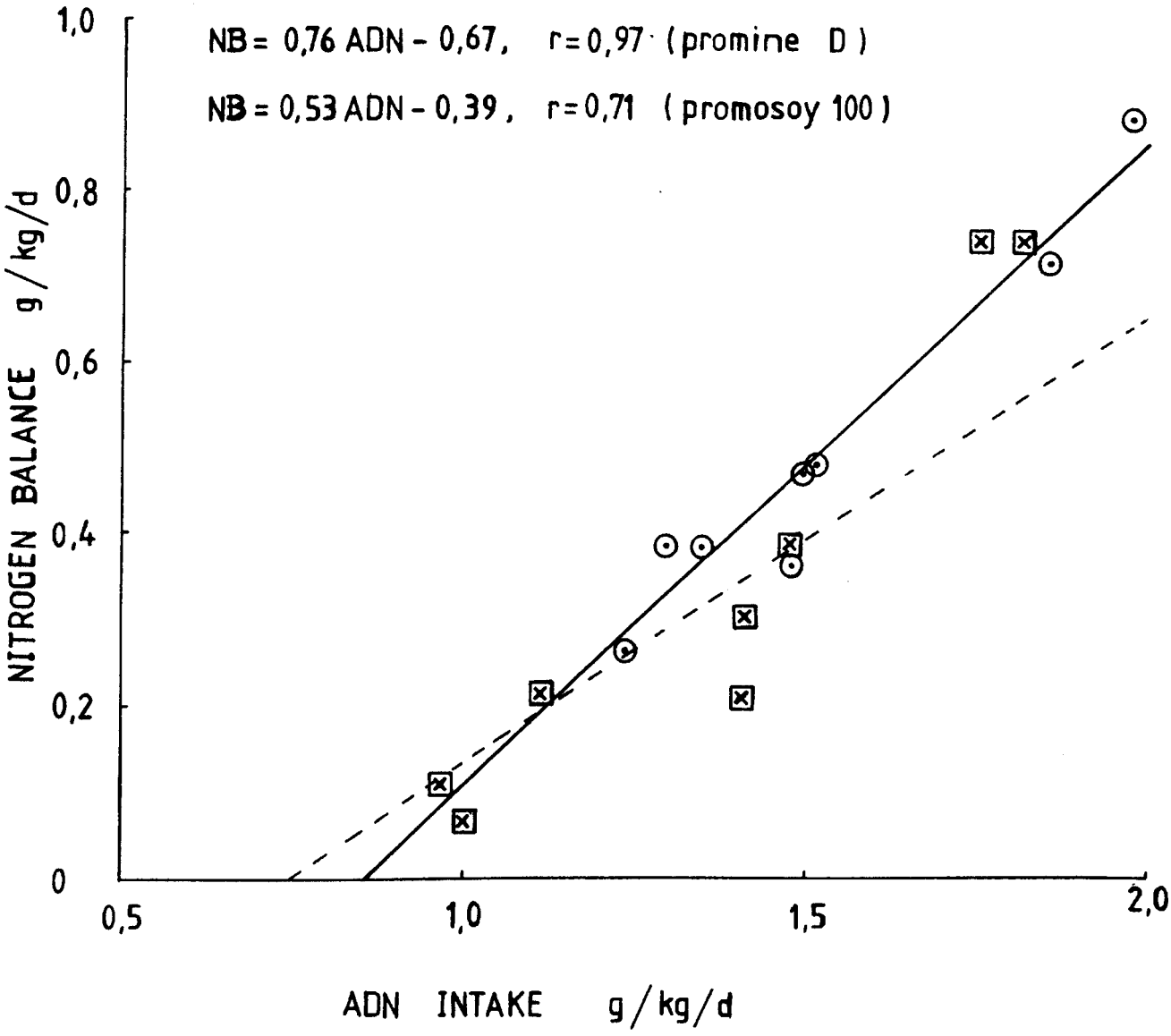


Fig. 8.1 Nitrogen balance as a function of ADN intake in rabbits fed promine D (⊙) and promosoy 100 (⊠) containing diets.

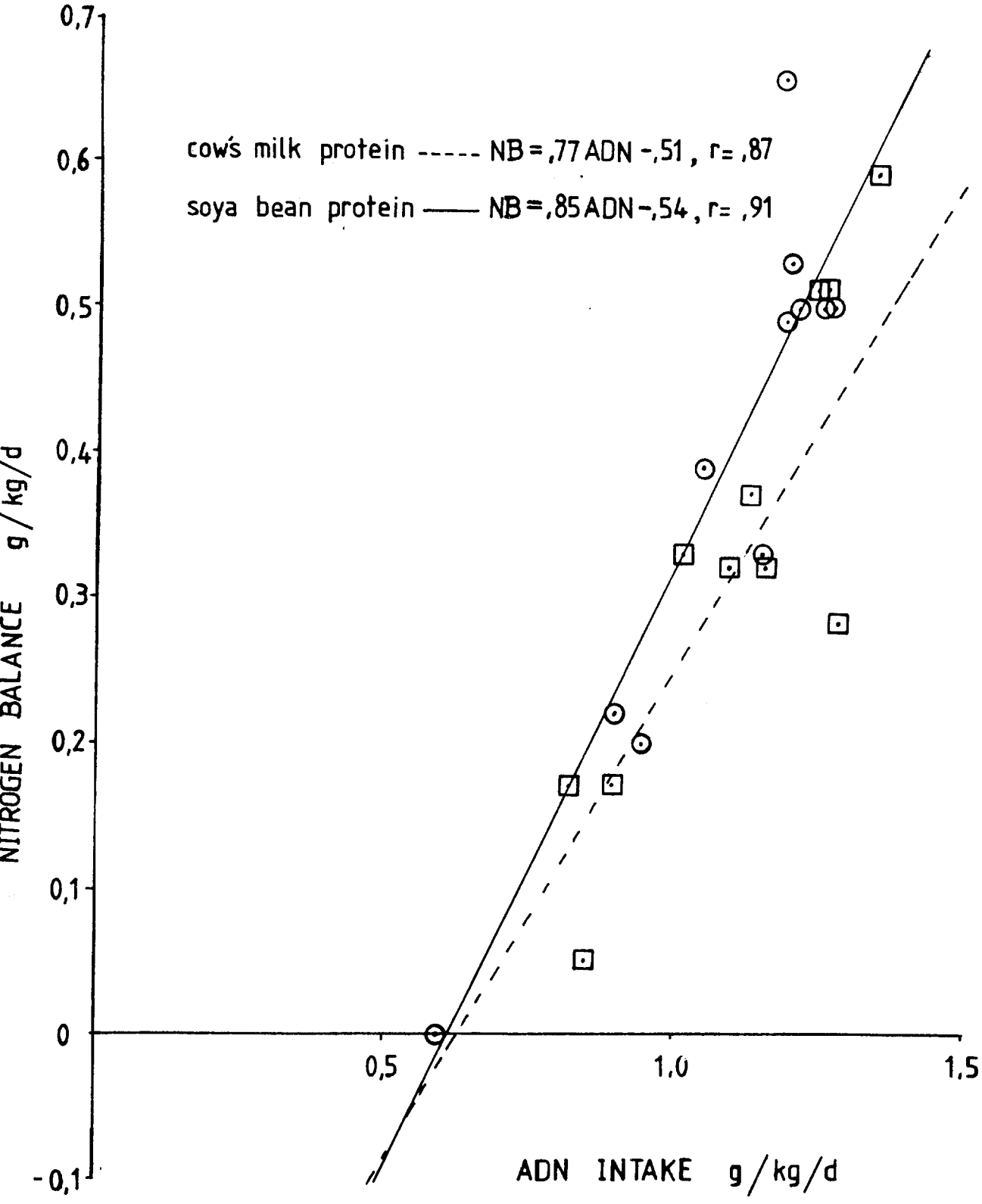


Fig. 8.2 Nitrogen balance as a function of ADN intake in rabbits fed soya protein (⊙) or cow's milk protein (⊡) containing diets.

BIBLIOGRAPHY

1. C.W.M. ADAMS (1964). Atherosclerosis in man, other animals and birds. Biological Reviews 39, 372-424.

2. B.A. KOTTKE, M.T. RAVI SUBBIAH (1978). Pathogenesis of atherosclerosis. Mayo Clin. Proc. 53; 35-48.

3. A.M. GOTTO, Jr., D. YESHURUN, M.E. DEBAKEY (1976). Atherosclerotic cardiovascular disease. J. Chronic Dis. 29; 677-682.

4. R.W. WISSLER, D. VESSELINOVITCH, G.S. GETZ (1976). Abnormalities of the arterial wall and its metabolism in atherogenesis. Prog. in Cardiovasc. Dis. 18; 341-369.

5. J.C. GEER, M.D. HAUST (1972). Smooth muscle cells in atheroma, Monograph on atherosclerosis. No. 2. p. 1-88, Karger, Basel.

6. N. WOOLF (1977). Atheroma: Aspects of atherogenesis, Br. J. Hosp. Med. 18; 286-295.

7. H.C. McGILL Jr. (1968). Introduction to the geographic pathology of atherosclerosis. Lab. Invest. 18; 465-467.

8. G.A. GRESHAM, A.N. HOWARD (1963). Comparative histopathology of atherosclerotic lesion, J. Ath. Res. 3; 161-177.

9. R. VIRCHOW, (1856). Phlogose und Thrombose in geffessystem. Gessamelte Abhandlungen zur wissenshaftlichen medicin. p. 458.

10. F. MARCHAND, (1904). Über arteriosklerose, Verhandl. d. 21 Kongr f. innere Med. 21; 23-40.

11. D.B. ZILVERSMITT, C.C. SWEELEY, H.A. NEWMAN (1961). Fatty acid composition of serum and aortic intimal lipids in rabbits fed low and high cholesterol diets. Circulation Res. 9; 235-241.

12. F.P. BELL, I.L. ADAMSON, A.S. GALLUS, C.J. SCHWARTZ (1973). In Atherosclerosis, III. edited by G. Schettler, A. Weizel. Springer-Verlag, Berlin, Heidelburg, N.Y. p. 235.

13. J.B. SOMER, C.J. SCHWARTZ (1971). Focal ^{3}H-cholesterol uptake in the pig aorta. Atherosclerosis 13; 293.

14. S. BJÖRKERUD, G. BONDJERS (1971). Arterial repair and atherosclerosis after mechanical injury, I. Permeability and light microscopic characteristics of endothelium in non-atherosclerotic and atherosclerotic lesions. Atherosclerosis. 13; 355-363.

15. S. MOORE (1978). Thrombosclerosis in normolipermic rabbits. A result of continued endothelial damage. Lab. Invest. 29; 478.

16. H.R. BAUMGARTNER (1974). In Thrombosis, Pathogenesis and clinical trials. (Eds. Deutsch, E., Brinkhous, K.M., Lechner, K. and Hinhom, S.). Schaltner-Verlag, Stuttgart. p.91.

17. M.B. STEMERMAN, E. ROSS. (1972). Experimental atherosclerosis. I. Fibrous plaque formation in primates, an electron microscopic study. J. Exp. Med., 136; 769.

18. T.H. SPAET, M.B. STEMERMAN, F.J. VEITH, I. LEJNIEKS (1975). Intimal injury and regrowth in the rabbit aorta: Medial smooth muscle cells as a source of neointima. Circ. Res. 36; 58.

19. J.A. FISHMAN, F.B. RYAN, M.J. KARNOVSKY (1975). Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myo-intimal thickening. Lab. Invest. 32; 339.

20. L.A. HARKER, R. ROSS, S.J. SLICHTER, C.R. SCOTT (1976). Homocystine induced atherosclerosis: the role of endothelial cell injury and platelet response in its genesis. J. Clin. Invest. 58; 731.

21. P. CONSTANTINIDES (1965). Experimental atherosclerosis. p. 20.

22. E. GAYNOR, C.A. BOUVIER, T.H. SPAET (1970). Vascular lesion: possible pathogenic basis of the generalized Shartzman reaction. Science. 170; 986.

23. R.G. GERRITY, H.K. NAITO, M. RICHARDSON, C.J. SCHWARTZ (1979). Dietary induced atherogenesis in swine: Morphology of the intima in prelesion stages. Am. J. Pathol. 95; 775.

24. R. ROSS, L. HARKER (1976). Hyperlipidaemia and atherosclerosis: chronic hyperlipidaemia initiates and maintains lesions by endothelial cell desquamation and lipid accumulation. Science. 193; 1094.

25. D.L. FRY (1973). Responses of arterial wall to certain physical factors. Ciba. Found. Symp. 12; 127-148.

26. W.E. STEHBENS (1974). Haemodynamic production of lipid deposition, intimal tears, mural dissection and thrombosis in the blood vessel wall. Proc. Royal Soc. London (Biol). 185; 357.

27. C.R. MINICK (1976). Immunologid arterial injury in atherogenesis. Ann. N.Y. Acad. Sci. 275; 210-227.

28. D.R. ALONSO, P.K. STAVEK, C.R. MINICK (1977). Studies on the pathogenesis of atheroarteriosclerosis induced in rabbit cardiac allografts by synergy of graft rejection and hypercholesterolaemia. Am. J. Path. 87; 415-442.

29. C.R. MINICK, M.B. STEMERMAN, W. INSULL, Jr. (1977). Effect of regenerated endothelium on lipid accumulation in the arterial wall. Proc. Natl. Acad. Sci. USA 74 (4): 1742.

30. C.R. MINICK, M.B. STEMERMAN, W. INSULL (1979). Role of endothelium and hypercholesterolaemia in intimal thickening and lipid accumulation. Am. J. Path. 95; 131.

31. A.L. ROBERTSON, Jr. (1979). Arterial endothelium in the initial stages of atherogenesis. Atherosclerosis V., A.M. Gotto, Jr., L.G. Smith, B. Allen (eds.). Springer-Verlag, N.Y. 103-111.

32. C. VON ROKITANSKY (1852). A manual of pathological anatomy. Sydenham Society, 261-72.

33. J.B. DUGUID (1946). Thrombosis as a factor in pathogenesis of coronory atherosclerosis. J. Path. Bact. 58; 207-212.

34. M. HAMBURG, J. SVENSSON, B. SAMUELSSON (1974). Prostaglandin endoperoxidase. A new concept concerning the mode of action and release of prostaglandins. Proc. Nat. Acad. Sci. USA. 71; 3824.

35. R.J. GRYGLEWSKI, S. BUNTING, S. MONCADA, R.J. FLOWER, J.R. VANE (1976). Arterial walls generate from prostaglandin endoperoxidase a substance (Prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibit platelet aggregation. Prostaglandins. 12; 897-913.

36. R.J. GRYGLEWSKI (1979). Prostacyclin and atherosclerosis - A hypothesis. p.762-765. Atherosclerosis V. A Gotto, L.C. Smith, B. Allen (eds.). Springer-Verlag, N.Y., Heidelberg, Berlin.

37. J.F. MUSTARD, M.A. PACKHAM (1970). Factors influencing platelet function: adhesion, release and aggregation. Pharmacol. Rev. 22; 97-187.

38. H.J. WEISS (1975). Platelet physiology and abnormalities of platelet function. New Eng. J. Med. 293; 531-541, 580-588.

39. H.J. DAY, A.T. ANG, H. HOLMSEN (1972). Platelet release reaction during clotting of native platelet rich plasma. Proc. Soc. Exp. Biol. Med. 139; 717-721.

40. R. ROSS, J.A. GLOMSET (1973). Atherosclerosis and the arterial smooth muscle cell. Science. 180; 1332-1339.

41. R. ROSS, J. GOMSET, B. KARIYA, L. HARKER. A platelet dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro (1974). Proc. Natl. Acad. Sci. 71; 1207-1210.

42. R. ROSS, J.A. GLOMSET (1976). The pathogenesis of atherosclerosis. New Eng. J. Med. 295; 369-377, 420-425.

43. M.A. GIMBRONE, Jr. (1979). Endothelial dysfunction and the pathogenesis of atherosclerosis. Atherosclerosis V. A. Gotto, L.S. Smith, B. Allen (eds.) Springer-Verlag, N.Y., Heidelburg, Berlin, 415-425.

44. R.N. POSTEN, D.F. DAVIES (1974). Immunity and inflammation in the pathogenesis of atherosclerosis. 19; 353-367.

45. L. LEVY (1967). A form of immunological atherosclerosis, the reticulo endothelial system and atherosclerosis. ed. M.R. di Luzzio, R. Paoletti. p.426.

46. H.V. LAMBERTSON, Jr., K.E. FRITZ (1974). Immunological enhancement of atherogenesis in rabbits. Arch. Path. 98; 9-16.

47. C.R. MINICK, G.E. MURPHY, W.G. CAMPBELL (1966). Experimental induction of athero-arteriosclerosis by the synergy of allergic injury to arteries and lipid rich diet. J. Exp. Med. 124; 635.

48. P. CLOPATH (1978). Immunologic arterial injury in swine: In. Peptides of the biological fluids. H. Peters (Ed.) Proceedings of the 26th Colloquium. Pergamon press, N.Y. 437-440.

49. A.N. HOWARD, J. PATELSKI, D.E. BOWYER, G.A. GRESHAM (1971). Atherosclerosis induced in hypercholesterolaemic baboons. Atherosclerosis. 14; 17-29.

50. N.J. ALEXANDER, T.B. CLARKSON (1978). Vasectomy increases the severity of diet induced atherosclerosis in macaca fascicularis. Science. 201; 538-541.

51. T.B. CLARKSON, N.J. ALEXANDER (1979). The effect of long term vasectomy on the occurence and the extent of arteriosclerosis in Rhesus monkeys fed monkey chow. Ped. Proc. 38; 1346.

52. P. CONSTANTINIDES, M. ROBINSON (1969). Ultrastructural injury of the arterial endothelium: Effects of vasoactive amines. Arch. Path. 88; 106-112.

53. C.G. COCHRANE, D. HAWKINS (1968). III. Factors governing the ability of circulating immune complexes to localise in blood vessels. J. Exp. Med. 127; 137-154.

54. P.M. HENSON, C.G. COCHRANE (1975). The effect of complement depletion of experimental tissue injury. Ann. N.Y. Acad. Sci. 256; 426-440.

55. P.M. HENSON (1974). Complement dependent platelet and PMN-leucocyte reactions. Transplant Proc. 6; 27-31.

56. P.A. WARD (1975). Complement dependent phlogistic factors in rheumatoid synovial fluids. Ann. N.Y. Acad. Sci. 256; 169-174.

57. P. GEERTINGER, H. SØRENSON (1973). Complement and atherosclerosis. Atherosclerosis. 18; 65-71.

58. I. GIGLI, J.W. MASON, R.W. COLEMAN, K.F. AUSTEN, B. ROBERT (1968). Interaction of kallikrien with the C_1 esterase inhibitor. J. Immunol. 101; 814-815.

59. E.A. KABAT, M.M. MAYER (1967). Experimental immunochemistry. 2nd edn. Thomas, Springfield, p.133.

60. K. INOUE, S. NOJIMA, T. TOMIZAWA (1965). The specificity of cardiolipin in serological reaction. J. Biochem. 57; 824-826.

61. O. GÖTZE, H.J. MÜLLER-EBERHARD (1971). The C_3 activator system. An alternate pathway of complement activation. J. Exp. Med. 134; 90-

62. C.G. CARO, J.M. FITZ-GERALD, R.C. SCHROTER (1969). Arterial wall shear and distribution of early atheroma in man. Nature (London), 223; 1159-

63. G.E. BIRCH, C.Y. TSUI, J.M. HARB (1971). Pathological changes of aorta and coronary arteries of mice injected with coxackie B_4 virus. Proc. Soc. Exp. Biol. Med. 137, 657-

64. C.G. COCHRANE, F.J. DIXON (1969). Cell and tissue damage through antrogen-antibody complexes. Calif. Med. 111; 99-112.

65. P.M. HENSON, C.G. COCHRANE (1971). Acute immune complex disease in rabbits. J. Exp. Med. 133; 554-571.

66. P.M. HENSON (1970). Release of vasoactive amines from rabbit platelets induced by sensitised mononuclear leucocytes and antigen. J. Exp. Med. 131; 287-306.

67. P.M. HENSON (1977). Activation of rabbit platelets by platelet activating factor derived from IgE sensitised basophils. J. Clin. Invest. 60; 481-490.

68. W.T. KNIKER, C.G. COCHRANE (1965). Pathogenic factors in vascular lesions of experimental serum sickness. J. Exp. Med. 122; 83-98.

69. T. URIHARA, J.Z. MOVAT (1966). The role of PMN-leucocyte lysosomes in tissue injury, inflammation and hypersensitivity. Exp. Mole. Path. 5; 539-558.

70. U.E. NYDEGGER (1979). Biological properties and detection of immune complexes in animal and human pathology. Rev. Physiol. Biochem. Pharmac. 85; 64-125.

71. W.T. KNIKER, C.G. COCHRANE (1968). The localization of circulating immune complexes in experimental serum sickness. J. Exp. Med. 127; 119-

72. S. GERÖ, J. SZEKELY, E. SZONDY, E. SEREGELYI (1975). Immunological studies with aortic and venous tissue antigens. Arterial Wall. 3; 89-92.

73. A.N. KLIMOV, Yu. N. ZUBLHITSKY, V.A. NAGARNEV (1979). Immunochemical aspects of atherosclerosis. Atherosclerosis Rev. 4; 119-156.

74. L. SCEBAT, J. RENAIS, W. GROULT, L. IRIS, J. LENÈGRE (1967). Lésions artérielles produites chez le lapin par des injections de Broyat d'aorte de rat. Arch. Mal. Coeur. 9; No. 2.

75. A.M. ROBERT, Y. GROSGOGEAT, V. REVERDY, B. ROBERT, L. ROBERT (1971). Lésions arterielles produites chez le lapin pas immunisation avec l'élastin et les glycoproteines de structure de l'aorta, etudes boichimique et morphologiques. Atherosclerosis. 13; 427-

76. H.W. INTROP, F. MILGROM (1969). Rabbit auto-antibodies to aorta. J. Immunol. 103; 544.

77. M. TUSKIEWICZ, E. PLESZCZYŃSKA, J. TOMASZEWSKI, J. LOPATYŃSKI, J. HANZHK (1972). Auto-organ antibodies in rabbits fed an atherogenic diet. Arch. Immun. Ther. Exp. 20; 485.

78. J. POKORNY, J. TEZKOVA (1962). Significance of immunological studies in peripheral obliterating vascular disease. Cir. Res. 11; 961.

79. N. APOSTALAKIS, K.D. VOIGT (1966). Preparation of anti serum against a glycoportein isolated from sclerotic media. J. Atherscler. Res. 6; 102-

80. S. GERÖ (1969). Allergy and auto immune factors. In F.G. Schettler and G.S. Boyd (eds.) Atherosclerosis, Elsevier, Amsterdam. p.455-464.

81. V. BEAUMONT, J.L. BEAUMONT (1968). L'hyperlipidémiè expérimentale par immunization chez le lapin. Pathol. Biol. (Paris). 16; 869.

82. S. GERÖ, L. JACOB, J. GERGELY, J. SZEKELY, S. VIRAG, K. FARKAS, A. CZUPPON (1960). Inhibition of cholesterol atherosclerosis by immunization with β-lipoprotein. Lancet i; 1119.

83. J.L. BEAUMONT (1969). Gamma-globulin et hyperlipidaemies. L'hyperlipidémie par auto anti corps. Ann. Biol. Chem. 27; 611.

84. J.L. BEAUMONT (1970). Autoimmune hyperlipidemia, an atherogenic metabolic disease of immune origin. Rev. Eur. Etud. Clin. Biol. 15; 1037-1041.

85. L.A. LEWIS, A.L. ROBERTSON (1974). Hyperimmuno-globulinemia-lipoprotein and atherogenesis. In: G. Schettler, A. Weizel (eds.) Atherosclerosis III, Springer-Verlag, Heidelburg, p.595-603.

86. G. NOSEDA, W. RIESON, E. SCHLUMF, A. MORELL (1972). Hypolipoproteinemia associated with auto antibodies against β-lipoporteins. Eur. J. Clin. Invest. 2; 342-347.

87. Z. JEZKOVA, J. POKORNY (1967). The appearance of a wide variety of antibodies in atherosclerosis. Angiologica. 4; 359-

88. P. ZEEK (1932). Studies in atherosclerosis. I. Conditions in childhood which predispose to early development of atherosclerosis. II. Atheroma and its sequelae in rheumatic heart disease. Am. J. Med. Sci. 184; 350-356, 356-364.

89. B.H. BUKLEY, W.C. ROBERTS (1975). The heart in systemic lupus erythematosus and the changes induced in it by corticosteroid therapy. Am. J. Med. 58; 243-264.

90. A.K. RIDER, J.C. COPELAND, S.A. HUNT, J. MASON, M.J. SPECTER, R.A. WINKLE, C.P. BIEBER, M.E. BILLINGHAM, E. DONG, R.B. GREIPP, J.C. STROEDER, E.B. STINSON, D.C. HARRISON, N.E. SHUANWAY (1975). The status of cardiac transplantation. Circulation. 52; 531-539.

91. M.M. GERTLER, M.M. DRISKELL,E.F. BLAND, S.M. GARN, J. LERMAN, S.A. LEVINE, H.B. SPRAGUE, P.D. WHITE (1951). Clinical aspects of coronary heart disease. An analysis of 100 cases in patients 23 to 40 years of age with myocardial infarction. J. Am. Med. Assn. 146; 1291-1295.

92. F.T. HATCH, P.K. REISSELL, T.M.W. POON-KING, G.P. CANELLOS, R.S. LEES, C.M. HAGOPIAN (1966). A study of the patients and comparison with age matched healthy men. Circulation. 33; 679-703.

93. N.S. SCRIMSHAW, M.A. GUZMAN (1968). Diet and atherosclerosis. Lab. Invest. 18; 623-628.

94. G.J. MILLER, N.E. MILLER (1975). Plasma high density lipoprotein concentration and development of ischaemic heart disease. Lancet. i; 16-19.

95. W.B. KANNEL, W.P. CASTELLI, T. GORDEN, P.M. McNAMARA (1971). Serum cholesterol, lipoprotein and the risk of coronary heart disease. Ann. Intern. Med. 74; 1-12.

96. T. GORDEN, W.P. CASTELLI, M.C. HJORTLAND, W.B. KANNEL, T.R. DAWBER (1977). High density lipoprotein as a protective factor against coronary heart disease - the Framingham study. Am. J. Med. 62; 707-714.

97. E. SMITH (1980). Comment - HDL - Should we be chasing it now?. J. Human Nutr. 34; 59-62.

98. J.W. GOFMAN, O. DELALLA, F. GLAZIER, N.K. FREEMAN, F.T. LINDGREN, A.V. NICHOLS, B. STRISOWER, A.R. TAMPLIN (1954). Serum-lipoprotein-transport system in health metabolic disorders, atherosclerosis and coronary heart disease. Plasma. 2; 413-484.

99. J.W. GOFMAN, L. RUBIN, J.P. McGINLEY, H.B. JONES (1954). Hyperlipo-porteinemia. Am. J. Med. 17; 514-520.

100. J.L. GOLDSTEIN, M.S. BROWN (1977). The low density lipoprotein path-way and its relation to atherosclerosis. Ann. Rev. Biochem. 46; 897-930.

101. J. GROEN, B.K. TJIONG, C.G. KAMMINGA, A.F. WILLEBRANDS (1952). The influence of nutrition, individuality and some other factors including various forms of stress on serum cholesterol. An experiment of 9 months duration in 60 normal human volunteers. Voeding. 13; 556-587.

102. A. KEYS (1953). Atherosclerosis: A problem in newer public health. J. Mt. Sinai Hosp. 20; 118-139.

103. L.W. KINSELL, J. PATRIDGE, L. BOLING, S. MARGEN, G. MICHAELS (1952). Dietary modification of serum cholesterol and phospholipid levels. J. Clin. Endocrinol. Metab. 12; 909-913.

104. G.F. LAMBERT, J.P. MILLER, R.T. OLSEN, D.V. FROST (1958). Hyper-cholesterolaemia and atherosclerosis induced in rabbits by purified high fat rations devoid of cholesterol. Proc. Soc. Exp. Biol. 97; 544-549.

105. H. MALMROS, G. WIGAND (1959). Atherosclerosis and deficiency of essential fatty acids. Lancet. ii; 749-751.

106. D. KRITCHEVSKY, S.A. TEPPER (1968). The influence of chow components. J. Atheros. Res. 8; 357-369.

107. D. KRITCHEVSKY (1976). Comparison of peanut, corn, coconut oil and butter. Exp. and Mole. Path. 24; 375-391.

108. E.H. AHRENS, Jr., D.H. BLANKENHORN, T.T. TSALTAS (1954). Effects on human serum lipids of substituting plant for animal fat in diet. Proc. Soc. Exp. Biol. Med. 86; 872-878.

109. D.M. HEGSTEAD, R.B. McGANDY, M.L. MYERS, F.J. STARE (1965). Quantitative effects of dietary fat on serum cholesterol in man. Am. J. Clin. Nutr. 17; 281-295.

110. A. KEYS, J.T. ANDERSON, F. GRANDE (1965). Serum cholesterol response to changes in the diet. Metabolism. 14; 759-765.

111. W.O. CASTER (1969). Studies of butterfat as related to human nutrition. Food. Prod. Develop. 3; 23, 26, 28, 65.

112. S.M. GRUNDY, E.H. AHRENS Jr. (1970). The effect of unsaturated dietary fats on absorption, excretion, synthesis and distribution of cholesterol in man. J. Clin. Invest. 49; 1135-1152.

113. A.C. TIDWELL, J.L. POPE, P. GIFFORD (1966). Metabolism of polyunsaturated fatty acids in serum cholesterol levels in the rat. J. Nutr. 88; 111-114.

114. J.H. MOORE, D.L. WILLIAMS (1964). The effect of diet on the level of plasma cholesterol and the degree of atheromatous degeneration in the rabbit. Br. J. Nutr. 18; 253-273.

115. N.N. ANITSCHOW (1967). In Cowdry's atherosclerosis. A survey of the problem. H.T. Blumenthal (ed.) 2nd edition. p.21-44. Charles C. Thomas, Springfield 111.

116. J.M.R. BEVERIDGE, W.F. CONNELL, H.L. HAUST, G.A. MEYER (1959). Dietary cholesterol and plasma cholesterol levels in man. Canad. J. Biochem. Physiol. 37; 575-582.

117. W.E. CONNOR, R.E. HODGES, R.E. BLEILER (1961). Serum lipids in man receiving high cholesterol and cholesterol free diets. J. Clin. Invest. 40; 894-901.

118. M.F. OLIVER (1976). Dietary cholesterol, plasma cholesterol and coronary heart disease. Br. Heart. J. 38; 214-218.

119. H.B. LOFLAND, Jr., T.B. CLARKSON, R.W. St. CLAIR, N.O.M. LEHNER (1972). Studies on the regulation of plasma cholesterol levels in squirrel monkeys of two genotypes. J. Lipid Res. 18; 39-47.

120. J. YUDKIN (1972). Sucrose and cardiovascular disease. Proc. Nutr. Soc. 31; 331-337.

121. D. KRITCHEVSKY, P. SALLATA (1968). The influence of various carbohydrates. J. Ath. Res. 8; 697-703.

122. D. KRITCHEVSKY, S.A. TEPPER, M. KITTAGAWA (1973). 3. Comparison of fructose and lactose and other carbohydrates. Nutr. Rep. Int. 7; 193-202.

123. D. KRITCHEVSKY, L.M. DAVIDSON, I.L. SHIPARO, H.K. KIM, M. KITAGAWA, S. MALHOTRA, P.P. NAIR, T.B. CLARKSON, I. BERSOHN, P.A.D. WINTER (1974). Lipid metabolism and experimental atherosclerosis in baboons. Influence of cholesterol-free, semi-synthetic diets. Am. J. Clin. Nutr. 27; 29-50.

124. D. KRITCHEVSKY, L.M. DAVIDSON, J.J. VANDER WATT, P.A.D. WINTER, I. BERSOHN (1974). Hypercholesterolaemia and atherosclerosis induced in vervet monkeys by cholesterol free, semi-synthetic diets. S. Afr. Med. J. 48; 2413-2414.

125. D.P. BURKITT (1973). Some diseases characteristic of modern western civilization. Br. Med. J. i; 274-278.

126. H. TROWELL (1972). Ischaemic heart disease and dietary fibre. Am. J. Clin. Nutr. 25; 926-932.

127. D. KRITCHEVSKY, J.A. STORY (1974). Binding of bile salts in vitro by non-nutritive fibre. J. Nutr. 104; 458-462.

128. J.A. STORY, D. KRITCHEVSKY (1976). Comparison of binding of various bile salts in vitro by several types of fibre. J. Nutr. 106; 1292-1294.

129. M.A. EASTWOOD, D. HAMILTON (1968). Studies on the adsorption of bile salts to non-absorbed components of diet. Biochem. Biophys. Acta. 152; 165-

130. B.H. ERSHOFF (1963). Effects of pectin, nutritive fibre and other complex carbohydrates on hypercholesterolaemia and atherosclerosis. Exp. Med. Surg. 21; 108-112.

131. P.N. DURRINGTON, A.P. MANNING, C.H. BOLTON, M. HARTOG (1976). Effect of pectin on serum lipids and lipoproteins, whole gut transit time, and stool weight. Lancet II. 394-396.

132. G.A. LEVEILLE, H.E. SAMBERLICH (1966). Mechanism of cholesterol depressing effect of pectin in cholesterol fed rat. J. Nutr. 88; 209-214.

133. B.H. ERSHOFF, A.F. WELLS (1962). Effects of guargum, locust bean gum and carrageenin on liver cholesterol of cholesterol fed rat. Proc. Soc. Exp. Biol. Med. 110; 580-582.

134. J.E. GARVIN, T. FORMAN, W.R. EISEMAN, C.R. PHILLIPS (1965). The lowering of human serum cholesterol by an oral hydrophylic colloid. Proc. Soc. Exp. Biol. Med. 120; 744-

135. D.J.A. JENKINS, A.R. LEEDS, C. NEWTON, J.H. CUMMINGS (1975). Effects of pectin, guar-gum and wheat-fibre on serum cholesterol. Lancet. i; 1116-1117.

136. R.M. KAY, A.S. TRUSWELL (1977). The effects of wheat fibre on plasma lipids and fecal steroid excretion in man. Br. J. Nutr. 37; 227-

137. S. TARPILA, T.A. MIETTINEN, L. METSARANTA (1978). Effect of bran on serum cholesterol, faecal mass, fat, bile acids and neutrol sterols and biliary lipids in patients with diverticular disease of the colon. Gut. 19; 137.

138. A. IGNATOWSKI (1908). Influence de la nourriture animale sur l' organisme des lapins. Arch. Med. Exp. 20; 1-20.

139. L.H. NEWBURGH, T.L. SQUIRE (1923). The production of atherosclerosis in rabbits by feeding diets rich in meat. Arch. Intern. Med. 26; 38-40.

140. N. ANITSCHOW, S. CHALATOW (1913). Über experimentelle cholesterinsteatose und ihre bedenhung für die Enstehung eineger pathologischen prozesse. Zbl. allg. Path. Anat. 24; 1-9.

141. A.N. HOWARD, G.A. GRESHAM (1965). The prevention of rabbit atherosclerosis by soya bean meal. J. Ath. Res. 5; 330-337.

142. T.B. CLARKSON (1971). Animal models for experimental atherosclerosis. Nth Car. Med J. 32; 88-98.

143. K.K. CARROLL (1971). Plasma cholesterol levels and liver cholesterol biosynthesis in rabbits fed commercial or semi-synthetic diets with or without added fats or oils. Atherosclerosis. 13; 67-76.

144. R. FUMAGALLI, R. PAOLETTI, A.N. HOWARD (1978). Hypocholesterolaemic effect of soya. Life Sci. 22; 947-952.

145. R.M.G. HAMILTON, K.K. CARROLL (1976). Plasma cholesterol levels in rabbits fed low fat, low cholesterol diets: Effects of dietary protein, carbohydrate and fibre from different sources. Atherosclerosis. 24; 47-62.

146. M.W. HUFF, K.K. CARROLL (1977). Effect of dietary protein on plasma cholesterol levels and cholesterol oxidation in rabbits. Fed. Proc. 36; 4402.

147. K.K. CARROLL (1978). Dietary proteins in relation to plasma cholesterol levels in atherosclerosis. Nutr. Rev. 36; 1-5.

148. K.K. CARROLL, R.M.G. HAMILTON (1975). Effect of dietary protein and carbohydrate on plasma cholesterol levels in relation to atherosclerosis. J. Food Sci. 40; 18-23.

149. K.K. CARROLL (1978). The role of dietary protein in hypercholesterolaemia and atherosclerosis. Lipids. 13; 360-365.

150. D.R. MEEKER, H.D. KESTEN (1941). Effect of high protein diets on experimental atherosclerosis of rabbits. Arch. Path. 31; 147-162.

151. D. KRITCHEVSKY, R.R. KOLMAN, R.M. GUTTMACHER, M. FORBES (1959). Influence of dietary carbohydrate and protein on serum and liver cholesterol in germ free chickens. Arch. Biochem. Biophys. 85; 444-451.

152. D.N. KIM, K.T. LEE, J.M. REINER, W.A. THOMAS (1979). Studies of hypocholesterolaemic effect of soy protein in swine. Proceedings of VIIth international Symp. on drugs affecting lipid metabolism, Milan. p. 273.

153. T.B. CLARKSON, R.W. PRICHARD, H.B. LOFLAND, H.O. GOODMAN (1962). Interactions among dietary fat, proteins and cholesterol in atherosclerosis-susceptible pigeons. Effects on coronary atherosclerosis. Circ. Res. 11; 400-404.

154. C.C. MIDDLETON, T.B. CLARKSON, H.B. LOFLAND, R.W. PRICHARD (1967). Diet and atherosclerosis of squirrel monkeys. Arch. Pathol. 83; 145-153.

155. J.P. STRONG, H.C. McGILL, Jr. (1967). Diet and experimental atherosclerosis in baboons. Am. J. Pathol. 50; 669-690.

156. A.H.M. TERPESTRA, L. HARKES, F.H. VAN DER VEEN (1980). Effect of different levels of dietary casein on serum cholesterol concentration in rabbit fed semi-purified diets. VII. International Symposium on Drugs affecting lipid metabolism. p.106.

157. G.A. LEVEILLE, H.E. SAUBERLICH (1961). The influence of dietary protein level on serum protein components and cholesterol in the young chick. J. Nutr. 74; 500-

158. Y. NAGATA, J. IMAIZUMI, M. SUGANO (1980). Effect of soya bean protein and casein on serum cholesterol levels in rats. Pro. J. Nutr. 44; 113-121.

159. M.B. KATAN, L. VROOMEN, R.J.J. HERMUS (1980). Reduction of casein induced hypercholesterolaemia and atherosclerosis in rabbits and rats by dietary glycine. Proc. VII international symposium on drugs affecting lipid metabolism. Milan. p.104.

160. N.R. YADEV, I.E. LEINER (1977). Reduction of serum cholesterol in rats fed vegetable protein or an equivalent amino acid mixture. Nutr. Rep. Int. 16; 385.

161. C. SAUTIER, C. DOUCET, C. FLAMENT, D. LEMONNIER (1979). Effect of soy protein and saponin on serum, tissue and faeces steroids in the rat. Atherosclerosis. 34; 233-241.

162. J. YUDKIN (1957). Diet and coronary thrombosis. Hypothesis and fact. Lancet. II; 155-162.

163. J. YERUSHALMY, H.E. HILLEBOE (1957). Fat in the diet and mortality from heart disease: A methodologic note. N.Y. State J. Med. 57; 2343-2354.

164. R.E. OLSEN, J.W. VESTER, D. GURSEY, N. DAVIS, D. LONGMAN (1958). The effect of low protein diets upon serum cholesterol in man. Am. J. Clin. Nutr. 6; 310-324.

165. G.R. WALKER, E.H. MORSE, V.A. OVERLEY (1960). The effect of animal protein and vegetable protein diets having the same fat content on the serum lipid levels of young women. J. Nutr. 72; 317-321.

166. K.K. CARROLL, P. GIOVENNETTI, M.W. HUFF, O. MOASE, D.C.K. ROBERTS, B.M. WOLFE (1978). Hypercholesterolaemic effect of substituting soy protein for animal protein in the diets of healthy young women. Amer. J. Clin. Nutr. 31; 1312-1321.

167. C.R. SIRTORI, E. AGRADI, F. CONTI, O. MONTERO, E. GATTI (1977). Soya bean protein diet in the treatment of type II-hyperlipoprotenemia. Lancet, I; 275-277.

168. C.R. SIRTORI, E. GATTI, O. MONTERO, F. CONTI, E. AGRADI, E. TREMOLI, M. SIRTORI, L. FRATERRIGO, L. TARAZZI, D. KRITCHEVSKY (1979). Clinical experience with the soya bean protein diet in the treatment of hypercholesterolaemia. Am. J. Clin. Nutr. 32; 1645-1658.

169. G.C. DESCOVICH, A. GADDI, G. MANNINO, L. CATTIN, U. SENIN, C. CARUZZO, C. FRAGIACOMO, M. SIRTORI, C. CEVEDI, M.S. BENASSI, L. COLOMBO, G. FONTANA, E. MANNARINO, E. BERTELLI, G. NOSEDA, C.R. SIRTORI (1980). Multicentre study of soya bean protein diet for outpatient hypercholesterolaemic patients. Lancet. II; 709-712.

170. J.T. ANDERSON, F. GRANDE, A. KEYS (1971). Effect on mans serum lipids of two proteins with different amino acid composition. Am. J. Clin. Nutr. 24; 524-530.

171. J.D. POTTER, R.J. ILLMAN, G.D. CALVERT, D.G. OAKENFULL, D.L. TOPPING (1980). Soya saponins, plasma lipids, lipoproteins and faecal bile acids: A double blind cross-over study. Nutr. Rep. Int. 22; 521-528.

172. J.M.A. VAN RAAIJ, M.B. KATAN, J.G. A.J. HAUTVAST (1979). Effects of casein versus soy protein diets on serum cholesterol and lipoprotein in young healthy volunteers. Proc. of VII International symposium of Drugs affecting lipid metabolism. p.100.

173. G. NOSEDA, C. FRAGIACOMO, C.G.C. DESCOVICH, R. FUMAGALLI, F. BERNINI, C.R. SIRTORI (1980). Clinical studies on the mechanism of action of the soya bean protein diet. Proc. VII international symposium on drugs affecting lipid metabolism.

174. C.R. SIRTORI, G. DESCOVICH, G. NOSEDA (1980). Textured soy protein and serum cholesterol. Lancet. i; 149.

175. K.K. CARROLL, M.W. HUFF (1977). Influence of dietary fat and protein on plasma cholesterol levels in the early post-natal period, in Manning and Haust, Atherosclerosis, pp.638-643 (Plenum Press, N.Y.).

176. M.W. HUFF, K.K. CARROLL (1980). Effects of dietary protein on turnover, oxidation and absorption of cholesterol and on steroid excretion in rabbits. J. Lipid Res. 21; 546-558.

177. D. KRITCHEVSKY, S.A. TEPPER, J.A. STORY (1979). Influence of soy protein and casein on atherosclerosis in rabbits. Fed. Proc. 37; 747.

178. D. KRITCHEVSKY (1979). Soya saponins and plasma cholesterol. Lancet, I; 610.

179. M.W. HUFF, R.M.G. HAMILTON, K.K. CARROLL (1977). Plasma cholesterol levels in rabbits fed low fat, low cholesterol, semi-purified diets: Effects of dietary protein, protein hydrolysates and amino acid mixtures. Atherosclerosis. 28; 187-195.

180. M.W. HUFF, K.K. CARROLL (1980). Effects of dietary proteins and amino acid mixtures on plasma cholesterol levels in rabbits. J. Nutr. 110; 1676-1685.

181. B.I. WEIGENSBERG, H.C. STARY, G.C. McMILLAN (1964). Effect of lysine deficiency on cholesterol atherosclerosis in rabbits. Exp. Mol. Path. 3; 444-454.

182. C.I. JAROWSKI, P. PYTELEWSKI (1975). Utility of fasting essential amino acid plasma levels in formulation of nutritionally adequate diets. III. Lowering of rat serum cholesterol level by lysine supplementation. J. Pharmac. Sci. 64; 690-691.

183. P.K. RAJA, C.I. JAROWSKI (1975). Utility of fasting essential amino acid plasma levels in formulation of nutritionally adequate diets. IV. Lowering of human plasma cholesterol and triglyceride levels by lysine and tryptophane supplementation. J. Pharmac. Sci. 64; 691-692.

184. M. SUGANO, Y. NAGATA, K. IMAIZUMI (1980). Hypocholesterolaemic action of soy protein isolate in rats. Proceedings of the VII international symposium on drugs affecting lipid metabolism. p.100.

185. R.E. OLSEN, M.Z. NICHAMAN, J. NITTKA, J.A. EAGLES (1970). Effect of amino acid diets upon serum lipids in man. Am. J. Clin. Nutr. 23; 1614-1625.

186. R.E. OLSEN, G. BAZZANO, J.A. D'ELISA (1970). The effect of large amounts of glutamic acid upon serum lipids and sterol metabolism in man. Trans. Am. Physicians. 83; 196-210.

187. J.D. GARLICH, G. BAZZANO, R.E. OLSEN (1970). Changes in plasma free amino acid concentrations in human subjects on hypercholesterolaemic diets. Am. J. Clin. Nutr. 23; 1626-1683.

188. B.L. COLES, I. McDONALD (1972). The influence of dietary carbohydrate, lipid interelationships. Nutr. Metab. 14; 238-244.

189. L. KOFLER (1972). Die Saponine, Vienna, Verlag von Juluis Springer.

190. MERCK INDEX (1976). 9th Edn. p.8120. Rahway, Merck.

191. H.A. NEWMAN, F.A. KUMMEROV, H.H. SCOTT (1957). Dietary saponin, a factor which may reduce liver and serum cholesterol levels. Poultry Sci. 37; 42-45.

192. P. GRIMINGER, H. FISHER (1958). Dietary saponin and plasma cholesterol in the chicken. Proc. Soc. Exp. Biol. Med. 99; 424-426.

193. B. MORGAN, M. HEALD, S.G. BROOKS, J.L. TEE, J. GREEN (1972). The interactions between dietary saponin, cholesterol and related sterols in the chick. Poultry Sci. 51; 677-682.

194. M.R. MALINOW, P. McLAUGHLIN, G.O. KOHLER, A.L. LIVINGSTON (1977). Prevention of elevated cholesterolemia in monkeys by alfalfa saponins. 29; 105-110.

195. D.G. OAKENFULL, D.E. FENWICK, R.L. HOOD, D.L. TOPPING, R.J. ILLMAN, G.B. STORER (1979). Effects of saponin on bile acids and plasma lipids in the rat. Br. J. Nutr. 42; 209-216.

196. D.L. TOPPING, G.D. CALVERT, G.B. STORER, R.J. ILLMAN, D.G. OAKENFULL, R.A. WELLER (1980). Effects of dietary saponins on fecal bile acids and neutral sterols, plasma lipids and lipoprotein turnover in the pig. Am. J. Clin. Nutr. 33; 783-786.

197. D.G. OAKENFULL, D.E. FENWICK (1978). Adsorption of bile salts from aqueous solutions by plant fibre and cholestyramine. Br. J. Nutr. 40; 299-309.

198. M.R. MALINOW, P. McLAUGHLIN, L. PAPWORTH, C. STAFFORD, G.O. KOHLER, A.L. LIVINGSTON, P.R. CHEEKE (1977). Effect of alfalfa saponins on intestinal cholesterol absorption in rats. Am. J. Clin. Nutr. 30; 2061.

199. P.R. CHEEKE (1976). Nutritional and physiological properties of saponins. Nutr. Rep. Int. 13; 315-324.

200. J.D. POTTER, D.L. TOPPING, D. OAKENFULL (1979). Soya products, saponins and plasma cholesterol. Lancet. i; 223.

201. R.M. ROTHBERG (1969). Immunoglobulin and specific antibody synthesis during first week of life of premature infants. J.Paediat. 75; 391-399.

202. W.A. WALKER, A.L. WARSHAW (1972). Intestinal uptake of macro molecules: Effect of oral immunisation. Science. 177; 608-610.

203. F.W.R. BRAMBELL (1970). The transmission of passive immunity from mother to young. Frontiers of biology. In Neuberger and E.L. Tatum (eds.). Vol. 18 Amsterdam, North Holland Publishing Co.

204. W.A. HEMMINGS (1977b). Absorption of bovine IgG by the intestine of adult rabbit. I.R.C.S. Med. Sci. 5; 286-

205. W. WALKER, K.J. ISSELBACHER, K.J. BLOCH (1973). Intestinal uptake of macro molecules. J. Immunol. 111; 221.

206. A.L. WARSHAW, A. WALKER, K.J. ISSELBACHER (1974). Protein uptake by the intestine, evidence for absorption of intact molecules. Gastro-enterology. 66; 987.

207. E. DANFORTH, R.O. MOORE (1959). Intestinal absorption of insulin in the rat. Endocrin. 65; 118-123.

208. H. SUSSMAN, A. DAVIDSON, M. WALZER (1928). Absorption of undigested proteins in adult human beings. Arch. Intern. Med. 42; 409-414.

209. W.A. WALKER, K.J. ISSELBACHER (1977). Intestinal antibodies. New Eng. J. Med. 297; 767-773.

210. C. ANDRÉ, R. LAMBERT, H. BAZIN, J.F. HEREMANS (1974). Interference of oral immunization of the intestinal absorption of heterologous albumin. Eur. J. Immunol. 4; 701-704.

211. W.A. WALKER, K.J. ISSELBACHER (1974). Immunological control of soluble protein absorption from the small intestine, A gut surface phenomenon. Am. J. Clin. Nutr. 27; 1434-1440.

212. R.M. ROTHBERG, S.C. KRAFT, R.S. FARR (1967). Similarities between rabbit antibodies produced following ingestion of serum albumin following parentaral immunization. J. Immunol. 98; 386-395.

213. R.H. SMITH, J.W. SISSONS (1975). The effect of different feeds, including those containing soya bean products, on the passage of digesta from the obomassum of the pre-ruminant calf. Br. J. Nutr. 33; 329-349.

214. M.E.J. BARRATT, P.J. STRATCHEN, P. PORTER (1978). Antibody mechanisms implicated in digestive disturbances following ingestion of soya porteins in calves and piglets. Clin. Exp. Immunol. 31(2); 305-312.

215. P.J. GALLAGHER, C.A. MUIR, T.G. TAYLOR (1978). Immunological aspects of arterial disease. Atherosclerosis. 31; 361-363.

216. S. AHLSTEDT, B. CARLSSON, S.P. FÄLLSTRÖM, L.Å. HANSON, J. HOLMGREN, G. LIDID-JANSON, B.S. LINDBLAD, U. JODAL, B. KAIYSER, S. SOHL ÅLKERLUND, C. WADSWORTH (1977). Antibodies in human serum or milk induced enterbacteria and food proteins. Immunology of the gut. Ciba Symp. 46; 115-134.

217. P.F. HANSEN (1970). Milk proteins, chem and mol. biol. 1; 45-123.

218. R.D.A. PETERSON, R.A. GOOD (1963). Antibodies to cow's milk proteins; their presence and significance. Ped. 31; 209-221.

219. R.M. ROTHBERG, R.F. FARR (1965). Anti-bovine serum albumin and anti-α-lactalbumin in the serum of children and adults. Pediat. 35; 371-388.

220. I. McDONALD (1978). Clinical effects of consuming milk and its products. Proc. Nutr. Soc. 37; 241.

221. R. WRIGHT, K.B. TAYLOR, S.C. TRUELOVE (1962). Circulating antibodies to cow's milk protein and gluten in the newborn. Br. Med. J. ii; 513-515.

222. M. GUNTHER, R. CHEEKE, R.H. MATHEWS, R.R.A. COOMBS (1962). Immune responses in infants to cow's milk proteins taken by mouth. Int. Archs. Allergy Appl. Imm. 21; 257-278.

223. J.F. SOOTHILL (1976). Allergy and infant feeding. Proc. Nutr. Soc. 35; 283-284.

224. W. DAVIES (1958). Cow's milk allergy in infancy. Arch. Dis. Childhd. 33; 265-268.

225. S. FREIER (1976). Paediatric gastro-intestinal allergy. J. Hum. Nut. 30; 187-192.

226. M.E. AMENT, C.E. RUBIN (1972). Soy portein - another cause of the flat intestinal lesion. Gastroenterology. 62; 227-234.

227. R.P. MATTHIESEN, W. BRAUN (1974). Soy protein - a latent allergen. Dtsch. med. Wschr. 99; 2175-2177.

228. E.Z. MORTIMER (1961). Analphylaxis following ingestion of soy bean. J. Paediat. 58; 90-92.

229. W.W. DUKE (1934). Soy bean as a possible source of allergy. J. Allergy. 5, 300.

230. G.R. OSBORN (1963). Incubation period of coronary thrombosis. p.177 London.

231. R.W.D. TURNER (1976). Breast feeding is best for coronary protection. Lancet. ii; 693-694.

232. D.F. DAVIES, B.W.G. REES, A.P. JOHNSON, P.C. ELWOOD, M. ABERNETHY (1974). Food antibodies and myocardial infarction. Lancet. i; 1012-1014.

233. B.B. SCOTT, M.L. SWINBURNE, P. McGUFFIN, M.S. LOWOSKY (1976). Dietary antibodies and myocardial infarction. Lancet. ii; 125-126.

234. A. TOIVANEN, M.K. VILJANEN, E. SAVILAHTI (1976). IgM and IgG anti-milk antibodies measured by radio immunoassay in myocardial infarction. Lancet. II; 125-126.

235. M.J. GIBNEY, P.J. GALLAGHER, G.P. SHARRATT, H.S. BENNING, T.G. TAYLOR, J.M. PITTS (1980). Antibodies to heated milk protein in coronary heart disease. Atherosclerosis. 37; 151-155.

236. J.C. SEGALL (1977). Is milk a coronary health hazard? Br. J. Prevent. & Soc. Med. 31; 81-85.

237. J.C. ANNAND (1967). Hypothesis - Heated milk protein and thrombosis. J. Ath. Res. 7; 797-801.

238. J.C. ANNAND (1972). Further evidence in the case against heated milk portein. J. Ath. Res. 15; 129-133.

239. M. DELIRE, C.L. LAMBIASO, P.L. MASSON (1978). Circulating immune complexes in infants fed on cow's milk. Nature. 272; 632.

240. J.L. BEAUMONT, V. BEAUMONT, M. ANTONUCCI (1969). Presence d'une auto anticorps anti-β-lipoprotéines dans le sérum d'un lapin avant une hyperlipidèmie par immunisation. C.R. Acad. Sci. 268 D; 1830-1835.

241. G. FUST, E. SZONDY, J. SZEKELY, I. NANAI, S. GERÖ (1978). Studies on the occurrence of circulating immune complexes in vascular diseases. Atherosclerosis. 29; 181-190.

242. J. HARKAVY, E. PERLMAN (1964). Tobacco allergy in coronary heart disease. N.Y. State J. Med. 64; 1287-1296.

243. G.C. BECKER, T. DUBIN, H.P. WIEDEMAN (1976). Hypersensitivity to tobacco antigen. Proc. Natl. Acad. Sci. (USA). 73; 1712-1716.

244. G.R. WARNICK, J.J. ALBERS (1978). A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. J. Lipid. Res. 19; 65-76.

245. R.E. BOWMAN, R.C. WOLF (1962). Observations on Zak cholesterol reaction: Glacial acetic acid purity, a more sensitive absorbance peak and bromide enhancement. Clin. Chem. 8; 296-301.

246. W.W. WELLS, M. MAKITA (1962). The quantitative analysis of faecal neutral sterols by gas-liquid chromatography. Analyt. Biochem. 4; 204-212.

247. J. FOLCH, M. LEES, G.H. SLOANE-STANLEY (1957). A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226; 497-500.

248. S.V. BOYDEN (1951). The absorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by anti-protein sera. J. Exp. Med. 93; 107-120.

249. E. ENGVALL, P. PERLMANN (1972). Enzyme linked immunosorbant assay. ELISA iii. Quantification of specific antibodies by enzyme labelled anti-globulin in antigen coated tubes. J. Immunol. 109; 129-135.

250. S. AVRAMEAS, T. TERNYNCK (1971). Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochem. 8; 1175-1179.

251. J.F. MOWBRAY, E.J. HOLBOROW, A.V. HOFFBRAND, P.P. SEAH, L. FRY (1973). Circulating immune complexes in Dermatitis herpetiformis. Lancet, 1; 400-401.

252. P. KING, J.P. ROYLE (1972). Analogous vein grafting in atheromatous rabbits by cholesterol feeding. J. Exp. Med. 23; 69-76.

253. G.W. SNEDECOR, W.G. COCHRANE (1967). Statistical methods, 6th ed. Iowa State Univ. Press, Ames, IA.

254. M. VON WINKLE, L. LEVY (1968). Effect of removal of cholesterol diet upon serum sickness of cholesterol induced atheroscherosis. J. Exp. Med. 128; 497-515.

255. E.A. BELTON, A.S. TRUSWELL (1978). Effect of casein compared with soya bean protein isolate on plasma cholesterol in the rabbit. Proc. Nutr. Soc. 37; 12A.

256. J.T. PRIOR, D.M. KURTZ, D.D. ZIEGLER (1961). The hypercholesteremic rabbit. An aid to understanding arteriosclerosis in man? Arch. Pathol. 71; 672-684.

257. C.H. BAILEY (1916). Atheroma and other lesions produced in rabbits by cholesterol feeding. J. Exp. Med. 23; 69-76.

258. F. PARKER, G.F. ODLAND (1966). A correlative histochemical, biochemical and electron microscopic study of experimental atherosicerosis in rabbit aorta with special reference to myo-intimal cells. Am. J. Path. 48; 197-239.

259. L.A. ERHART, D. HOLDERBRAUN (1977). Stimulation of aortic protein synthesis in experimental rabbit atherosclerosis. Atherosclerosis. 27; 477-485.

260. K.G. McCULLAGH, L.A. ERHART (1974). Increased arterial collagen synthesis in experimental canine atherosclerosis. Atherosclerosis. 19; 13-28.

261. K.G. McCULLAGH, L.A. ERHART (1977). Enhanced synthesis and accumulation of collagen in cholesterol aggrevated pigeon atherosclerosis. Atherosclerosis. 26; 341-352.

262. B. ROKOSOVA, J.P. BENTLEY (1979). Biosynthesis of aortic collagen and glycosamino-glycans following immunological injury. Atherosclerosis. 32; 359-365.

263. S.M. BRAGDON (1952). Spontaneous atherosclerosis in the rabbit. Circulation. 5; 641-646.

264. E.A. SCHENK, E. GAMAN, A.S. FEIGENBAUM (1966). Spontaneous aortic lesions in rabbits, 1. Morphological characteristics. Circ. Res. 19; 80-88.

265. F.M. BURNETT (1976). Tolerance and hyporesponsiveness in readings from Scientific American, Immunology, Ch. II. 116-122, W.H. Freeman, San Francisco.

266. P. PORTER (1976). Immunoglobulin mechanisms in health and nutrition from birth to weaning. Proc. Nutr. Soc. 35; 273-282.

267. A.E. STUART, A.E DAVIDSON (1964). Effect of simple lipids on antibody formation after injection of foreign red cells. J. Path. Bact. 87; 305-316.

268. P.E. KORENBLAT, R.M. ROTHBERG, P. MINDEN, R.S. FARR (1968). Immune response of human adults after oral and parenteral exposure to bovine serum albumin. J. Allergy. 41; 226-223.

269. C. HEMMINGS, W.A. HEMMINGS (1979). The passage of macro molecules derived from dietary protein across the placenta of rats. In protein transmission through living membranes. W.A. Hemmings, Elsevier, North Holland Biomedical press.

270. W.A. HEMMINGS (1957). Protein selection in the yolk sac splanenopleur of the rabbit; the total uptake estimated as loss from the uterus. Proc. R. Soc. B. 148; 76.

271. A.C. KULANGARA (1980). The demonstration of ingested wheat antigens in human breast milk. I.R.C.S. Med. Sci. 8; 19.

272. J.W. GERRARD (1979). Allergy in breast fed babies to ingredients in breast milk. Ann. Allergy. 42; 69-72.

273. A.E. BEER, R.E. BILLINGHAM, S. HEAD (1974). The immunologic significance of the mammary gland. J. Invest. Dermat. 63; 65-74.

274. D.B.L. McCLELAND, T.T. McDONALD (1976). Antibodies to cow's milk protein in human colostrum. Lancet. 2; 1251-1252.

275. J.W.M. LAWTON, K.F. SHORTRIDGE (1977). Protection factors in human breast milk and colostrum. Lancet. 1; 253.

276. M.J. HENSCHEL (1973). Comparison of the development of proteolytic activity in the obomassum of the preruminant calf with that in the stomach of the young rabbit and guinea-pig. Br. J. Nutr. 30; 285-296.

277. R.T. SMITH, R.A. BRIDGES (1958). Immunological responsiveness in rabbits produced by neonatal injections of defined antigens. J. Exp. Med. 108; 227-250.

278. R.T. SMITH (1961). Immunological tolerance of non-living antigens. Adv. in Immunol. 1; 67-129.

279. M. BURNETT (1959). The clonal selection theory of acquired immunity. Cambridge University Press.

280. D.W. DRESSER, N.A. MITCHISON (1968). The mechanisms of immunological paralysis. Ad. Immunol. 8; 129-181.

281. M.F.O. WOODRUFF, L.O. SIMPSON (1955). Induction of tolerance to skin homografts in rats by injection of cells from prospective donor soon after birth. Br. J. Exp. Path. 36; 494.

282. J.B. AUST, C. MARTINEZ, J.J. BILTNER, R.A. GOOD (1956). Tolerance in pure strains of new-born mice to tumor homografts. Proc. Soc. Exp. Biol. and Med. 92; 27.

283. R.B. TAYLOR (1976). Suppressor cells in humoral immunity and tolerance. Br. Med. Bull. 32; 152-157.

284. J.H. HUMPHREY (1976). Some reflections on principles and mechanisms of immunological tolerance. Br. Med. Bull. 32; 182-184.

285. C. ANDRÉ, J.F. HEREMANS, J.P. VAERMAN, C.L. CAMBIASO (1975). A mechanism of the induction of immunological tolerance by antigen feeding: Antigen-antibody complexes. J. Exp. Med. 142; 1509-1519.

286. C. ANDRÉ, F. ANDRÉ, J.P. VAERMAN (1978). Biological function of antigen - IgA antibody complexes: in vivo and in vitro interference with intestinal absorption and tolerogenic effect. In antigen absorption of the gut. W.A. Hemmings (ed.) M.T.P. Press, Lancaster. p.73.

287. J.W. SHRADER, G.J.V. NOSSAL (1974). Effector cell blockade. A new mechanism of immune hypoactivity induced by multi-valent antigens. J. Exp. Med. 139; 1582-

288. E.T. SWARBRICK, C.R. STOKES, J.F. SOOTHILL (1979). Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. Gut. 20; 121-125.

289. G.L. ASHERSON, M. ZEMBALA, M.A.C.C. PERERA, B. MEYHEW, W.R. THOMAS (1977). Production of immunity and unresponsiveness in the mouse by feeding contact sensitising agents and the role of suppressor cells in the peyer's patches mesentetic lymph nodes and other lymphoid tissues. Cell Immunol. 33; 145-155.

290. J. NGAN, L.S. KIND (1978). Suppressor T cells for IgG and IgE in peyer's patches of mice made tolerant by the oral administration of ovalbumin. J. Immunol. 120; 861-865.

291. H.M. CANTOR, A.E. DUMONT (1967). Hepatic suppression of sensitization to antigen absorbed into the portal system. Nature. 215; 744-745.

292. R. AUERBACH, S. CLARK (1975). Immunological tolerance: Transmission from mother to offspring. Science. 189; 811-812.

293. J.F. HALSEY, D.C. BENJAMIN (1978). Induction of immunologic tolerance in nursing neonates by absorption of tolerogen from colostrum. J. Immunol. 116; 1204-1207.

294. H.J. ROTHENBACHER, S. TUFTS (1964). Coronary arteriosis in a horse. J. Am. Vet. Med. Assoc. 145; 132.

295. R. FINLAYSON, C. SYMONS (1964). Comparative 'atheroma' in sheep. J. Pathol. Bacteriol. 88; 73.

296. C.F. SIMPSON, R.F. JACKSON (1963). Aortic sclerosis in a cat. J. Am. Vet. Med. Assoc. 142; 282.

297. K. HELLSTRÖM, J. SJOVALL, G. WIGAND (1962). Influence of semi-synthetic diets and type of fat on the turnover of deoxycholic and in rabbits. J. Lipid. Res. 3; 405-412.

298. T.A. MIETTINEN, E.H. AHRENS, Jr., J.M. GRUNDY (1965). Quantitation isolation and gas-liquid chromatographic analysis of total dietary and faecal neutral steroids. J. Lipid. Res. 6; 411-424.

299. M.R. MALINOW, P. McLAUGHLIN, C. STAFFORD, A.L. LIVINGSTON, G.O. KOHLER, P.R. CHEEKE (1979). Comparative effects of alfalfa saponin and alfalfa fibre on cholesterol absorption in rats. Am. J. Clin. Nutr. 32; 1810-1812.

300. D.C.K. ROBERTS, M.E. STALMACH, K.K. CARROLL (1979). Plasma lipoprotein turnover in rabbits fed low fat, cholesterol free diets containing casein or soya protein isolate. Proc. 3rd Eur. Nutr. Conf. in Var foda. 31; Suppl. 247.

301. J.R. SABINE (1977). Cholesterol, N.Y.: Marcel Dekker.

302. J.M. DIETSCHY, J.D. WILSON (1970). Regulation of cholesterol metabolism. New Eng. J. Med. 282; 1128-1138, 1179-1183, 1241-1249.

303. W.G. BRYDON, K. TADESSE, M.A. EASTWOOD, M.E. LAWSON (1980). The effect of dietary fibre on bile acid metabolism in rats. Br. J. Nutr. 43; 101-106.

304. T.A. MIETTINEN (1979). Effect of dietary fibre on serum lipids and cholesterol metabolism in man. Atherosclerosis V. A.M. Gotto, L.C. Smith, B. Allen (eds.). Springer-Verlag, p.311-315.

305. W.T. BEHER, K.K. CASAZZA, A.M. FILUS, M.E. BEHER, J. BERTASIUS. (1970). Effects of accumulated tissue cholesterol on bile acid metabolism in hypophysectomized rats and hamsters. Atherosclerosis. 12; 383-392.

306. W.T. BEHER, G.D. BAKER, D.G. PENNEY (1963). A comparative study of the effects of bile acids on cholesterol metabolism in the mouse, rat, hamster and guinea-pig. J. Nutr. 79; 523-530.

307. D.L. TOPPING, R.P. TRIMBLE, R.J. ILLMAN, J.D. POTTER, D.G. OAKENFULL (1980). Prevention of dietary hypercholesterolaemia in the rat by soy flour high and low in saponins. Nutr. Rep. Int. 22; 513-520.

308. S. KIRIYAMA, Y. OKASAKI, A. YASIDA (1969). Hypocholesterolemic effect of polysaccharides and polysaccharide rich food stuffs in cholesterol fed rats. J. Nutr. 197; 382-388.

309. J.D. POTTER, R.J. ILLMAN, G.D. CALVERT, D.G. OAKENFULL, D.L. TOPPING (1980). Soya saponins, plasma lipids, lipoproteins and fecal bile acids: A double blind cross over study. Nutr. Rep. Int. 22; 521-528.

310. K.S. SHURPALEKAR, T.R. DORAISWAMY, O.E. SUNDARAVALLI, M.N. RAO (1971). Effects of inclusion of cellulose in an atherogenic diet on the blood lipids of children. Nature. 232; 554.

311. O.W. PORTMAN, D. MURPHY (1958). Excretion of bile acids and β-hydroxy-sterols by rats. Arch. Biochem. 76; 367.

312. K.W. HAETON (1972). Bile salts in health and disease. London, Churchill, Livingstone.

313. J. DAVIDSON, D. SPREADBURY (1975). Nutrition of the New Zealand White rabbit. Proc. Nutr. Soc. 34; 75-83.

314. J.W.G. PORTER (1969). Digestion in the pre-ruminant animal. Proc. Nutr. Soc. 28; 115-120.

315. B.S. PLATT, D.S. MILLER (1958). The quantity and quality of protein for human nutrition. Proc. Nutr. Soc. 17; 156.

316. J. AGUILERA, J. BOZA, J. VERELA (1970). Effect of amount of energy in the diet on nitrogen balance in rabbits. Anals. de Edalogia. Y. Agrobiologica. 29; 709-718.

317. R.H. BARNES, E. KWONG (1964). In "The role of the gastro intestinal tract in protein metabolism". H.N. Munro (ed.) p.41 (Blackwell, Oxford).

318. L.G. KENNEDY, T.V. HERSHBERGER (1974). Protein quality for the non-ruminant herbivore. J. Animal. Sci. 39; 506-511.

319. D.M. WALKER, R.D. KIRK (1975). The utilization by pre-ruminant lambs of isolated soya bean protein in low protein milk replacers. Aust. J. Agri. Res. 26; 1037-1052.

320. W.G. POND, W. SNYDER, J.T. SNOOK, E.F. WALKER, D.A. McNEIL, B.R. STILLING (1971). Relative utilization of casein, fish protein concentrate, and isolated soya bean protein for growth and pancreatic enzyme generation of the protein - calorie malnourished baby pig. J. Nutr. 101; 1193-1200.

321. A.D.L. GORRILL, J.W.G. NICHOLSON (1969). Growth, digestibility and nitrogen retention by calves fed milk replacers containing milk and soya bean proteins supplemented with methionine. Canad. J. Animal Sci. 49; 315-321.

322. E. GAMAN, H. FISHER (1970). The essentiallity of Arginine, lysine, methionine for the growing rabbit. Nutr. Rep. Int. 1; 57-63.

323. G.A. LEVIELLE, A.S. FEIGENBAUM, H. FISHER (1960). The effect of dietary protein, fat and cholesterol on plasma cholesterol and serum protein components of the growing chick. Arch. Bioch. Biphys. 86; 67-70.

324. M. KOKATNUR, N.T. RAND, F.A. KUMMEROW, H.M. SCOTT (1958). Effect of dietary protein and fat on changes of serum cholesterol of mature birds. J. Nutr. 64; 177.