AN INVESTIGATION INTO THE IMMUNOLOGICAL THEORY OF ATHEROGENESIS IN RABBITS

by

Carol Ann Muir, BSc (Hons)

A thesis submitted for the degree of Doctor of Philosophy
the Faculty of Science, University of Southampton

October 1979

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

NUTRITION

Doctor of Philosophy

AN INVESTIGATION INTO THE IMMUNOLOGICAL THEORY OF ATHEROGENESIS IN RABBITS

by Carol Ann Muir

There is evidence to show that injections of foreign proteins in conjunction with high fat diets will produce atherosclerosis in rabbits. Furthermore, it has been suggested that patients with heart disease have higher levels of circulating milk antibodies than controls and that immunological injury might initiate the process which eventually leads to atherosclerosis.

An experimental programme was designed to investigate the possibility that a regular intake of food antigens (soya bean proteins) together with a high intake of fat might produce atherosclerosis, and an arterial disease was produced by feeding cholesterol-free, semi-synthetic diets to rabbits for eleven months. The histological features of this disease were unusual in that there was evidence of an inflammatory infiltrate with mononuclear cells and occasional giant cells. In most of the rabbits food antibodies were detected and there were significant positive correlations between plasma cholesterol levels and atherosclerosis scores and between food antibody titres and atherosclerosis scores.

The above experiment was repeated to investigate more closely the immunological processes that might be involved in the development of arteriosclerosis using similar diets. The rabbits sacrificed after three months on the diets showed no arterial disease, but after nine to twelve months on the diets some disease was evident, more predominant in the nine than in the twelve month feeding trial. The arterial disease produced in the first experiment and the level of food antibodies was also lower. There was a significant correlation between plasma cholesterol levels and the atherosclerosis scores and between immune complex levels and atherosclerosis scores. A further experiment with low unsaturated fat, high starch diets was carried out. No arterial disease was produced in these rabbits, which produced food antibodies but had normal plasma cholesterol levels.

Clearly, from the first experiment food antigens appear to play a role in the atherogenic process but due to the lesser extent of the disease in the second experiment this role could not be confirmed, probably because the rabbits were immunologically tolerant to the soya proteins as their mother's diet had also contained soya. The weight of evidence suggests that soluble immune complexes may be responsible for causing injury to the vessel walls. There is a need for further investigation into the effect of feeding food antigens on atherogenesis.

ACKNOWLEDGEMENTS

I would like to express my thanks to the following individuals and institutions without whose help this thesis would never have been completed:

Special thanks are due to Professor T. G. Taylor for his diligent supervision and guidance of the project

The University of Southampton for their financial support.

Doctor P. J. Gallagher for his help and guidance in all the pathological aspects of this thesis.

Mr. C. Bunce and the Animal House staff for their diligence in attending to the animals.

Miss C. Brant and Miss P. McDonnell for their efficient typing.

My parents for their continued interest throughout my formal education and my husband for his patience and support.

ABBREVIATIONS

App	Appendix
BSA	Bovine serum albumin
Ca	Calcium
CO	Coconut oil
Expt	Experiment
Fig	Figure
g	Gram
i.u.	International units
kcal	Kilocalorie
kg	Kilogram
l	Litre
MO	Maize oil
µmol	Micromole
mg	Milligram
ml	Millilitre
mmol	Millimole
nm	Nanometer
OD	Optical Density
PMN	Polymorphonuclear leucocyte
PD	Promine D
Ps	Promosoy 100
Vit	Vitamin

CONTENTS

ATHEROSCLEROSIS

1 LITERATURE REVIEW

Introduction

Atherosclerosis is a pathological condition of the arteries characterised by a variable combination of changes in the intima including focal damage and the accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits.[1,2,3] It is further characterised by focal intimal thickening consisting of proliferated connective tissue and medial changes.[4] The disease is both degenerative and proliferative.[5]

The Lesions in Atherosclerosis

The lesions which characterise the early development of atherosclerosis in humans are called fatty streaks. These are recognisable as tiny deposits of lipid which are visible in the intima of large arteries.[6] The next stage is recognisable as gelatinous lesions which are seen under the microscope as oedematous separation of somewhat thickened collagen fibres. The archetypal lesion is the fibrolipid plaque which is an area considerably elevated above the surface of the intima and is seen microscopically as a yellow lipid-rich basal pool covered by a connective tissue cap of variable thickness.[4] The ultimate stage is ulceration, which shows adherent thrombi, and fibrous tissue associated with damage to the muscle and elastic tissue.[7]

The occurrence of large quantities of plasma-derived lipids and other constituents in the lesions suggest that the process of development must be accompanied by either a focal increase in permeability of the endothelial barrier or a decrease in the mechanisms which keep blood constituents out of the arterial wall. Several theories have been put forward as to how the process leading to the development of atherosclerosis can be initiated.

Thrombogenic Theory of Atherogenesis

The Encrustation Theory[8] postulates that plaques are merely transformed mural thrombi. This has been subsequently modified[9] to posulate that encrustation of fine platelet deposits and films of fibrin on the endothelium provokes an inflammatory reaction. According to this revised theory, now called the Thrombogenic Theory[9], the thrombi would become incorporated into the arterial wall due to the endothelial cells growing over the thrombus. An invasion of vascular cells from underneath[10] would then scavenge away the thrombic mass and replace it by scar tissue. The method by which fat deposition subsequently occurs remains unclear. No intermediate stages consisting of thrombi in the atheroma itself can be found.

Filtration Theory

The second theory to be put forward is based on the thesis that most of the arterial wall is nourished by diffusion from the lumen. Under normal circumstances, most of the lipid molecules in the percolating fluid pass straight through the wall and drain into the lymphatic system. In cases of hyperlipidaemia, the lipid molecules do not all pass through but a proportion become trapped and hence cause an inflammatory reaction in the tissue surrounding them. This theory was first called the Lipid Imbibition Theory[11] but is more commonly known as the Lipid Filtration Theory[12].

This theory has been accepted for many years without serious challenge. The fact that the arterial wall depends on diffusion for its nourishment could explain the way in which the fat was deposited. In addition, the fat that is deposited is not generated in the arterial wall, but comes from the plasma[13].

The Smooth Muscle Cell

A more complete understanding of the disease requires an appreciation of the function of the components of the arterial wall. The arterial smooth muscle cell plays a fundamental

role in the response of the arterial wall to endothelial damage.[15,16,17] The smooth muscle cell is versatile and metaplasia enables these cells to become endothelial-like cells, or as fibrocytes to produce collagen and ground substances. They are prone to fatty change and accumulate intracellular lipid in the presence of an increased concentration of extracellular lipoprotein, becoming fatty foam cells. They may also promote the deposition of lipid in the extracellular matrix.

When endothelial damage occurs, the smooth muscle cells migrate to the intima and there they can proliferate. This proliferation is dependent on a platelet factor becoming released.[1,18,19] Platelets adhere to the focal areas of the injured endothelium[19,20] and release the platelet constituents into the subendothelial space[21,22] causing proliferation of the smooth muscle cells.

Response-to-Injury Hypothesis

This led to another theory of atherogenesis to be proposed; the so-called Response-to-Injury Hypothesis.[23,24] This theory encompasses the two previous theories. It is based on the similarity between atherosclerosis and the response of the artery to experimental injury. Factors such as hyperlipidaemia, hormone dysfunction and the increased shear stress in hypertension may injure the endothelium and alter the nature of the endothelial barrier to the passage of blood constituents into the arterial wall.

The relationship between the cells in the endothelium and between endothelial cells and the underlying connective tissue is thus altered allowing desquamation of the endothelium and exposing the subendothelial tissue to the plasma. The platelets in the plasma will then adhere to the subendothelial collagen, aggregate[21,25,26,27] and then release the contents of their granules.[26] This infiltration of plasma constituents such as platelets, plasma lipoproteins and hormones leads to proliferation of arterial smooth-muscle cells which can lead to the formation of a connective tissue matrix and the deposition of lipid within and between the

cells. If the instigator of injury is then withdrawn, the lesion thus produced will regress. However, if injury to the endothelium persists, or is repeated, further proliferation of smooth-muscle cells and accumulation of connective tissue and lipid will occur.

Thus, the progression or regression of the lesion is held in a critical balance. Risk factors can possibly interfere with this balance by tipping it towards cell proliferation and destruction. They may not only initiate an injury, but also convert what might have been a limited tissue response to injury into what is recognised as atherosclerosis.

Risk Factors

Many risk factors are recognised as being conducive to the development of atherosclerosis. These factors can be split into two main categories: dietary and others. Dietary factors which predispose the body to atherosclerosis include saturated fat,[28] dietary fibre,[29,30,31] protein,[32] carbohydrates especially sucrose[33] and trace elements.[34] The other factors include hypercholesterolaemia[35] and hypertension, which might owe its effect to the action of Angiotensin II.[36,37] Excessive smoking is a risk factor working either through carbon monoxide or the nicotine produced when smoking[38] or through the allerginicity of tobacco.[39] Haemodynamic stress,[40] heredity, diabetes mellitus,[41] maleness,[42] disturbances in the hormonal balance and disease either bacterial or viral[43] are considered to be risk factors in certain individuals.

It can be seen that many factors have been suggested to predispose an individual to atherosclerosis. However, a single risk factor seldom shows a good correlation with atherosclerosis.[14,44,45,46]

The Role of Diet in Atherogenesis

(i) Cholesterol:

Most of the initial research was conducted to demonstrate lipid deposition in the arterial wall and the involvement of hypercholesterolaemia. Hypercholesterolaemia was

considered to be the best indicator of atherosclerosis,[46] although a strong relationship was not always shown.[47] The earliest work on rabbits showed that diets containing meat, milk and eggs produced an arterial disease[48] and subsequently, feeding fat and egg yolk also produced a disease.[49] Both these types of diets contain cholesterol and these experiments led many researchers to utilise the cholesterol-fed rabbit as the experimental model for atherosclerosis.[50,51] This model produced fatty streaks similar to human streaks and also fat in the intima lying between the endothelium and the internal elastica. No lesions resembling the human fibrous plaques or other advanced lesions were found.[50] It was further criticised because the advanced disease it produced was grossly dissimilar to that produced in humans.[52,53,54,55] The disease produced in the cholesterol-fed rabbit was likened to a cholesterol storage disease[1] due to the massive accumulation of cholesterol in the body organs and tissues.[52,53] The plasma cholesterol levels in these rabbits were up to forty times higher than normal plasma cholesterol levels.[52,56]

(ii) Fat:

The fact that the cholesterol-fed rabbit produced a cholesterol storage disease and not a human type atherosclerosis led researchers to investigate other means of producing an arterial disease without the use of dietary cholesterol. It is well established that semi-synthetic diets containing saturated fats but no cholesterol can induce atherosclerosis.[28,57,58] However, a commercial diet seemed to protect rabbits from atherosclerosis as the addition, to the commercial diet, of saturated fat did not induce the disease.[46] It was shown that the complete commercial diet was required for this protection,[59] therefore saturated fat cannot alone produce the disease.

Work was carried out to establish which fats were most atherogenic when fed with cholesterol-free, semi-synthetic diets to rabbits.[47,60] Coconut oil, which is a highly saturated oil, was shown to increase cholesterol and

triglyceride levels in the plasma. It was also shown to be most atherogenic when compared with peanut oil, maize oil or butter fat. Maize oil, a predominantly unsaturated oil, was found to be the least atherogenic. Thus, it was shown that saturated fats are more atherogenic than unsaturated fats. Investigations were then initiated to ascertain which other dietary factors could influence the development of atherosclerosis.

(iii) Carbohydrate:

Carbohydrates, especially sucrose[32] have been implicated as risk factors; high levels were fed to rabbits in order to see if they would have an influence on the development of arterial disease. Starch, fructose and sucrose were found to be most atherogenic with glucose as the least.[61,62] The carbohydrate constituent of the diet has been shown to effect plasma triglyceride levels[63] but not cholesterol levels.[64]

(iv) Fibre:

Some types of fibre have been shown to be protective when included in a potentially atherogenic diet.[20,30,31] Some sources of roughage like wheat straw and peat were found to reduce both plasma cholesterol levels and the degree of atherosis substantially, whereas other sources such as cellophane and solkafloc tended to give higher plasma cholesterol levels and higher degrees of atherosis in rabbits.[29] The protective effect of fibre depends on the quantity and type of roughage included in the diet.[65]

(v) Protein:

The other major component shown to be involved in atherosis is the protein. In humans the incidence of atherosclerosis was shown to be correlated with protein.[32,66] Many of the early feeding trials which investigated the use of protein to produce atherosclerosis used proteins such as meat, milk, eggs[48] and powdered beef,[67] all of which contained cholesterol which had been shown to produce an arterial disease in rabbits.[48,49] Other research produced

an arterial disease in rabbits with proteins which did not contain cholesterol.[51,68,69] Animal proteins were found to be more hypercholesteraemic and atherogenic than plant proteins.[51,69,70,71]

(vi) Interaction of dietary components:

Although these dietary components can individually affect the degree of atherosclerosis, some dietary factors interact in ways which may negate or exacerbate deleterious effects. Interactions between protein and fibre have been established. Casein, a milk protein, was found to be more atherogenic than soya protein when fed with most fibre sources, but they were found to be of equal atherogenicity when fed with alfalfa.[72] Casein was also shown to give higher cholesterol levels and a greater degree of atherosclerosis than soya protein or soy protein isolate when fed with dextrose or wheat starch but not with potato starch.[73] The increase in cholesterol levels produced by casein could not be prevented when the fat in the diet was unsaturated,[74] neither could it be prevented by increasing the fibre content of the diet[75] even though fibre has been shown to be hypocholesterolaemic.[47]

The results of these studies show the importance of considering the diet as a whole and taking into account interactions between dietary components.

Endothelial Injury

Although it has been shown that by the use of cholesterol-free diets fed to rabbits a disease resembling atherosclerosis can be produced,[51,68,69] these experiments do not answer the question of how the initial injury actually occurs. It is known that hyperlipidaemia can cause arterial injury, but damage can be produced when plasma lipid levels are normal.[10]

As endothelial damage plays a critical role in the pathogenesis of atherosclerosis and the earliest fat deposits occur at the site of injury[23,76,77] in cholesterol-fed rabbits,

the effects of agents which would induce endothelial injury were studied.[78] After direct perfusion of the arteries with agents thought to cause injury, sections of arteries were examined with an electron microscope. It was shown that the inter-endothelial cell junctions, which are composed of glycoproteins, are destroyed by extremes in pH or anoxia.[78] The endothelial cells contain myosin in their cytoplasm and have been shown to be contractile.[3,79] The cells contract in response to extremes in pH, changes in osmolarity and anoxia.[78]

Arterial damage has been produced by abrasion with a catheter[3], and the intima in pigs was thickened to twelve times normal thickness by gentle abrasion.[80] Other work has shown that chemical injury can cause endothelial cell loss.[23,81] A major criticism of inducing endothelial damage by these methods is that the doses of the agents required to cause injury are far greater than those occurring spontaneously.[82]

Immunological Theory

An explanation of the mechanism by which arterial injury could arise has been put forward in a new hypothesis called the Immunological Theory.[90] Red blood cells were shown to have a negative charge,[83] which was decreased in ischaemic vascular disease.[84] This decrease in charge was positively correlated with platelet stickiness[85] and was found to be independent of serum cholesterol levels.[83,86] This behaviour was attributed to alterations in plasma activity[87] and the discovery that an increase in gamma globulin in patients with heart disease compared to control patients[88] suggested that the stickiness of blood particles could be caused by immunoadherence.[89]

It was then considered how this increase in gamma globulin could come about and the mechanisms by which injury could be caused to the arterial wall; this injury increasing endothelial permeability, hence, lipid entry to the intima.[90]

Absorption of Dietary Protein

It is well established that dietary proteins can be antigenic and produce specific antibodies.[91] Consequently, it was suggested[92] that an allergy to certain compounds, for example,

antibiotics and other drugs, food proteins or an individual's own tissues, may be a causal factor in arterial disease. It was little considered or supported by experiments at this time.

Absorption of whole dietary protein across the gut in antigenic quantities has been shown.[93] This can occur in a mature mammalian intestine,[94-98] through which 2% of the protein remains immunologically intact[99,100] although it is insignificant nutritionally. However, if this absorption is normal it need not lead to disease, as the absorption of intact protein across the small intestine causes local antibody production. Further absorption of antigens is inhibited by these antibodies present either on the intestinal surface or possibly within the intestinal lumen.[101,102] Thus deleterious consequences of antigen-antibody interaction[102] are prevented; these actions will be discussed later.

Experimental Serum Sickness

Experimental evidence supporting the immunological theory has been produced by inducing an immune-complex disease in rabbits: 50% of rabbits receiving a diet containing 1% cholesterol and given one injection of bovine serum albumin rapidly developed aortic lesions, compared to 20% receiving the injection alone and 1% receiving cholesterol alone.[103] Recent evidence has shown that administration of a single antigen can cause a reaction in the blood vessels manifested in the stimulation of vascular connective tissue synthesis,[104] confirming the evidence that vascular injury in vivo can be caused independently of cholesterol.[103] This immunologically-induced vascular injury potentiated atherogenesis in rabbits fed a cholesterol diet[105] and in baboons fed a high fat diet.[106]

These results were reproduced in cholesterol-fed rabbits by administering four injections of horse serum.[107,108] The cholesterol diet plus injections produced fatty proliferative arterial degeneration, which resembled human lesions, the injections alone gave proliferative changes, and fatty lesions were produced when the cholesterol with no injections was fed to the rabbits.[107]

Mechanisms of Immunological Injury

Therefore, it has been shown that the synergy of immunological injury and hypercholesterolaemia can lead to atherosclerosis in rabbits. These lesions are similar to human lesions and the immune response to foreign proteins occurs normally in humans.[108] In these experiments, injections of antigenic foreign proteins produced immune-complex disease because the antigen persisted in the circulation until an antibody response occurred. The antigen and antibody then combined to form pathogenic immune complexes. In animals, serum sickness can accelerate the development of arterial disease.[107,109,110]

It has been shown that the increase in vascular permeability was brought about in part by vaso-active amines and histamine[10] released from tissue reservoirs such as mast cells and platelets by the action of immune complexes.[111] Mast cells have been shown to accumulate around chronic inflammations and in atherosclerosis.[112] The damage to the arterial wall can cause a deposition of large molecules in the arterial wall and this could be prevented by antagonists of histamine.[113] The action of immune complexes in rabbits causes complement dependent accumulation of platelets, mast cells and neutrophils and aggregate anaphylaxis.[114] This leads to cell lysis,[115] and then to the secretion of vaso-active amines which cause an increase in vascular permeability as a result of the contraction of endothelial cells.

Non immune processes can stimulate complement components[116] which can cause neutrophil mediated inflammation reactions[117] and a hypothesis has been put forward that involves complement in the pathogenesis of arteriosclerosis.[118] Complement is composed of a group of nine proteins which circulate in the blood in an inactive form. The system can be activated specifically by infection and non-specifically by aggregates of macromolecules,[119] enzymes,[120] lipoproteins,[121] viscosity and turbulence.[112] It has been shown that experimentally induced infection can produce arterial damage reminiscent of arteriosclerosis.[123] There is evidence that activated complement can cause arterial damage.[124]

Even in complement-depleted animals, arterial damage can occur, hence, there is more than one mechanism involved by which immune complexes can cause increased permeability. Immune complexes can deposit in glomeruli in complement-depleted animals.[125] In rabbits after an injection to induce serum sickness, immune complexes appear in the serum. In addition, IgE antibody is formed which binds to the surface of basophils. When these basophils combine with the specific antigen a soluble factor,[126] called platelet activating factor, is released which can clump platelets and stimulate the release of vaso-active amines.[127] This leads to increased permeability in the endothelium, allowing immune complexes greater than 19S [111] in size to be deposited in the arterial wall. The deposited complexes can then induce inflammatory injury.

The presence of polymorphonuclear lymphocytes (PMN) are essential for the development of cardiovascular lesions in serum sickness.[128,129,130] Disruption of the internal elastic lamella was associated with the presence of PMN. It seemed that the internal elastic lamella acted as a barrier to the outward spread of inflammation and that this is an important substrate of PMN action.

The damage produced by immune complexes in the absence of lipid feeding results in a necrotising arteritis, with endothelial proliferation, subendothelial fibrinoid deposition, medial necrosis and PMN infiltration.[113,128] Complement and PMNs are required in the rabbit for full arterial damage to occur.

This destruction of the wall may lead to the liberation of proteins with autoantigenicity. The presence of antibodies directed against antigens derived from the arterial wall has been noted.[131,132] Since the elastic elements of the arterial wall are the most changed in atherogenesis, investigators have tried to show the presence of antielastin antibodies. When human and porcine aortic elastin was injected into rabbits more than 70% of the rabbits showed macroscopic lesions compared to none in the uninjected controls. All the lesions were characterised by early massive destruction

of the elastic tissue, which became fragmented and calcified.[133] Arterial lesions were produced in 70% of rabbits immunised with homologous aorta but only in 25% injected with liver or heart extracts.[134] The rabbits injected with homologous aorta produced aortic antoantibodies.[135] Another study has shown antoantibodies were produced against heart, kidney and splenic tissues in rabbits fed cholesterol. However, the antibody titres were not correlated with the degree of aortic lesions.[136]

Autoimmune reactions have been implicated in atherogenesis.[137] In human arterial disease, autoantibodies against aortae are present[138] but only in the advanced disease. However, elastin antibodies were found to be lower in heart disease patients than normal patients.[139] It was considered that autoantibodies were secondary to the disease state and that advanced stages may be accelerated by their presence. Autoantibodies arise from the appearance of new antigens which might occur as a consequence of atherosclerotic change.[140] Glycoprotein was isolated from a sclerotic human media and used to immunise rabbits. The antibodies so produced were found not to react with glycoprotein from a normal artery,[141] showing that only the altered determinants were immunogenic.

Antigenicity of Food Proteins

The significance of producing an arterial disease in rabbits using immunological methods can be seen when the incidence of an immune response to common food proteins is examined. Antibodies produced against cow's milk proteins[142,143] and some foods[142,144,145,146] have been detected in healthy children. An allergy to food proteins has been displayed in a large number of infants under the age of one[147-153] and in small children.[154] This allergy is shown to be maximal in small children and that, as age increases, the severity decreases.[155] Alongside cow's milk protein allergies there has been a rising incidence of allergy to soya proteins in infants[151,156-158] children[159] and adults.[157,160]

Epidemiology

Heart disease is more prevalent in humans who have been fed on cow's milk as infants than in those who were breast-fed.[161] Abnormal arteries in infants examined at autopsy were found more in infants that were given cow's milk rather than being breast-fed, conversely, normal arteries were found to come more from breast-fed infants.[162]

In a survey of the occurrence of milk antibodies, using a passive haemagglutination test for measuring antibodies, it was found that patients with ischaemic heart disease had an increased titre when compared to control patients.[163,164] However, using different assays for detecting antibodies, these results were refuted in other studies which showed no relationship between ischaemic heart disease and antibodies to milk proteins.[165,166]

Circumstantial evidence for the involvement of milk protein in the aetiology of atherosclerosis has been produced. It was shown that an increase in the incidence of ischaemic heart disease occurred soon after the introduction of Holder Pasteurisation, which involves heating milk for a relatively long period of time.[167] The consumption of milk proteins is positively correlated with ischaemic heart disease, an example being Finland which has the highest coronary mortality rate and the highest per capita intake of total milk.[168,169] It was suggested that a high intake of milk might be harmful to health, but that this might not only be due to the antigenicity of the milk proteins (3.28g/100ml milk), but also the level of fat (3.79g/100ml milk) in milk.

However, milk proteins might not be the only antigens involved, and an immune response might be induced by other antigens. A heart transplant recipient, who at the time of the transplant operation had hypercholesterolaemia, was found nearly two years later to have severe atheroma.[170] It is thought that rejection of the heart was brought about by an immune response and, together with the hypercholesterolaemia, induced the development of atheroma. This type of arteriosclerosis has been called graft arteriosclerosis (GAS), which, in the above case, led to atherosclerosis. However, it has

been shown that GAS and spontaneous arteriosclerosis (SAS) are similar, and it is suggested that a unifying hypothesis can explain their similarity.[15] Both SAS and GAS are typified by the accumulation of modified smooth muscle cells, which is non-specific and would occur with many forms of arterial injury. However, in GAS, the cause of damage to the endothelium is suggested to be an immune reaction and it is hypothesised that the same might occur in SAS.

As circulating immune complexes are thought to be the pathogenic form of the immune response then their detection in humans would be of prime importance. Circulating immune complexes have been detected in infants who are fed cow's milk.[171] The presence of immune complexes containing β-lipoprotein as the antigen with its respective antibody has been noted in patients with autoimmune lipoproteinaemia,[172] and in rabbits fed an atherogenic diet.[172,173] The occurrence of immune complexes was shown to be greater in patients with vascular disease than in control patients.[174]

Another common antigen which might be implicated in the development of an immune response is tobacco, since there is evidence that antibodies are raised to tobacco.[39] It was shown that 68% of patients with thrombo-angiitis obliterans had antibodies to tobacco. This disease is related to atherosclerosis in that the lesions in the advance stages are similar in both diseases.

Experiments have shown that a synergy between foreign protein injections and subsequent fat feeding will produce atherosclerosis in rabbits.[104-110] This disease produced in rabbits is broadly similar to the human disease. A series of experiments is required to establish if dietary proteins, shown to produce an immune response, with a high fat content in the diet will also produce atherosclerosis in rabbits.

CHAPTER 2

EXPERIMENT 1

2.1 Materials and Methods

Sixteen New Zealand White rabbits were weaned from a commercial stock diet (App.1, Table 1), (Labsure Animal Foods, Christopher Hill Group Limited, P.O. Box 6, Castle Street, Poole) and were allocated in groups of four, to one of four isonitrogenous, isoenergetic diets for eleven months. These diets are shown in Appendix 1, Table 2. They were high fat diets containing 190g/kg diet of either maize oil (MO) (C.P.U. (U.K.) Limited, Trafford Park, Manchester) which contains predominately unsaturated fatty acids or coconut oil (CO) (Keira Trading Company, West Midlands) which is composed predominantely of saturated fatty acids. The diets contained 20% dry weight of either one of two soya proteins. One was a soy protein concentrate called Promosoy 100 (Ps), (Central Soya, Chicago, Ill., USA) which has been shown to be non-antigenic in preruminant calves.[180] The other protein was a soy protein isolate called Promine D (PD) , (Central Soya, Chicago, Ill., USA) which has been shown to be highly antigenic [180,181] in preruminant calves.

PD is a sodium proteinate and no NaCl was added to the diets in which PD was included; however, it was necessary to supplement this protein with potassium in the form of KCl. All diets contained 150g wheat bran/kg diet as the source of fibre.

There were thus four dietary treatments identified thus: Ps-MO, Ps-CO, PD-MO, PD-CO. The diet Ps-MO containing the non-antigenic protein and the non-atherogenic fat was regarded as the control diet. However, two additional rabbits were fed the stock diet throughout the experiments to provide further controls.

The ingredients of the diets were mixed and then moistened with water, pelleted and dried overnight at 36°C. During the course of the feeding trial the rabbits were given food and water ad libitum. Food intake and weight gain were recorded each week.

2.1.1 Blood Analyses

Fasting blood samples were taken at monthly intervals from the ear vein of the rabbits using Heparin (BDH Chemicals, Poole, Dorset) as anti-coagulant when plasma was required. Blood taken for serum was allowed to clot at room temperature. All samples were then spun at full speed on a bench centrifuge (Measuring & Scientific Equipment Limited, Sussex).

Total cholesterol and triglyceride concentrations (App.1, III) were determined with an Auto Analyser II (Technicon Instruments Company Limited, Basingstoke, Hampshire) using Clinical Method AAII No.24 (1972).

Antibodies to the soya proteins were determined by a turbidimetric assay (App.1, I) which utilises the fact that when antigen and antibody combine in solution a precipitate is formed. On the final bleeding a passive haemagglutination assay was also used (App.1, II).

2.1.2 Tissue Studies

The animals were killed with an injection of Nembutal (Sigma, London). A large portion of the aorta was removed, stained for lipid using Sudan Black (George T. Gurr Limited, London) and then scored macroscopically on a 1-5 scale, 1 being no atheroma and 5 being very advanced lesions (App.1, IV). Samples of abdominal aorta, kidneys and one renal artery were removed for calcium analysis by atomic absorption spectrophotometry (App.1, V). Other samples of renal and carotid

arteries and aorta were fixed in buffered formalin for histological examination (App.1, VI). All animals with a score of 3 or more were examined histologically by a pathologist who had no knowledge of the treatment the animals had received.

2.2 Results

The experimental results, which are tabulated fully at the end of this chapter, were statistically analysed using the analysis of variance technique. There were no differences in food intake (Table 2.1) or live weight gain (Fig.2.1, Table 2.2) between the rabbits on each of the four diets. Towards the end of the experiment some rabbits appeared to have digestive disturbances. Mainly as a result of this, one rabbit was lost from each dietary treatment before the completion of the experiment. The plasma cholesterol levels were high (6.46-15.52 mmol/l) in three of these rabbits lost and of these, two had high aorta scores (PsCO, PDMO). The other rabbit (PsMO) had sores on its face which would not heal, the plasma cholesterol in this rabbit was low (0.9 mmol/l).

2.2.1 Plasma Cholesterol and Triglycerides

The plasma cholesterol concentrations measured were variable from month to month. At six months, shown in Table 2.3, the plasma cholesterol levels showed no difference between the four treatments; however, at the final bleeding there were significant differences in plasma cholesterol levels between diets, the CO-fed rabbits having significantly higher levels than the MO-fed animals. No differences were found between the two proteins.

The plasma triglycerides levels also showed great variability from month to month and there were no differences due to protein or fat at either six months or the final bleeding. These results are shown in Table 2.3.

2.2.2 Food Antibodies: Turbidimetric Assay

The results shown in Table 2.4 are the measurements taken in the last three months of life. No statistical differences were obtained on analysis except in the eleventh month where there was a significant interaction ($p<0.05$). This indicates that when Ps was fed, MO gave lower titres than CO but that when PD was fed the reverse occurred. In all the measurements of food antibody titre by this method PsMO gave the lowest titres.

Using the mean titre for the last three months, analysis of variance indicated that PD gave significantly higher mean antibody titres than Ps ($p<0.05$) and the fat versus protein interaction was also significant ($p<0.05$) as in the eleventh month titres. Antibodies to the two proteins were undetectable in the rabbits fed the stock diet.

2.2.3 Food Antibodies: Haemagglutination Assay

The results shown in Table 2.4 were obtained from the final blood samples taken at death, or in the case of those that died unexpectedly, the penultimate sample. There were no significant differences between the proteins or the fats. However, seven rabbits in the groups fed PD had a measurable food antibody titre whereas in only three of the rabbits in the groups fed on Ps could food antibodies be detected. The rabbits fed the commercial stock diet had no detectable antibodies to either protein.

2.2.4 Aorta Scores

These results are shown in Table 2.4. Appreciable arterial disease was detected in 75% of the rabbits. Main treatment differences were found to be non-significant; however, there was a significant interaction ($p<0.05$) reflecting the fact that, when the proteins were fed in conjunction with MO, the scores

of PD-fed animals were higher than Ps-fed animals, but when CO was fed then Ps gave higher scores than PD. No atheroma was found in the rabbits fed the stock diet.

2.2.5 Liver Cholesterol and Liver Triglyceride

These results are shown in Table 2.5. No differences were seen between the four dietary treatments in the liver contents of either cholesterol or triglyceride. Total liver cholesterol ranged between 7.54-20.54 μmol/g and liver triglyceride ranged from 8.5-32.3 mg/g tissue.

2.2.6 Tissue Calcium

Macroscopically, at post mortem, some arteries appeared to be highly calcified but calcium was determined only in the tissues of the animals that survived to the end of the feeding trial. The results shown in Table 2.6 indicate no significant differences due to treatment in any of the vessels analysed. The inter-animal variation in calcium concentrations in the arteries was very large. A ranking-order procedure, followed by analysis of variance, showed that in the renal arteries there was no difference between the proteins but that the CO-fed animals had significantly more calcium than the MO-fed animals ($p<0.05$).

The calcium content of the kidneys was uniformly low suggesting that deposition of calcium in the arteries was not a result of generalised soft-tissue calcification.

2.2.7 Relationship Between Aorta Scores And Other Experimental Parameters

A summary of this data can be found in Tables 2.7 and 2.8. The product-moment correlation coefficients between aorta scores and the other experimental data are shown. There was a highly

significant negative correlation ($p<0.01$) between final live weight and aorta score. Live weight is a good index of general health and well-being in rabbits and this correlation suggests that advancing arterial disease was having an increasingly deleterious effect on the health of the experimental animals.

There were significant positive correlations between plasma cholesterol and plasma triglycerides and aorta score ($p<0.05$). There was a positive correlation which was not statistically significant between aorta score and food antibody titre by the haemagglutination assay but this was significant ($p<0.05$) using the turbidimetric assay.

There was a positive correlation between aorta score and calcium in the renal artery ($p<0.05$) but not in the other two tissues (aorta and kidney) that were analysed for calcium. Also, there was no correlation of aorta scores with liver lipids.

2.2.8 Histological Observations

Only animals which showed macroscopic changes were further examined microscopically. It can be seen from Table 2.9 that none of the animals in the PsMO group showed any of the changes observed in the other three dietary treatments and the arteries from these rabbits and those fed the stock diet were normal (Fig. 2.2). Significant microscopic changes were present in either the aorta, the renal or the carotid arteries of all of the animals which registered an atherosclerosis score of 3 or more. The chief morphological features were intimal thickening, intimal and medial calcification and fragmentation of the elastic lamellae (Fig. 2.3A). The extent of intimal thickening varied considerably from animal to animal, but was generally within the range of 25% to 100% of the thickness of the medial muscle. Intimal thickening was more prominent in the renal arteries (Fig. 2.3A) and

and calcification was more extensive in the aortae (Fig. 2.4) thus confirming the chemical analysis. Only small to moderate amounts of fat were identified in appropriately stained frozen sections. No cholesterol clefts or aggregates of foamy macrophages were identified.

It can be seen from Table 2.9 that in six rabbits there were significant numbers of chronic inflammatory cells. These were mainly present in the renal (Fig. 2.3A) or carotid (Fig. 2.5) arterial walls and were also present to a lesser degree in one aorta (Fig. 2.6). Lymphocytes and macrophages could be clearly recognised, but plasma cells, eosinophils and neutrophil polymorphs were absent. In four of the rabbits (three of which were fed PD), there were appreciable numbers of giant cells (Fig. 2.3B) and a single focal cluster of giant cells was noted in two more rabbits fed Promine D. The giant cells were centred around both internal and external elastic lamellae, often in associtation with disrupted fragments of elastin. These changes were extensive in the renal arteries (Fig. 2.3), but only focal in the aortae (Fig. 2.4) and carotid arteries (Fig. 2.5).

There was no evidence in any of the arterial or aortic sections of either a necrotising arteritis or a thrombus formation.

2.3 Discussion

Arteriosclerosis with hypercholesterolaemia has been produced in rabbits fed on cholesterol-free diets, high in soya proteins and both saturated and unsaturated fats. Published evidence has also shown that arteriosclerosis can be produced in this species with cholesterol-free diets,[182] and high in saturated fats.[47,58,183] There is also evidence to show that CO will produce extensive atheromatous degeneration of the aorta and coronary arteries but that MO will not produce these changes,[47,28,184-186]

and that by feeding hydrogenated CO, hypertriglyceridaemia will occur in rabbits.[184,187] However, the plasma triglyceride levels of the MO-fed rabbits were also high (2411.5 ± 646 mg/l) when compared to rabbits fed 15% CO for five months (1170 ± 150 mg/l).[187]

Most of the rabbits (75%) had elevated levels of plasma cholesterol. Those rabbits fed MO had raised cholesterol levels (2.76 ± 1.5 mmol/l) when compared to other work[47] where rabbits were fed 20% MO with 25% casein diet (0.78 ± 0.1 mmol/l) for 8.5 months. However, the rabbits fed CO had the same cholesterol levels (6.27 ± 1.7 mmol/l) as the rabbits[47] fed a diet containing 25% casein, 20% CO for 8.5 months (8.16 ± 0.59 mmol/l). Plant proteins have been shown to have no significant influence on cholesterol levels[73] and casein has been shown to increase the plasma cholesterol levels in rabbits.[73] The diets used here contained 150g wheat bran/kg diet and it is known that fibrous materials of plant origin exert a hypocholesterolaemic action in rabbits.[29] However, it was reported subsequently that wheat bran will not affect the hypercholesterolaemia in rabbits[211] produced by high fat diets.[184,188] Wheat straw has been shown to have a hypocholesterolaemic action in rabbits[29] and in the experiment reported above this might cancel out the hypercholesterolaemic effect of casein, thus making the cholesterol levels similar in the two experiments in the CO-fed animals.

Two of the rabbits (Table 2.10) fed PDMO had high levels of plasma cholesterol, plasma triglycerides and atherosclerosis score. These two rabbits also had high antibody titres (by the haemagglutination assay) and maybe the immune response was having some effect on the lipid levels. The increase in plasma lipids might be due to some interaction of PD with the unsaturated fat.

In planning this experiment it was anticipated that Ps would be of low antigenicity[180] and PD of high antigenicity.[180,181] This difference in antigenicity is thought to be due to the different ways in which the proteins are

processed. PD contains 900g protein/kg and consists largely of globulins prepared from the original heat-treated, solvent extracted soya-bean flakes by extracting with mild alkali followed by iso-electric precipitation at pH 4.5. The precipitated protein is then suspended in dilute sodium hydroxide at pH 7.0, separated and spray dried. Ps contains 600g protein/kg and is produced by extracting the soya-bean flakes with 60-80% ethanol at 65°C; the residue is steam-treated and ground to a meal.[180]

Thus, Ps would be expected to contain proteins of the same primary structure as those present in PD together with other less soluble ones, but their antigenicity might be reduced due to the alcohol treatment. Conversely the alkali treatment of PD may have enhanced the antigenicity of the native protein. Since both proteins evoked antibodies that precipitated with a solution of PD then the antigenic determinant must have been the same or similar.

The increased antigenicity of PD [180,181] was not shown conclusively; however, more rabbits fed PD (88%) had detectable food antibodies compared to rabbits fed Ps (38%). Arteriosclerosis was shown to be more predominant in rabbits that had produced antibodies, when the turbidimetric assay was used (Table 2.7). Three of the five rabbits with the highest scores also had high levels of food antibodies (Table 2.10). Four of these high scoring rabbits also had high levels of plasma cholesterol and plasma triglycerides.

Previous experiments have shown that atherosclerosis is produced by evoking an immune response in rabbits [128] fed on atherogenic diets [106,107] by injecting foreign proteins. The postulated mechanism by which the food antibodies can accelerate the development of atherosclerosis in rabbits is through the action of immune-complexes.[107,109,110] These complexes can adhere to the endothelial lining,[125] induce the release of vasoactive amines from platelets[111] and cause an increase in vascular permeability,[10] thus initiating the damage to the arterial wall.[113,128]

The histological picture observed in the animals in Experiment 1 is very different from that produced in the cholesterol-fed rabbit. In the cholesterol-fed rabbit a "cholesterol-storage disease" is produced and the plasma cholesterol levels would usually be ten to twenty times normal levels.[1] In rabbits fed a cholesterol-free, saturated fat diet an arterial disease is produced which is characterised by intimal proliferation with some evidence of lipid deposition.[60] The arterial disease produced in rabbits [106,107] by foreign protein injections and fat feeding was characterised by intimal thickening and fat deposition.

The disease produced in Experiment 1 was characterised by intimal thickening, calcification, fragmentations of the elastic lamellae and dense mononuclear infiltrates with giant cells. There was very little focal lipid deposition. The marked difference between the histological evidence in this experiment and that from other dietary induced arteriosclerosis experiments is the appearance in some rabbits of appreciable numbers of mononuclear and giant cells associated with the ruptured elastic lamellae. These histological features are strongly reminiscent of the human disease giant cell arteritis.

Four of the seven rabbits that had giant cells also had high aorta scores (Table 2.10). The other three rabbits with giant cells had moderate aorta scores showing a presence of atherosclerosis. Therefore, there might be some connection between the presence of giant cells and arteriosclerosis. The unusual pathological features of temporal arteritis are found superimposed on progressive intimal thickening and occasional discrete atherosclerotic plaque in the intima of aging arteries.[189] Fatal coronary disease in young people is associated with varying degrees of inflammation in the occluded arterial segment.[190]

The abundant mononuclear and giant cell infiltrates indicate an active inflammatory process. Although there

is no histological evidence in this experiment of an acute arteritis, the mixed chronic inflammatory infiltrate observed here is sometimes seen in an immune-complex mediated disease. Granulomata-containing giant cells, macrophages, epithelial cells and lymphocytes were recognised when immune-complexes at equivalence were injected intradermally into rats.[191]

The close association of the inflammatory infiltrate with fragmented elastica from the internal and external lamellae reinforces the possibility that damage might be mediated by either an antibody reacting directly with elastic tissues[189,192] or a cell-mediated response involving lymphocytes and macrophages reacting to the elastin.[189] Atherosclerosis has been produced by prolonged immunisation in rabbits with elastin-rich extracts of both procine and human aortic walls.[133]

The excessive amounts of calcium in the tissues raises the possibility of a disease induced by hypervitaminosis D. Some workers have produced arterial disease by giving large amounts of vitamin D,[194] but the present diets contained 2000 iu. vit. D_3/kg which is below the level at which harmful effects would be expected. The recommended level of vitamin D for rabbits is 744 iu. vit. D_3/kg diet.[196] A vitamin D_3 intake of 7260 iu/kg diet produced calcium deposits in the major arteries and calcification of the medium and small arteries of rabbits.[196] As the levels of vitamin D used in Experiment 1 are above the recommended levels[195] it cannot be completely rejected as a factor in this multifactorial disease.

In conclusion, an arterial disease has been produced in most of the rabbits fed a cholesterol-free, semi-sythetic diet. The disease produced was not essentially a fat storage disease and was characterised by intimal thickening, calcification and an inflammatory infiltrate with giant cells. It is clearly important to elucidate which, if any, immunological mechanisms are responsible for the arterial damage; the progression of the disease produced in this way must therefore be more closely analysed.

GRAPHS AND TABLES

Legend for graphs

Symbol	Diet
O	Promosoy 100 and maize oil (PsMO)
●	Promosoy 100 and coconut oil (PsCO)
Δ	Promine D and maize oil (PDMO)
▲	Promine D and coconut oil (PDCO)

Table 2.1: Food Intake (kcal/kg$^{0.75}$/week)

DIET	TIME (MONTHS) 0	2	4	6	8	10
PsMO	6.15±1.5	6.97±1.3	5.65±0.9	4.3±0.3	4.78±0.3	4.45±0.5
PsCO	4.03±1.2	6.08±0.4	5.02±0.3	3.61±0.2	4.15±0.5	3.74±0.4
PDMO	4.51±1.3	5.48±0.7	5.07±0.6	4.28±0.7	4.46±0.7	3.81±0.4
PDCO	4.85±1.1	5.02±0.5	4.72±0.4	3.35±0.3	3.71±0.2	3.77±0.8

Table 2.2: Live Weight Gain (kg)

DIET	TIME (MONTHS) 0	2	4	6	8	10	11
PsMO	1.44±0.1	3.0±0.1	3.37±0.2	3.58±0.2	3.59±0.1	3.69±0.1	3.85±0.1
PsCO	1.45±0.1	2.74±0.1	3.03±0.1	3.27±0.2	3.25±0.2	2.95±0.3	2.95±0.4
PDMO	1.42±0.1	2.68±0.1	2.96±0.2	3.01±0.2	3.15±0.2	3.1±0.3	3.23±0.4
PDCO	1.45±0.2	2.8±0.1	3.08±0.1	3.21±0.1	3.27±0.1	3.26±0.1	3.25±0.2

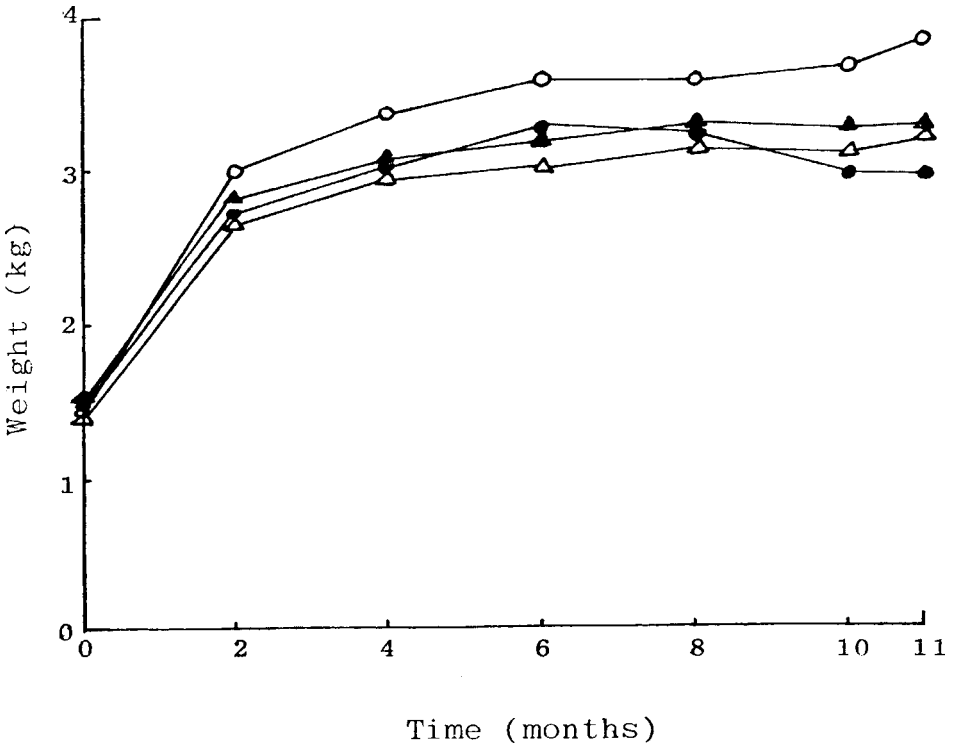


Figure 2.1: Weight Gain

Table 2.3: Plasma Cholesterol and Triglyceride

DIET	Plasma Cholesterol (mmol/l) TIME (MONTHS) 6	12	Plasma Triglyceride (mg/l) TIME (MONTHS) 6	12
PsMO	3.52±1.2	2.05±1.0	933±160	1665±510
PsCO	7.01±1.1	8.27±2.6*	828±110	2518±770
PDMO	5.29±1.7	3.48±1.2	1003±80	3158±1150
PDCO	5.19±1.8	4.26±0.8*	1105±270	1398±480

* Significantly greater than corresponding MO means ($p<0.05$)

Table 2.4: Food Antibody Titre and Aorta Score

DIET	Turbidimetric Assay TIME (MONTHS) 9	10	11	Mean	Haemagglutination Assay	AORTA SCORE
PsMO	7.3±3.1	6.8±4.0	12.0±1.3	8.0	320±320	2.0±0.4
PsCO	13.5±4.0	21.0±9.5	16.8±4.7	17.0	400±303	4.0±0.4
PDMO	15.5±2.3	34.8±8.7	23.8±3.1	25.0	320±174	3.5±0.7
PDCO	14.5±0.9	14.3±2.7	17.3±2.9	15.0	370±304	3.3±0.3
STOCK	0	0	0	0	0	0

Table 2.5: Liver Cholesterol and Triglyceride

DIET	Cholesterol (μmol/g)	Triglyceride (mg/g)
PsMO	9.3±1.8	10.5±2.0
PsCO	15.3±5.2	23.3±9.0
PDMO	8.8±1.3	14.1±5.0
PDCO	12.5±2.1	14.9±2.0

Table 2.6: Tissue Calcium

DIET	Calcium content of tissues (μmol/g) Renal Artery	Aorta	Kidneys
PsMO	15.0±5	70.8±10	10.8±5
PsCO	105.0±70*	218±67.5	6.8±2.5
PDMO	50.8±25	246±217	9.3±2.5
PDCO	71.8±2.5*	609±292.5	5.8±1.5

* Significantly higher than corresponding MO means ($p<0.05$)

Table 2.7: Relationship Between Aorta Scores and Other Experimental Data

	AORTA SCORE					
Parameter Measured	1	2	3	4	5	r^{Δ}
Number of Animals	1	2	7	4	2	
Final Live Weight (kg)	3.8	3.9±0.1	3.4±0.2	3.1±0.3	2.3±0.1	-0.74**
Plasma Cholesterol (mmol/l)	0.91	2.22±0.34	3.67±0.71	7.4±3.0	6.3±0.75	0.535*
Plasma Triglycerides (mg/l)	300	2347±87	1383±310	2475±768	4710±1730	0.56*
Antibody Titre † (Turbidimetric)	6	14±3	15±4	17±2	29±5	0.61*
Antibody Titre (Haemagglutination)	0	427±426.7	93.3±47	480±306	960±320	0.406

* Significant positive correlation with aorta score ($p<0.05$)
** Significant positive correlation with aorta score ($p<0.01$)
Δ correlation coefficient
† mean of final three months

Table 2.8: Relationship Between Aorta Scores and Other Experimental Data

AORTA SCORE						
Parameter Measured	1	2	3	4	5	r[△]
Number of Animals	0	3	6	3	1	
Aorta Calcium (μmol/g)	/	55.0±0.5	408±203	295.0±32	350	0.4
Renal Artery Calcium (μmol/g)	/	27.5±15	70.0±18	30.0±5	24.8	0.65**
Kidney Calcium (μmol/g)	/	7.5±2.5	10.0±2.5	5.0±0	12.5	-0.02
Liver Cholesterol (μmol/g)	/	10.7±1.0	9.88±1.8	15.9±5.2	9.88	0.16
Liver Triglyceride (mg/g)	/	9.8±1.5	16.4±5	13.0±4	20.4	0.273

** Significant positive correlation with aorta score ($p<0.01$)

△ correlation coefficient

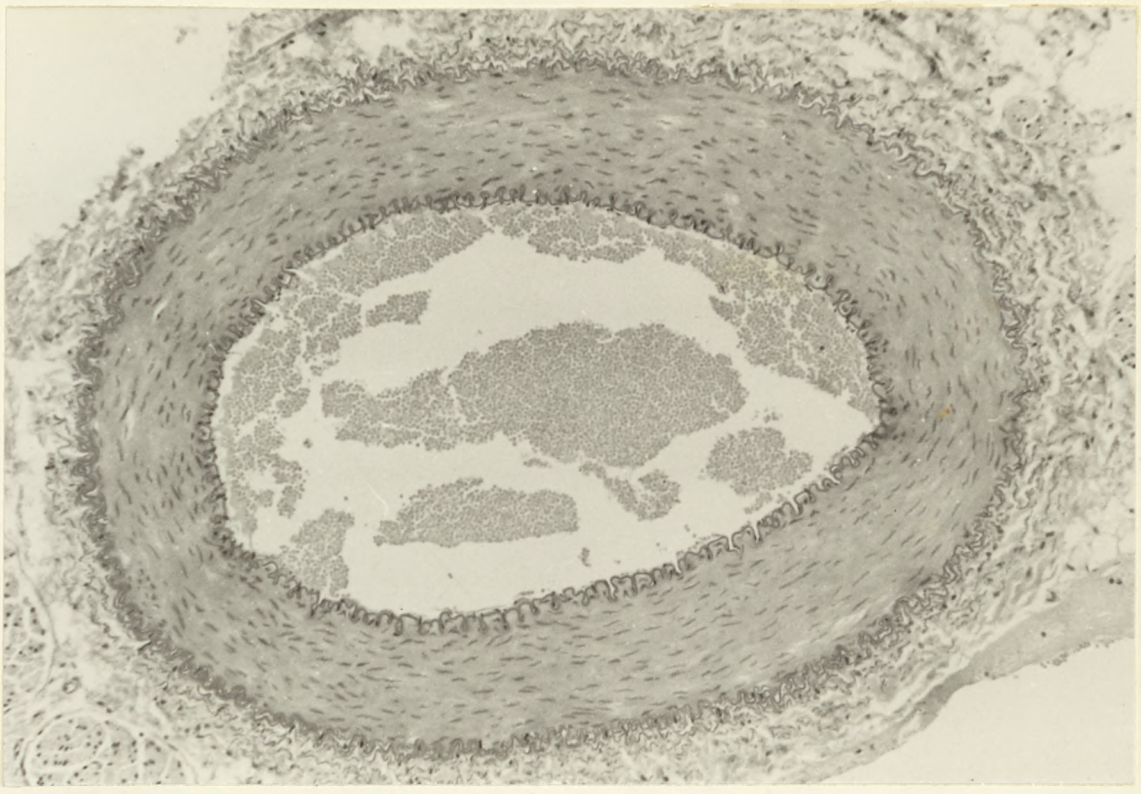
Table 2.9: Histological Changes

DIET	Intimal Thickening	Calcification	Inflammatory Reaction	Rupture of Elastica	Giant Cells
PsMO n=1	0	0	0	0	0
PsCO n=2	2	2	2	2	2
PDMO n=4	3	2	3	3	3
PDCO n=4	2	3	3	3	2

Table 2.10: Summary of Individual Results

Rabbit No.	DIET	Aorta Score	Final Antibody Titre	Haemagglutination Antibody Titre	Plasma Cholesterol (mmol/l)	Plasma Triglycerides (mg/l)	Presence of giant cells	Ca Content of tissue (μmol/g) Renal Art.	Aorta
1	PsMO	3	10	0	1.88	1500	-	32.5	92.5
9		1	11	0	0.91	300	-	/	/
10		2	11	1280	2.95	2390	-	5.0	65.0
11		3	15	0	2.46	2470	-	55.0	55.0
2	PsCO	3	16	320	3.59	1840	-	37.5	140.0
12		4	6	0	15.52	830	+	/	/
13		5	29	1280	5.53	2980	+	247.5	350.0
14		4	16	0	8.43	4420	-	30.0	165.0
5	PDMO	4	15	640	3.36	2850	+	27.5	680.0
15		3	25	80	1.73	1160	-	102.5	12.5
16		2	25	0	1.78	2180	+	22.5	45.0
17		5	30	640	7.03	6440	+	/	/
3	PDCO	3	23	80	6.47	590	+	/	/
18		3	10	80	3.98	630	-	120	1002
19		4	21	1280	2.30	1800	-	35	40.0
20		3	15	40	4.29	2570	+	60	785
6	STOCK	1	0	0	1.09	2100	-	20.0	12.5
7		1	0	0	3.85	1860	-	7.5	62.5

Underlined number representing high level.



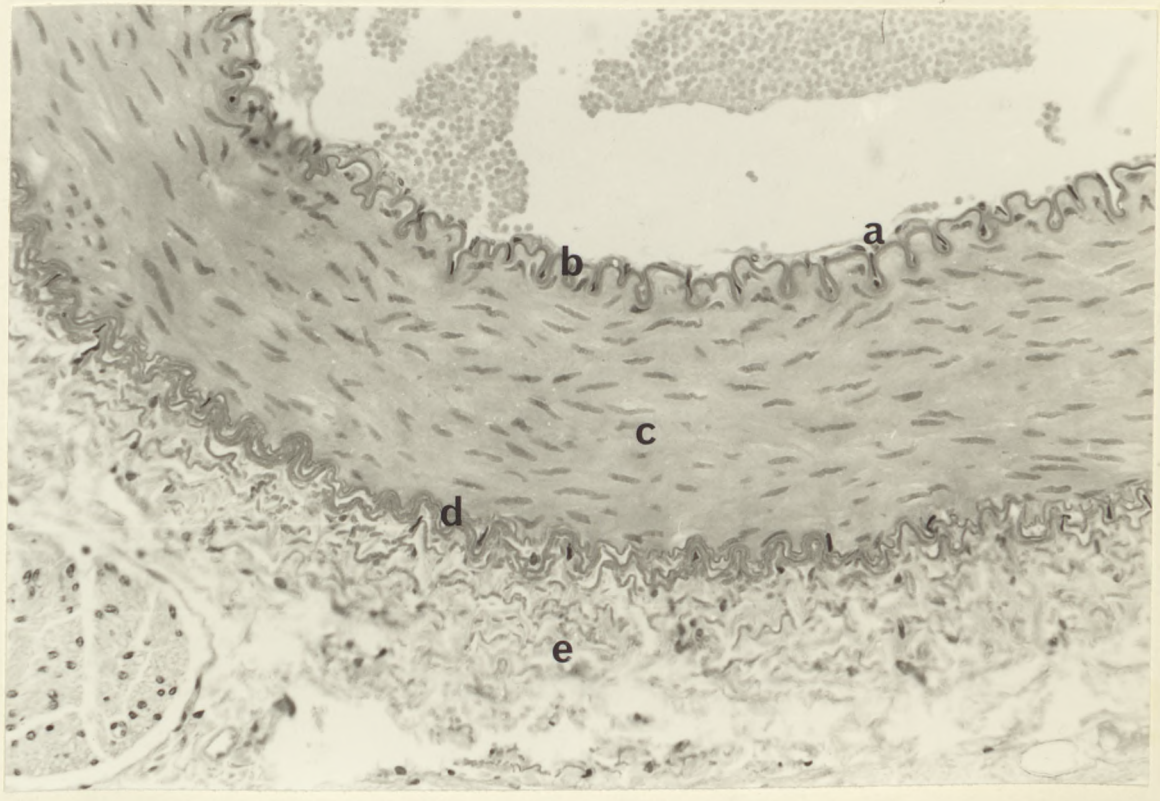
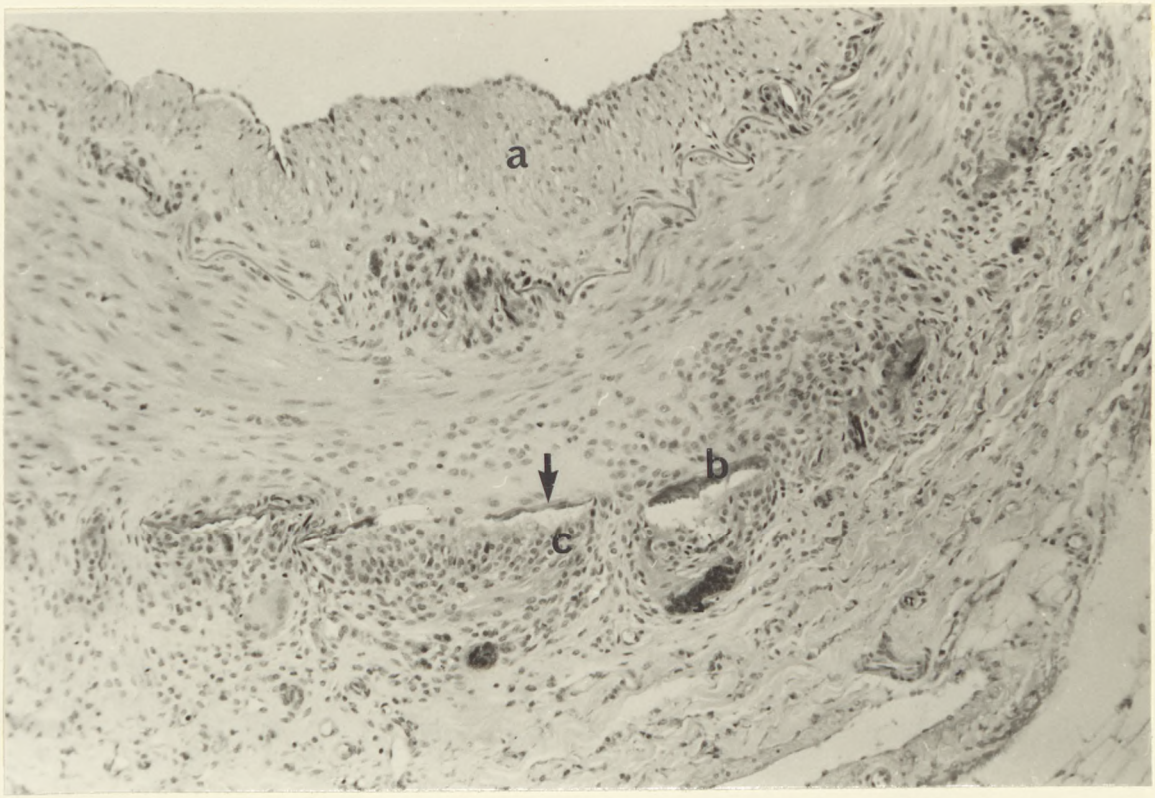


Fig 2.2 Normal renal artery from rabbit 7 fed on stock diet stained with:
A Haematoxylin and eosin (x140)
B Elastic van Gieson stain (x310). This section shows the endothelium (a), internal elastic lamella (b), muscular media (c), external elastic lamella (d) and adventitia (e).



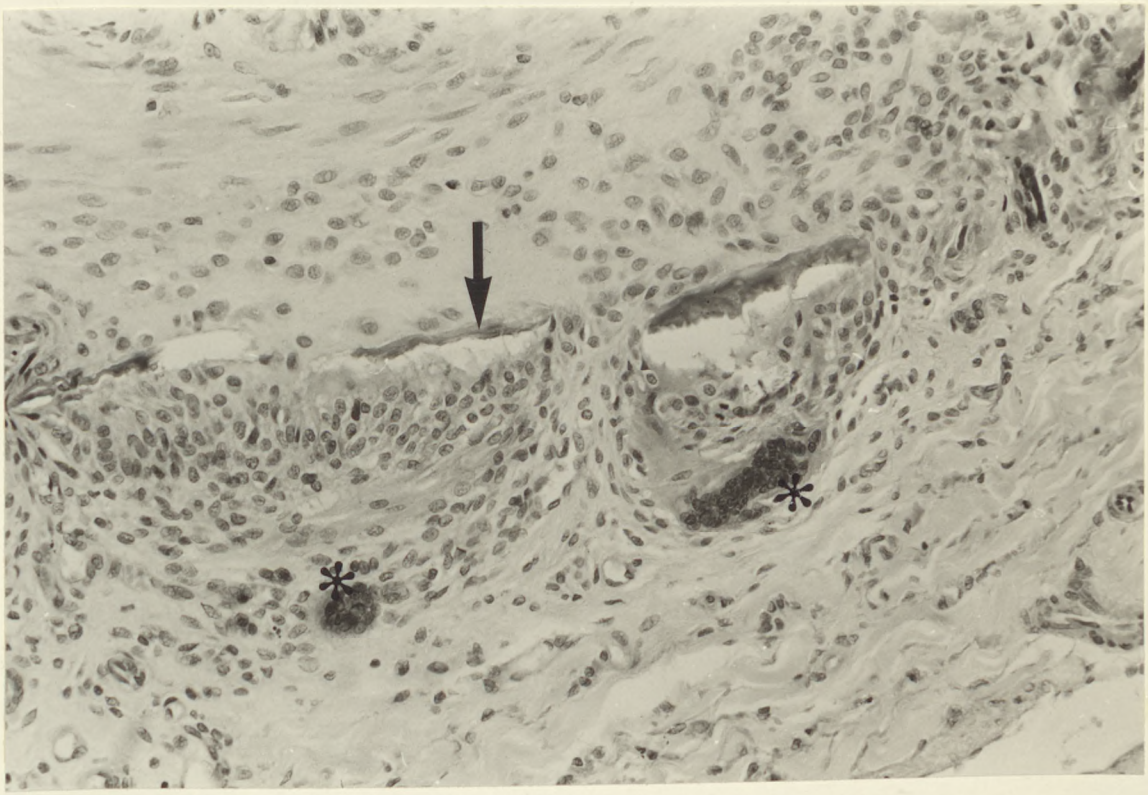


Fig 2.3 Renal artery from rabbit PDC0-20 stained with:
A Haematoxylin and eosin (x110). This section shows pronounced intimal thickening (a), calcification (b), an inflammatory cell infiltration (c) centred around the elastic lamella (arrowed).
B Haematoxylin and eosin (x330). A section showing the elastic lamella (arrowed) and giant cells (*).

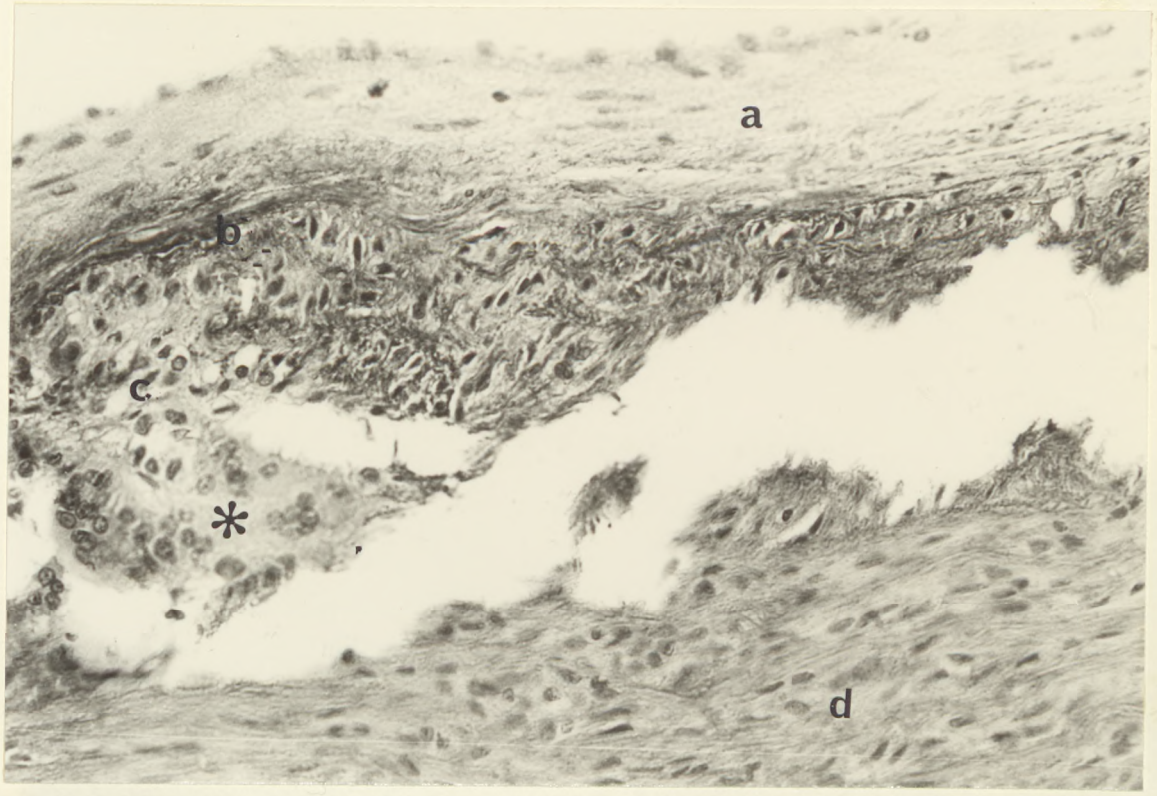


Fig 2.4 Aorta from rabbit PDMO-17 stained with haematoxylin and eosin (x330). A section showing slight intimal thickening (a), calcification around the superficial media (b), an inflammatory infiltrate (c) including giant cells (*) with a normal media (d).

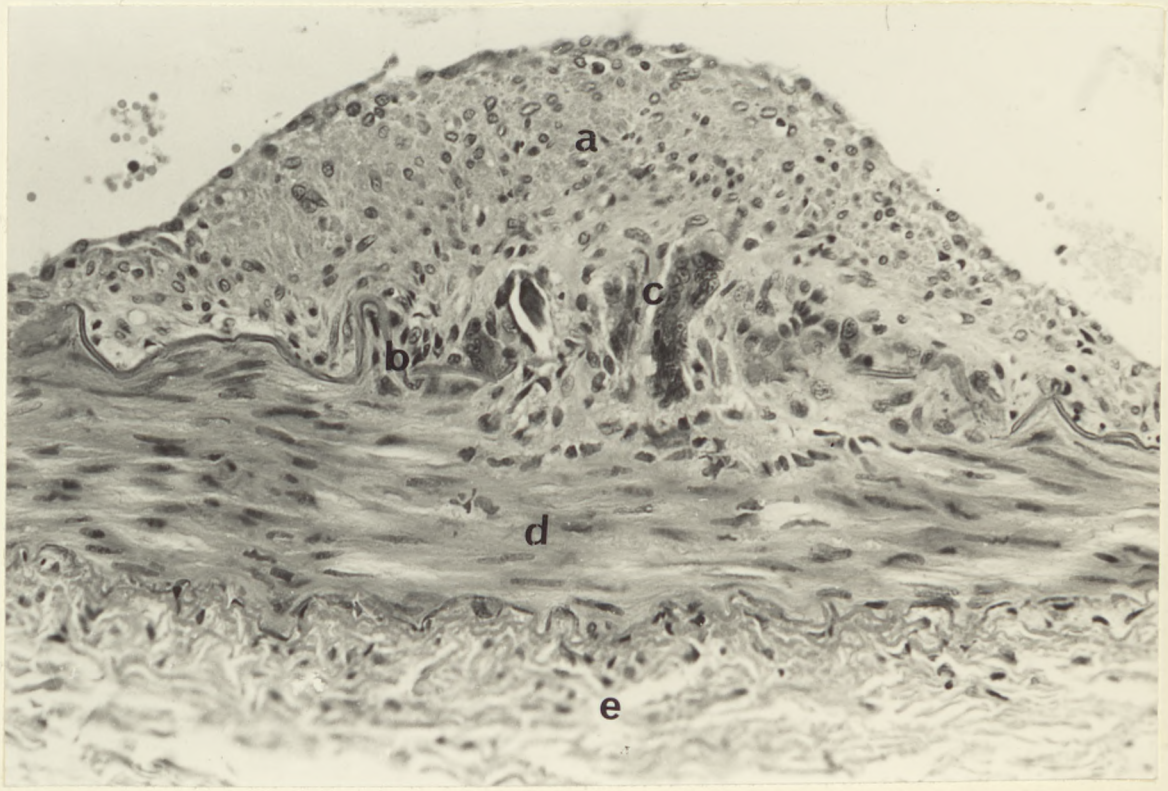


Fig 2.5 Carotid artery from rabbit PDMO-15 stained with haematoxylin and eosin (x340). This section shows focal intimal thickening (a), a fragmented internal elastic lamella (b), giant cells (c), a normal media (d) and adventitia (e).

Fig 2.6 Aorta from rabbit PDCO-3 stained with haematoxylin and eosin (x560). This section shows diffuse intimal thickening (i) with a dense inflammatory cell infiltrate in the media (*) with calcification of the residual elastic tissue (arrowed).

CHAPTER 3

EXPERIMENT 2

3.1 Introduction

Many questions remain unanswered from the results of the first experiment, in that no conclusive evidence could be shown for the cause of the extensive arterial disease. It was decided that the experiment should be repeated, by feeding to rabbits diets broadly similar to the ones used in Experiment 1, and examining more closely biochemical and histological events which take place during the development of arteriosclerosis.

3.2 Materials and Methods

Details of materials and methods are given in Appendix 1. Special note can be taken of the difference in the diets (App.1, Table 4) given in Experiment 2 compared to those given in Experiment 1 (App.1, Table 2). The vitamin D content was reduced from 2000iu. to 1000iu./kg diet as it seemed possible that the higher level of vitamin D might have been a factor in the calcification of some tissues in Experiment 1. Due to the high incidence of digestive disturbances in Experiment 1 the fibre content of the diet was increased from 150g bran/kg diet to 200g bran/kg diet. The diets were mixed and each one was given to five weanling rabbits as in Experiment 1. The biochemical analyses used in Experiment 1 were also used here (App.1, II, III, V). In addition to food antibodies, immune complexes (App.1, VIII) and total serum complement (App.1, VII) were measured. Serum calcium (App.1, V) was also monitored. In order to have a more detailed histological picture of the development of the arterial disease produced in rabbits by these dietary treatments the rabbits were to be sacrificed three, six, nine and twelve months after weaning onto the diets. The aorta scoring method (App.1, IX) was a modification of that used in the previous experiment in that the whole aorta from the aorta valves to the junction of the aorta with the iliac arteries was used. Organ weights were recorded to indicate the

health of various tissues. The animals were killed with a lethal dose of Nembutal (Sigma, London) and the tissues removed were processed as given in Appendix 1, X.

3.3 Results

The biochemical results for all four parts of this experiment showed similar trends, they were therefore pooled and the results from one part of the experiment are presented here as being representative of the whole experiment (the results from the other parts are given in App. 2). All the results were analysed statistically with the use of Analysis of Variance.

3.3.1 Food Intake, Weight Gain and Serum Calcium

It can be seen from Table 3.1 that the food intake, taken from the twelve month feeding trial, is similar in all four dietary treatments. Figure 3.1 (Table 3.2) shows the live weight gain, from the same feeding trial. It can be seen that all the rabbits gained weight at a good rate after weaning and reached a maximum weight at about five months after weaning, the weight gain being similar in all the treatments.

It can be observed (Fig. 3.2, Table 3.3) that there was a slight variation, from month to month, in the six month feeding trial in the serum calcium levels (2.4-3.0 mmol/l). However the levels were similar on all diets and there were no significant differences between treatments.

3.3.2 Plasma Cholesterol and Plasma Triglycerides

The plasma cholesterol levels from the nine month feeding trial are shown in Figure 3.3 (Table 3.4). The graph shows that from the first plasma sample taken at half a month after weaning the rabbits fed CO had significant higher plasma cholesterol levels than the MO-fed rabbits ($p<0.001$). It can also be seen that cholesterol levels in CO-fed animals remained higher throughout the feeding trial ($p<0.001$).

Plasma triglyceride levels from the nine month feeding trial are shown in Figure 3.4 (Table 3.5). There was a decline of plasma triglycerides in all four treatments for the first five months after weaning followed by a small increase before a constant level was attained. There were no significant differences in plasma triglyceride levels in the first month; however, a difference was observed in the second ($p<0.01$) third ($p<0.001$) fourth ($p<0.01$) and fifth ($p<0.01$) in that the CO-fed animals had significantly higher plasma triglyceride levels than the MO-fed animals.

3.3.3 Immunological Parameters

Food antibodies titres for the twelve month feeding trial are given in Figure 3.5 (Table 3.6). There were large variations from month to month especially in PD-CO fed rabbits and significant differences between treatments appeared only for months ten and eleven, in which the food antibodies in the rabbits fed PD were significantly higher than in those fed Ps ($p<0.05$ in both cases). The occurrence of antibodies to food proteins is greater in rabbits fed PD (70%) than in those fed Ps (27.5%). Levels of soluble immune complexes in the sera during the nine month feeding trial are shown in Figure 3.6 (Table 3.7). The immune complexes did not appear in the sera until the rabbits had been fed the diet for one month. Immune complexes increased gradually from the end of the first month in the groups fed PD and from the middle of the second month in the rabbits fed Ps. Significant differences were found between the two proteins for four and a half months between month 2.5 and month 7, the rabbits fed PD producing higher concentrations than those fed Ps. The level of significance was greatest ($p<0.001$) at three months and then fell off subsequently, until at the seventh month the

level of significance was $p<0.05$. There were no significant differences between the four treatments in the eighth and subsequent months.

The total serum complement levels are shown in Figure 3.7 (Table 3.8) from the nine month trial. The serum complement levels remained constant throughout the first seven months on the diet and then they appeared to rise slightly. No continuous significant differences were noted between the four treatments except for the seventh month where the rabbits fed MO had higher complement levels than those fed CO ($p<0.05$). This difference was not repeated in the following months.

3.3.4 Aorta Scores

The atherosclerotic scores for all three feeding trials that were terminated by killing the rabbits are given in Table 3.9. The rabbits which were fed the diets for only three months did not show any extensive lesions. Only one rabbit had a small lesion and this was in the group fed PD-CO. Rabbits were not killed after six months on the diets as those rabbits killed after nine months on the diet exhibited little evidence of an advanced disease. The nine and twelve month feeding trials both produced some lesions but generally the disease was not extensive. There were no significant differences between the four treatments in either of these two feeding trials.

3.3.5 Organ Weights and Liver Lipids

As an indication of the health and weights of selected organs were recorded; these are shown in Table 3.10 from the nine month trial. The weights for the right kidney,heart, spleen and adrenals were similar in all the rabbits and ranged from 8.5-9.8g,7.3-8.9g, 1.5-2.2g and 0.5-0.95g respectively. The rabbits fed CO had increased liver weights ($p<0.001$) ranging from 101-117g whereas those fed MO were normal, ranging from 77-82g weight.

The liver lipids from the twelve month feeding trial are shown in Table 3.11. There were no significant differences in the liver cholesterol between the four treatments. In contrast the liver triglycerides were higher in rabbits fed CO ($p<0.001$) than in those fed MO.

3.3.6 Tissue Calcium

Table 3.12 shows the tissue calcium levels recorded from the twelve month feeding trial except for aortic calcium values which are derived from the nine month feeding trial. The calcium contents of the carotid artery (5.17-9.67 µmol/g tissue), the renal artery (31.0-79.9 µmol/g) and kidney (1.45-4.52 µmol/g) were similar and no significant differences occurred between treatments. No significant differences could be detected in aorta calcium.

3.3.7 Relationship Between Aortic Scores And Other Experimental Data

The product moment correlation coefficient was calculated from the final data collected as an indication of any relationship between the amount of arterial disease and other experimental parameters. Table 3.13 shows that final live weight gain gives a negative correlation in both experiments although these are not significant. Also shown in Table 3.13 are the correlation coefficients between scores and organ weights. It can be seen that heart and spleen weights cannot be correlated with aorta scores. The liver weights showed a positive correlation with aorta score ($p<0.05$) in the twelve month feeding trial but in the nine month experiment this correlation was not significant, reflecting the fact that liver weight increases with advancing arterial disease. Adrenal weights were significantly increased ($p<0.001$) with advancing arterial disease in the nine month but not in the twelve month feeding trial.

Table 3.14 gives the relationship between aorta scores and lipid levels. Plasma cholesterol levels increased with the development of lesions in both feeding trials ($p<0.001$). Plasma triglycerides however only showed a positive correlation with aorta scores in the nine month feeding trial ($p<0.05$). The liver cholesterol and triglyceride levels were not significantly correlated to aorta scores.

Table 3.15 records the relationship between aorta scores and the immunological estimations. Food antibodies were not significantly correlated with advancing disease. Immune complexes on the other hand showed a strong positive correlation with aorta score in the nine month trial ($p<0.001$) and a positive correlation which was not significant in the twelve month feeding trial. Serum complement showed no correlation with aorta scores in either of the feeding trials.

The relationship between calcium determinations and aorta scores is shown in Table 3.16. No correlation was found between serum calcium levels and aorta score. With tissue calcium differences occurred between the two feeding trials. In the twelve month feeding trial aorta score did not show a relationship with the calcium content of any of the tissues. However, in the nine month feeding experiment a positive correlation was found between aorta scores and the calcium content of the carotid artery ($p<0.001$), the kidney ($p<0.001$) and the aorta ($p<0.001$). The calcium content of the renal artery did not show a correlation with aorta scores. The sex of the rabbits did not influence the amount of arterial disease produced. The male rabbits had a mean aorta score of 11.37 ± 0.96 and the females a mean score of 14.5 ± 1.8: these were not statistically different.

3.3.8 Histological Observations

The rabbits in the three month feeding trial had entirely normal arteries (Fig. 2.2) except for one rabbit fed PD-CO. This rabbit had a minimal foam cell accumulation and calcification in the subintimal region of the media in the carotid artery. The renal artery from this rabbit fed PD-CO was normal.

The animals fed the diets for nine months showed overall the most atheromatous disease. Some of the rabbits had firm depressed atheromatous areas with areas of normal aorta (Fig. 3.8), an aorta from one rabbit (Fig. 3.9) showed marked atherosclerotic fleckening.

From this group representative sections were examined from 1) the renal arteries of all the animals; 2) the carotid arteries of all those animals whose aortae contained atheromatous plaques; 3) the aortae of these same animals; 4) the kidneys and hearts of all the animals fed PD and of selected ones from those fed Ps.

A summary of the arterial histology can be seen in Table 3.17. The majority of the sections were entirely normal (as in Fig. 2.2). In occasional animals minimal intimal thickening and reduplication of the internal elastic lamellae could be seen. In two rabbits, one fed PDMO (Fig. 3.10) and one fed PDCO (Fig. 3.11), there could be seen more advanced intimal thickening, disruption of the internal elastic lamella and a slight associated inflammatory infiltrate (Fig. 3.12). In two rabbits one fed PDCO (Fig. 3.13) and one fed PDMO a single focus of giant cells was seen.

The histological appearances of the majority of lesions of the aortae were similar in all four dietary treatments. The plaques generally showed focal fibrous intimal thickening, underlying calcification of the elastic lamellae and small numbers of intimal or superficial medial foam

cells. The changes were similar to lesions previously reported as spontaneous atherosclerosis. However, in two of the animals a significant inflammatory infiltrate was also seen which included mononuclear (Fig. 3.14) and giant cells (Fig. 3.15B).

Occasional animals from all groups showed small foci of dystrophic mineralisation at the cortico-medullary junction of the kidney. This is a normal finding in the Southampton University laboratory colony. In two of the rabbits, those fed PD showing giant cells in the arteries, marked calcification was present. Most of the deposits were centred around the proximal renal tubules in the cortex and at the cortico-medullary junction. There was no evidence of glomerular disease in any animal. None of the animals showed myocardial changes. One rabbit fed PDCO had marked intimal thickening in the coronary artery, this rabbit also showed significant changes in other arteries (Table 3.17). All other animals had normal coronary arteries.

Sections of some tissues from rabbits at the end of the twelve month feeding trial were also examined histologically.

The arteries examined from this group were all normal. Representative sections of aortae were examined from all the animals that showed more than one diseased area. The histological changes did not vary significantly from group to group. The changes noted included: focal calcification of the superficial medial elastic tissue with slight fibrous intimal thickening. Occasional clusters of sub-intimal mononuclear cells were seen in two animals, one fed PsMO and one fed PDMO. It must be stressed that the degree of intimal thickening was only slight and that there was no evidence of a giant cell reaction.

The renal histology was normal and no myocardial changes were noted. All animals showed normal coronary arterial histology.

3.4 Discussion

An arterial disease with hypercholestolaemia was once again produced in rabbits by feeding cholesterol-free, semi-synthetic diets with soya proteins as the protein sources. The plasma cholesterol levels in animals fed CO were elevated (4.79-7.59 mmol/l) compared to the ones fed MO (1.5-2.5 mmol/l). This agrees with evidence that CO produces hypercholesterolaemia in rabbits.[47,28,184,186] CO has also been shown to produce hypertriglyceridaemia,[184,187] and here the plasma triglyceride levels from CO-fed rabbits were significantly higher than in rabbits fed MO for quite a long time during the feeding trial (Table 3.5).

The aim of this experiment was to look more closely at immunological events which occur during the development of the arterial disease. However as no disease was produced in the three month feeding trial and since no progression in the disease process occurred from the nine to the twelve month feeding trial no definite conclusions can be drawn about the relationship between the immunological events and the arterial disease.

The incidence of soya antibodies was greater in the PD than in the Ps rabbits. However, compared to the results obtained in Experiment 1 using the identical haemagglutination technique it can be seen that the titres were lower in Experiment 2. The reason for this remained unclear until the serum of some stock animals, that had been born one year previously, were examined for the presence of antibodies to soya. It was found that they had just detectable levels in their serum, and analysis of the stock diet used at that time was made. It was found that the mothers of the rabbits used in Experiment 1 were fed on a stock diet (App.1, Table 1) which used fish meal as the protein source whereas the rabbits used in Experiment 2 had mothers that were fed on a stock

diet (App.1, Table 3) which had soya as a protein source. It might be suggested that in the rabbits in Experiment 2, which had already been exposed nutritionally to soya protein, a state of immune tolerance existed.[197]

Despite this immune complexes assayed with the anticomplementary assay[198] were detected in the latter rabbits. Immune complexes are known to activate the complement system,[114] and so an increase in immune complexes levels might be associated with an increase in serum complement levels. However, the serum complement levels remained relatively constant throughout the nine month feeding trial.

The immune complex levels were strongly correlated with the advancing atherosclerosis in the nine month feeding trial in which the most advanced lesions occurred. This correlation was not reproduced in the twelve month part, in which the disease was less extensive.

In three of the rabbits (Table 3.19) there was evidence of a scanty giant cell infiltrate. However, these were either a small focus of giant cells or a single giant cell. This again suggests that since there was a reduction in the immune response compared to Experiment 1 giant cell arteritis might have an immunological origin in that immunofluorescent techniques have shown immunoglobulin deposits along the elastic lamella in arteries with temporal arteritis.[240] Two of the rabbits which had giant cells in their arteries also had a high atherosclerosis score (Table 3.18). All three had high levels of circulating immune complexes and one of them (PDCO-8) had high levels of plasma cholesterol and triglycerides. The two with giant cells in the PDMO group had food antibodies but compared to Experiment 1 (Table 2.10) these were not particularly high. The rabbits with the highest aorta scores also had high levels of plasma cholesterol (except PDMO-10) and immune complexes and one had a very high plasma triglyceride level (PDCO-8). The levels of food antibodies were low with only 30% of the rabbits in this feeding trial having food antibodies at the end of the experiment.

The content of calcium in the tissues of the rabbits in Experiment 2 (nine months) did not differ significantly from that in Experiment 1 except for the kidneys (Expt.1: 8.13 ± 1.63 µg/g; Expt.2:66.5 ± 16.1 µg/g ($p<0.001$)). The dietary vitamin D levels in Experiment 2 were 1000 iu vitamin D/kg diet compared to 2000 iu/kg in Experiment 1. This did not cause a reduction in arterial calcification. Therefore, the calcification in Experiment 1 was not the result of hypervitaminosis D-induced atherosclerosis.[194] The excessive calcification noted in the renal arteries and aortae of some of the animals in Experiment 1 (Table 2.10) might have been the result of advancing atherosclerosis.[1,2,3]

A limited amount of arterial disease was produced, however, and this could be attributed to increased immune complexes and high plasma cholesterol and triglyceride levels produced by feeding high fat, (cholesterol-free) semi-synthetic diets. No conclusions can be drawn as to the relative importance of these injurious agents as they all increased at the same time during the experiment, well before any arterial disease was detected.

GRAPHS AND TABLES

Legend for graphs

Symbol	Diet
O	Promosoy 100 and maize oil (PsMO)
●	Promosoy 100 and coconut oil (PsCO)
Δ	Promine D and maize oil (PDMO)
▲	Promine D and coconut oil (PDCO)

Table 3.1: Food Intake (kcal/$kg^{0.75}$/week)

DIET	TIME (MONTHS) 0	½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	6.6 ±0.9	6.23 ±0.8	6.65 ±0.1	4.72 ±0.2	5.29 ±0.3	5.19 ±0.2	4.88 ±0.7	3.98 ±0.2	4.09 ±0.4	4.45* ±0.5	4.48 ±0.4	3.96 ±0.3	3.71 ±0.5	3.67 ±0.3
PsCO	5.88 ±0.5	7.21 ±0.5	6.26 ±0.7	5.36 ±0.3	6.22 ±0.96	6.91 ±0.9	4.11 ±0.4	3.76 ±0.2	4.20 ±0.3	4.56* ±0.4	4.51 ±0.3	2.85 ±0.5	3.81 ±0.2	3.62 ±0.4
PDMO	6.77 ±0.7	7.77 ±0.4	5.53 ±0.4	4.43 ±0.2	4.62 ±0.2	5.33 ±0.2	4.39 ±0.3	3.8 ±0.2	4.13 ±0.2	2.89 ±0.6	4.21 ±0.3	3.91 ±0.8	4.46 ±0.3	3.94 ±0.5
PDCO	6.39 ±1.6	7.16 ±0.4	6.91 ±0.2	4.96 ±0.6	6.04 ±0.6	5.78 ±0.6	4.82 ±0.6	3.74 ±0.2	4.39 ±0.3	3.61 ±0.4	3.96 ±0.1	3.27 ±0.5	3.35 ±0.2	3.31 ±0.5

* Significantly greater than PD means ($p<0.05$)

Table 3.2: Live Weight (kg)

DIET	TIME (MONTHS) 0	½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	1.27 ±0.03	1.66 ±0.1	2.23 ±0.1	3.06 ±0.1	3.51 ±0.1	3.85 ±0.2	4.06 ±0.2	4.1 ±0.2	4.2 ±0.3	4.24 ±0.3	4.29 ±0.3	4.33 ±0.2	4.36 ±0.2	4.46 ±0.2
PsCO	1.4 ±0.02	1.79* ±0.05	2.17 ±0.1	3.08 ±0.1	3.51 ±0.1	3.76 ±0.1	3.96 ±0.1	4.0 ±0.1	4.05 ±0.1	4.08 ±0.1	4.16 ±0.1	4.19 ±0.1	4.2 ±0.1	4.19 ±0.1
PDMO	1.32 ±0.04	1.69 ±0.04	2.24 ±0.05	3.22 ±0.06	3.68 ±0.1	3.92 ±0.1	4.18 ±0.1	4.32 ±0.15	4.35 ±0.2	4.37 ±0.2	4.44 ±0.2	4.44 ±0.2	4.45 ±0.2	4.43 ±0.2
PDCO	1.49 ±0.1	1.89* ±0.1	2.39 ±0.1	3.25 ±0.2	3.67 ±0.2	3.98 ±0.2	4.21 ±0.2	4.3 ±0.3	4.38 ±0.25	4.44 ±0.3	4.43 ±0.3	4.33 ±0.2	4.23 ±0.2	4.21 ±0.2

* Significantly greater than MO means ($p<0.05$)

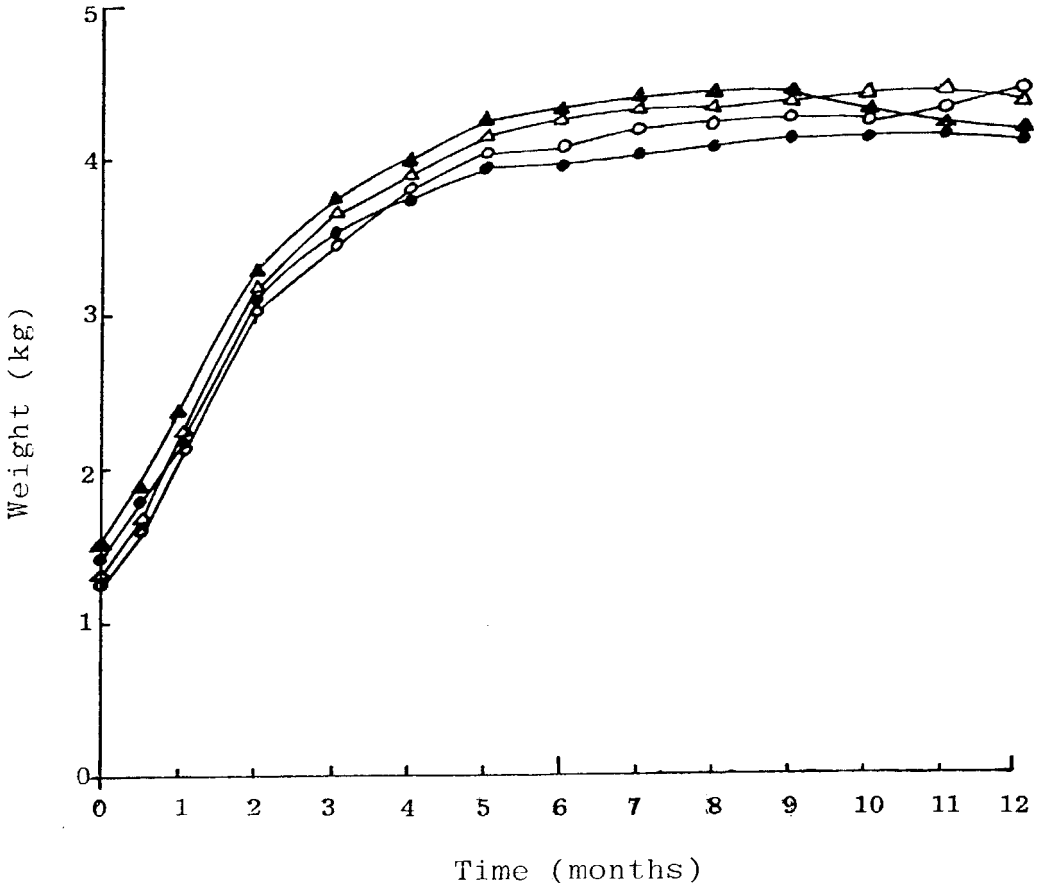


Figure 3.1: Weight Gain

Table 3.3: Serum Calcium (mmol/l)

DIET	TIME (MONTHS) 0	1	2	3	4	5	6
PsMO	2.97 ±0.27	2.87 ±0.3	2.77 ±0.12	2.78 ±0.1	2.62 ±0.3	2.69 ±0.2	2.71 ±0.2
PsCO	2.75 ±0.22	2.77 ±0.22	2.82 ±0.12	2.90 ±0.20	2.50 ±0.10	2.67 ±0.1	2.69 ±0.1
PDMO	2.55 ±0.2	2.57 ±0.2	2.66 ±0.1	2.8 ±0.07	2.7 ±0.1	2.71 ±0.05	2.73 ±0.05
PDCO	2.57 ±0.1	2.75 ±0.15	2.79 ±0.02	2.92 ±0.1	2.38 ±0.05	2.55 ±0.1	2.61 ±0.05

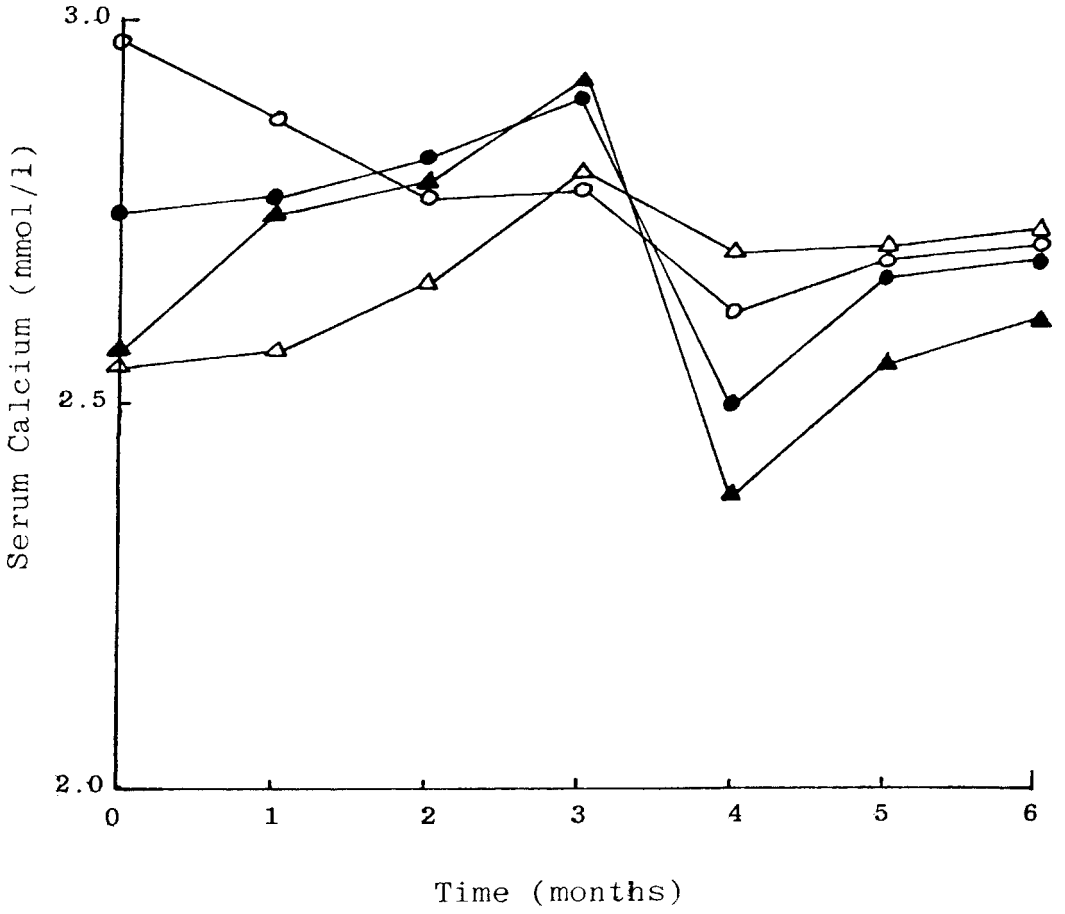


Figure 3.2: Serum Calcium

Table 3.4: Plasma Cholesterol (mmol/l)

DIET	TIME (MONTHS) ½	1	1½	2	2½	3	4	5	6	7	8	9
PsMO	1.89 ±0.2	1.89 ±0.1	1.76 ±0.13	2.04 ±0.27	1.63 ±0.22	1.23 ±0.17	1.33 ±0.19	1.55 ±0.36	1.76 ±0.29	.2.75 ±0.55	1.67 ±0.33	1.5 ±0.4
***PsCO	4.91 ±0.47	5.54 ±0.3	5.13 ±0.7	6.12 ±0.8	4.88 ±0.54	4.41 ±0.35	5.73 ±0.98	3.95 ±0.47	4.34 ±0.96	3.28 ±0.52	6.49 ±0.73	4.79 ±0.4
PDMO	2.24 ±0.18	2.16 ±0.2	1.94 ±0.25	2.17 ±0.18	2.10 ±0.32	1.50 ±0.17	1.81 ±0.13	1.43 ±0.24	1.71 ±0.19	2.68 ±0.5	2.30 ±0.53	2.51 ±0.78
***PDCO	3.72 ±0.34	3.34 ±0.36	3.87 ±0.3	4.94 ±0.49	5.14 ±0.76	4.58 ±0.64	6.32 ±0.92	5.66 ±1.18	6.89 ±1.45	9.73 ±1.63	7.03 ±1.37	7.59 ±2.36

***CO is significantly higher throughout experiment ($p<0.001$)

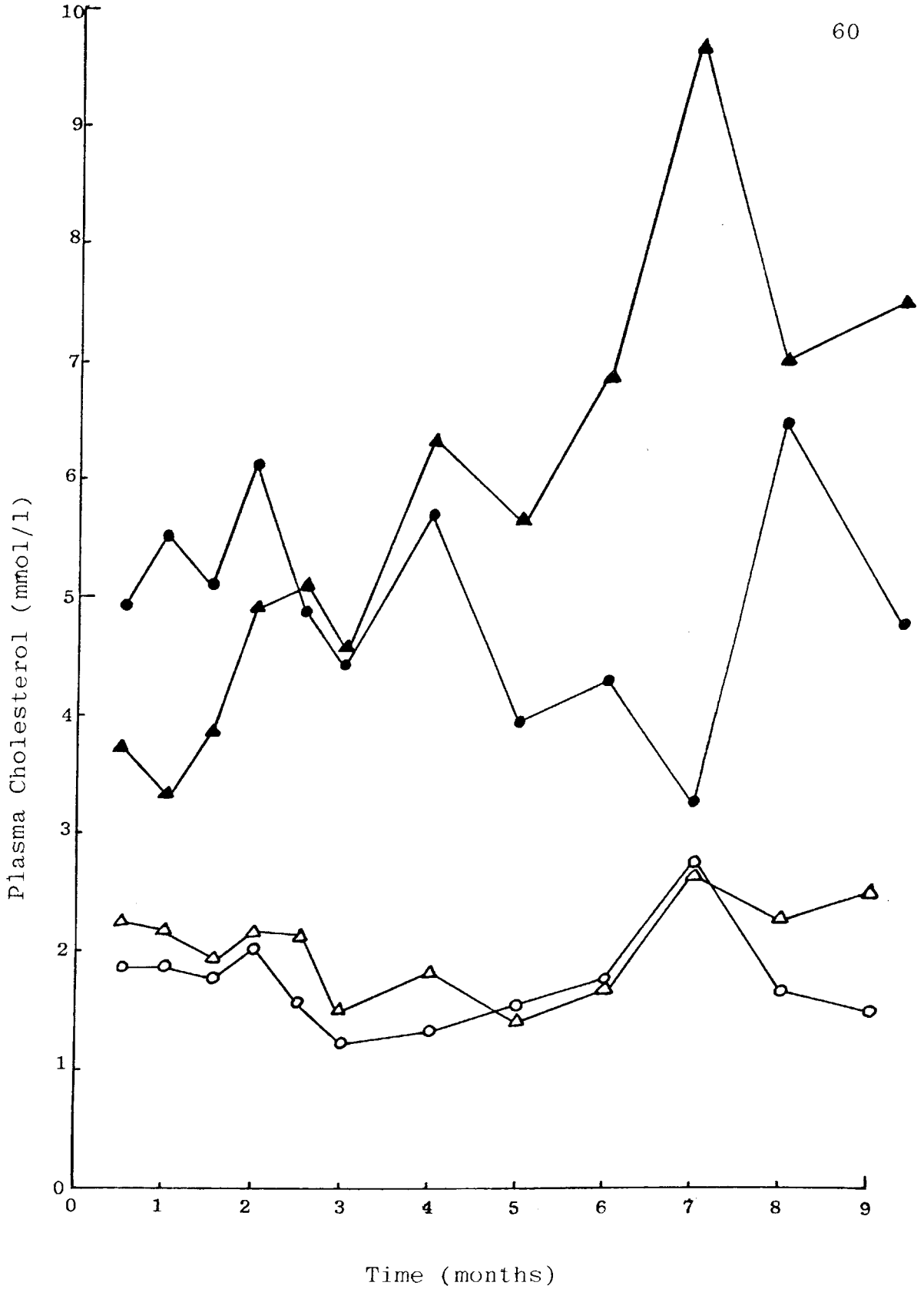


Figure 3.3: Plasma Cholesterol

Table 3.5: Plasma Triglycerides (mg/l)

DIET	TIME (MONTHS) ½	1	1½	2	2½	3	4	5	6	7	8	9
PsMO	772.4 ±246	670.0 ±108	274.2 ±35	286.2 ±18	290.1 ±17	210.7 ±18	104.4 ±18	160.1 ±33	182.0 ±38	218.4 ±50	244.3 ±13	286.1 ±5.0
PsCO	668.3 ±49	748.2 ±87	380.3* ±57	490.1** ±99	454.0** ±81	442.6*** ±100	344.6** ±99	320.5** ±84	464.1 ±75	540.1 ±75	378.1 ±36	385.2 ±58
PDMO	600.1 ±26	522.1 ±35	288.1 ±14	286.3 ±47	237.9 ±42	146.8 ±16	106.7 ±22	125.9 ±18	368.3 ±79	444.3 ±105	284.2 ±35	322.2 ±30
PDCO	696.5 ±49	606.3 ±48	354.2* ±21	388.6** ±36	336.8** ±36	340.2*** ±37	248.6** ±85	252.4** ±23	430.4 ±147	558.8 ±225	462.5 ±152	287.5 ±31

*
**Significantly higher than corresponding MO means ($p<0.05$)
***Significantly higher than corresponding MO means ($p<0.01$)
Significantly higher than corresponding MO means ($p<0.001$)

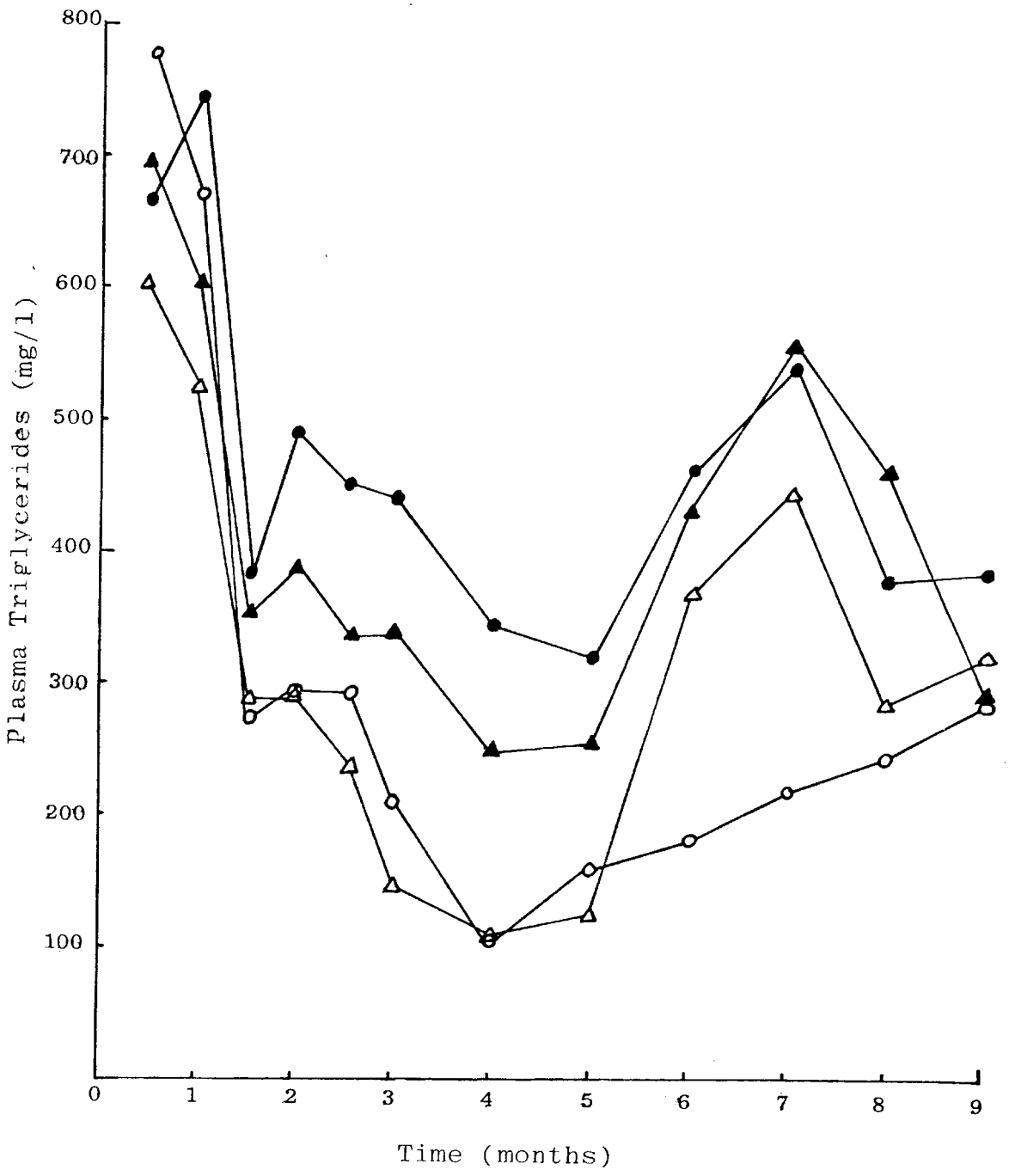


Figure 3.4: Plasma Triglycerides

Table 3.6: Food Antibody Titres

DIET	TIME (MONTHS) ½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	0	0	0	0	0	0	0	0	20.0 ±15.5	6.0 ±4.0	4.0 ±4.0	8.0 ±8.01	4.0 ±4.0
PsCO	14.0 ±6.8	0	2.0 ±2.01	0	8.0 ±8.01	8.0 ±8.01	8.0 ±8.01	8.0 ±4.9	4.0 ±2.5	10.0 ±7.7	4.0 ±4.0	16.0 ±16.01	8.0 ±8.01
PDMO	0	0	20.0 ±15.5	0	8.0 ±8.01	8.0 ±8.01	8.0 ±8.01	20.0 ±15.5	16.0 ±9.8	48.0 ±32	96.0* ±64	384.0* ±256	80.0 ±62
PDCO	66.0 ±64	0	8.0 ±8.01	0	192.0 ±128	192.0 ±128	96.0 ±64	64.0 ±39	26.0 ±15	104.0 ±61	144.0* ±73	290.0* ±146	64.0 ±30

* Significantly higher than corresponding Ps means ($p<0.05$)

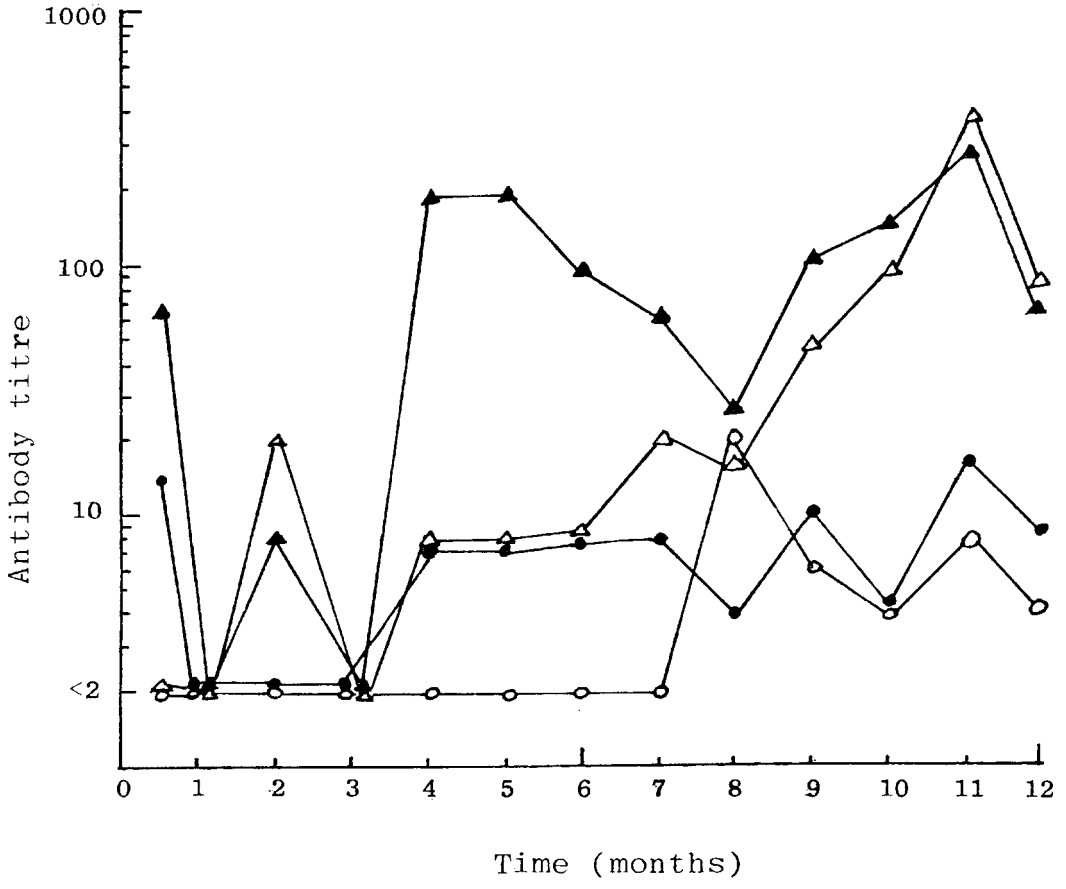


Figure 3.5: Food Antibodies

Table 3.7: Immune Complexes (CH50 units consumed)

DIET	TIME (MONTHS) ½	1	1½	2	2½	3	4	5	6	7	8	9
PsMO	0	0	0.8 ±0.1	0.52 ±0.2	0.48 ±0.08	0.26 ±0.08	0.57 ±0.2	0.67 ±0.15	0.68 ±0.1	0.94 ±0.1	1.1 ±0.05	1.17 ±0.05
PsCO	0	0	0.8 ±0.2	0.76 ±0.1	0.51 ±0.15	0.48 ±0.1	0.6 ±0.09	0.63 ±0.09	0.81 ±0.1	0.79 ±0.1	0.9 ±0.16	1.09 ±0.01
PDMO	0	0	0.97 ±0.16	0.88 ±0.2	0.87** ±0.2	0.85*** ±0.17	1.002** ±0.2	0.99** ±0.27	1.08* ±0.25	1.11* ±0.24	1.17 ±0.17	1.35 ±0.1
PDCO	0	0	0.85 ±0.1	0.87 ±0.15	0.896** ±0.14	1.08*** ±0.15	1.19** ±0.2	1.29** ±0.2	1.32* ±0.2	1.48* ±0.2	1.5 ±0.25	1.3 ±0.2

* Significantly higher than corresponding Ps means ($p<0.05$)
** Significantly higher than corresponding Ps means ($p<0.01$)
*** Significantly higher than corresponding Ps means ($p<0.001$)

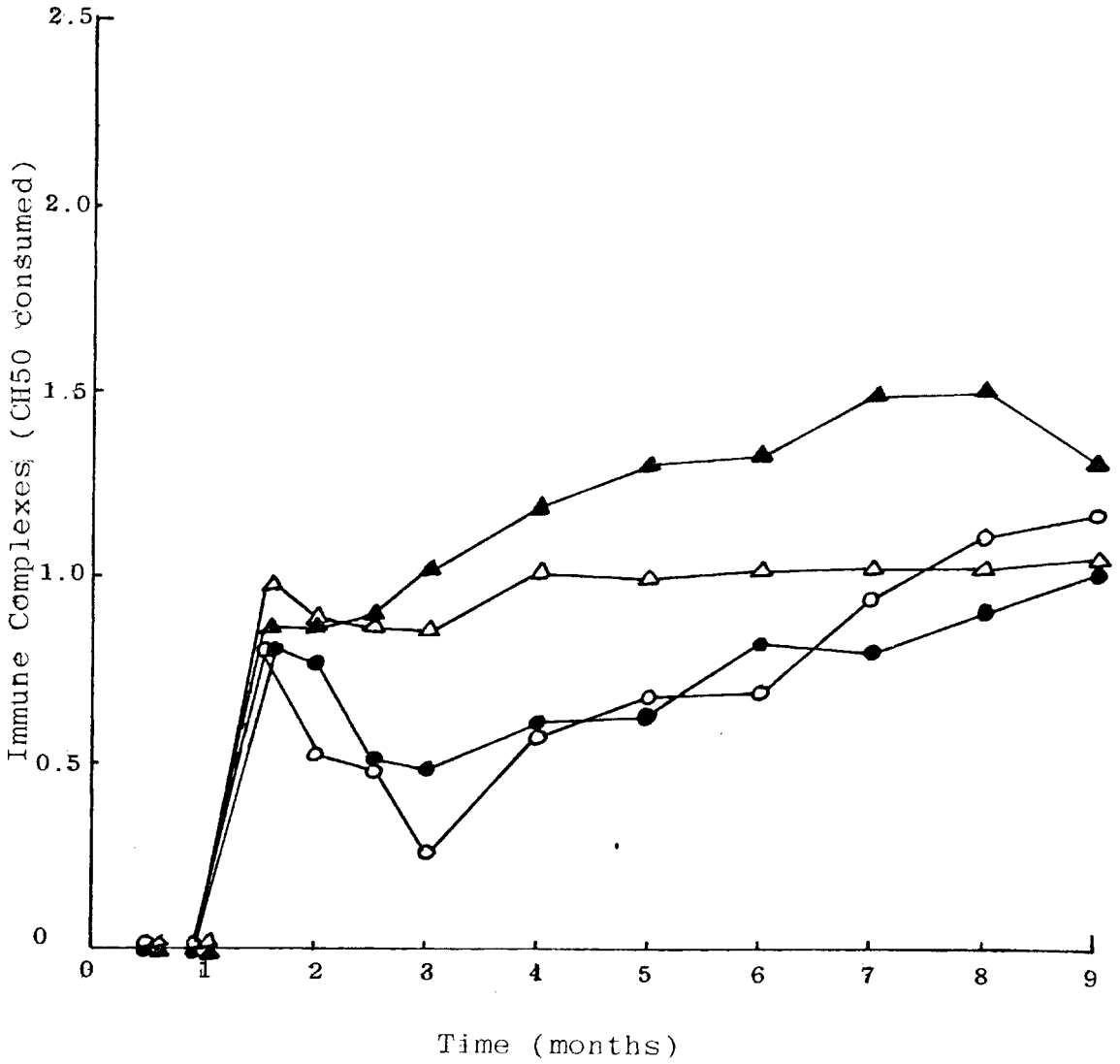


Figure 3.6: Immune Complexes

Table 3.8: Total Serum Complement (CH50/ml)

DIET	TIME (MONTHS) ½	1	1½	2	2½	3	4	5	6	7	8	9
PsMO	11.15 ±0.2	9.45 ±0.05	9.98 ±0.3	10.2 ±0.3	10.27 ±0.2	10.55 ±0.2	10.87 ±0.3	11.27 ±0.3	11.6 ±0.4	12.62* ±1.3	15.22 ±0.5	15.82 ±0.6
PsCO	10.75 ±0.4	9.42 ±0.1	10.68 ±0.5	10.87 ±0.4	11.22 ±0.5	11.52 ±0.4	11.87 ±0.5	12.32 ±0.8	13.12 ±0.9	11.07 ±0.9	20.98 ±11	19.8 ±5.7
PDMO	13.0 ±1.4	9.82 ±0.4	10.02 ±0.2	10.22 ±0.2	10.5 ±0.2	10.77 ±0.3	11.12 ±0.4	11.5 ±0.5	11.67 ±0.6	16.35* ±2.4	15.8 ±0.7	16.38 ±0.8
PDCO	11.3 ±0.2	9.52 ±0.1	9.82 ±0.05	10.05 ±0.05	10.25 ±0.11	10.85 ±0.1	11.25 ±0.2	11.77 ±0.3	12.97 ±0.9	11.52 ±0.7	19.15 ±3.1	18.8 ±2.3

* Significantly higher than corresponding CO means ($p<0.05$)

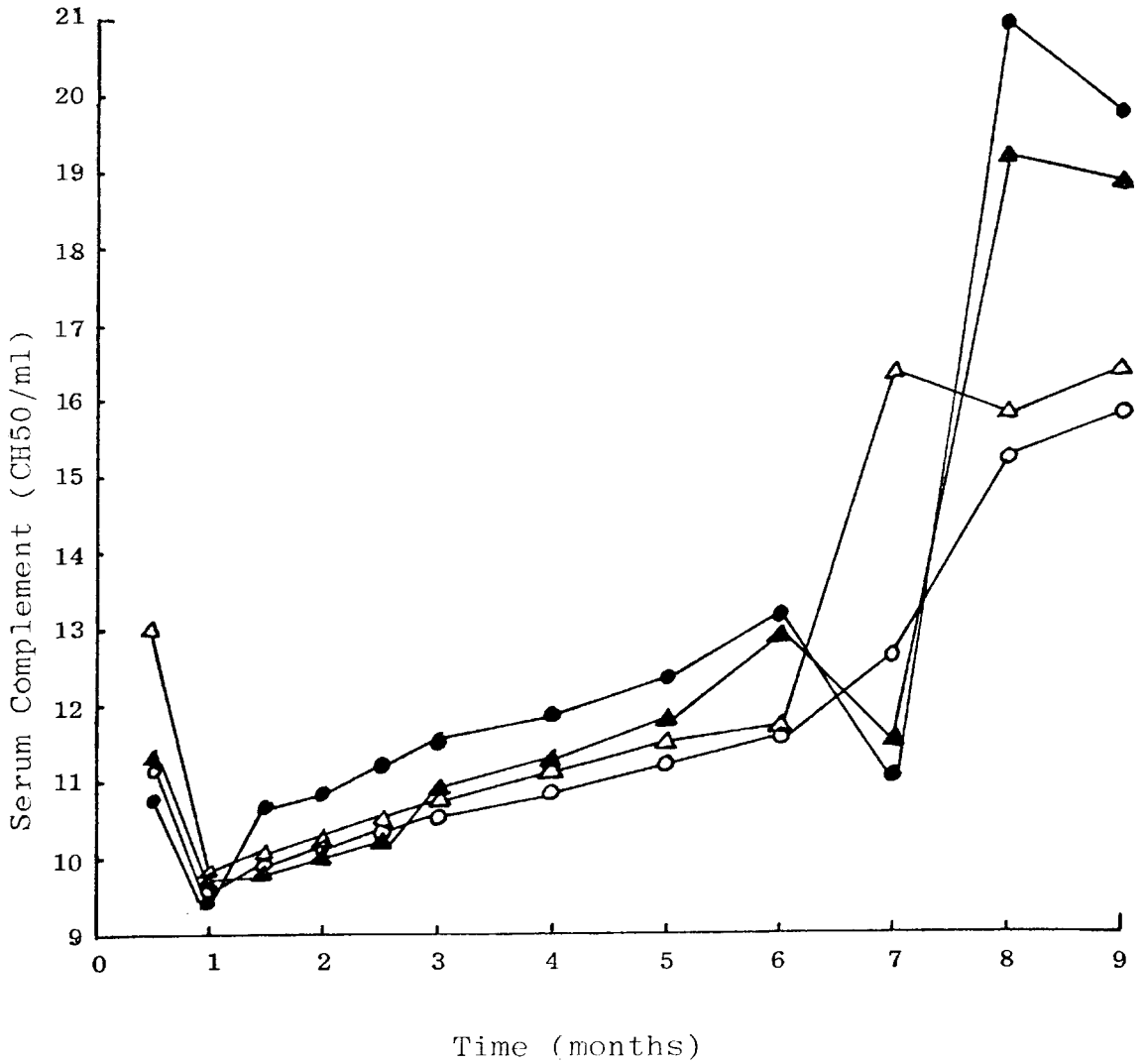


Figure 3.7: Serum Complement

Table 3.9: Atherosclerotic Score

EXPERIMENT 2 Time on Diet (months)				EXPERIMENT 3 Time on Diet (months)	
DIET	3	9	12	DIET	12
PsMO	8.0	10.8±2.1	9.2±1.0	Ps 2% MO	8.0
PsCO	8.0	10.0±1.1	14.6±3.8		
PDCO	8.0	12.2±3.2	12.4±1.2	PD 2% MO	9.4±0.6*
PDCO	8.6±0.6	20.8±5.4	13.2±0.7		

* Significantly greater than corresponding Ps means ($p<0.05$)

Table 3.10: Organ Weights (g) (Nine Months)

DIET	Kidney	Liver	Heart	Spleen	Adrenals
PsMO	9.35±0.4	77.29±5.1	8.86±0.8	2.18±0.3	0.53±0.07
PsCO	8.72±0.98	101±11.8***	7.78±0.5	2.16±0.3	0.7±0.04*
PDMO	8.56±0.4	81.94±5.1	7.68±1.1	1.87±0.4	0.74±0.05*
PDCO	9.79±1.3	117 ±4.9***	7.34±0.6	1.52±0.3	0.95±0.24

* Significantly higher than MO means when fed Ps, and CO means when PD fed ($p<0.05$)

*** Significantly higher than MO means ($p<0.001$)

Table 3.11: Liver Cholesterol and Triglycerides (twelve months)

DIET	Liver Cholesterol (μmol/g)	Liver Triglyceride (mg/g)
PsMO	13.4±5.1	13.2±1.0
PsCO	20.72±5.2	22.1 ±4.0***
PDMO	10.36±0.52	7.2±0.7
PDCO	15.02±2.1	24.3±3.0***

*** Significantly higher than corresponding MO means ($p<0.001$)

Table 3.12: Tissue Calcium (μmol/g tissue)

DIET	Carotid Artery	Renal Artery	Kidney	Aorta
PsMO	5.17±2.0	47.25±15	1.45±0.25	270.0±110
PsCO	6.65±1.5	31.0±15	2.4±0.5	297.0±213
PDMO	9.67±3.7	79.9±40	4.52±1.25	415.0±75
PDCO	9.17±4.0	59.0±15	2.22±0.5	582.0±203

Table 3.13: Relationship Between Aorta Scores and Final Live and Organ Weights

Time on Diet (months)	PARAMETER	ATHEROSCLEROSIS SCORE 0-8	8.1-16	16.1-24	24.1-32	32.1-40	r△
9	Number of animals	3	11	2	2	1	
12		5	12	1	2		
9	Final Live Weight (kg)	3.79±0.1	4.08±0.2	4.07±0.3	4.18±0.03	2.72	-0.275
12		4.21±0.07	4.36±0.1	4.47±0.3	3.96		-0.154
9	Kidney (g)	8.92±0.7	8.82±0.4	8.8±0.6	8.68±0.9	13.7	0.45*
12		8.84±0.2	9.65±0.5	9.5±0.4	9.65		0.159
9	Liver (g)	89.7±2.1	91.1±7.5	96.52±27.8	98.97±9.1	109.9	0.326
12		88.4±7.3	103±5.2	107±1.7	150		0.438*
9	Heart (g)	10.42±0.7	7.44±0.4	7.11±0.1	7.23±1.1	8.81	-0.165
12	Heart & Aorta(g)	12.94±0.9	13.15±0.8	13.29±0.6	8.57		-0.264
9	Spleen (g)	2.45±0.1	1.75±0.2	2.16±0.8	2.15±0.1	1.46	-0.047
12		1.03±0.2	1.99±0.2	1.53±0.02	0.91		-0.093
9	Adrenal (g)	0.56±0.1	0.69±0.03	0.54±0.04	0.82±0.1	1.64	0.661***
12		0.64±0.06	0.79±0.1	0.78±0.1	0.86		0.129

* Significant positive correlation with aorta score ($p<0.05$)

*** Significant positive correlation with aorta score ($p<0.001$)

△ Product Moment Correlation Coefficient

Table 3.14: Relationship Between Aorta Scores and Lipids

Time on Diet (months)	PARAMETER	ATHEROSCLEROSIC SCORE 0-8	8.1-16	16.1-24	24.1-32	32.1-40	r_λ
9	Plasma Cholesterol (mmol/l)	2.44±0.8	3.0±0.6	5.06±3.22	9.8±4.8	8.43	0.747***
12		1.97±0.43	2.47±0.28	2.71±1.63	8.02		0.705***
9	Plasma Triglycerides (mg/l)	295±5.0	357±28	275±5.0	220 ±10	1060	0.463*
12		474±12	488±15	450±70	600		0.232
9	Liver Cholesterol (µmol/g)	13.3±2.6	12.17±1.3	14.8±7.8	20.98±7.8	15.13	0.444
12		13.6±2.6	13.99±2.6	13.0±2.6	36.18		0.385
9	Liver Triglycerides (mg/g)	20.1±2.2	21.0±4.0	25.5±15	21.3±9.5	10.2	-0.06
12		20.1±5.0	15.8±2.7	12.5±2.8	19.4		-0.012

* Significant positive correlation with aorta score ($p<0.05$)

*** Significant positive correlation with aorta score ($p<0.001$)

λ Product Moment Correlation Coefficent

Table 3.15: Relationship Between Aorta Scores and Immunological Data

Time on Diet (months)	PARAMETER recorded	ATHEROSCLEROTIC SCORE 0-8	8.1-16	16.1-24	24.1-32	32.1-40	r[λ]
9	Food	0	15.0±8.3	0±0	20.0±20.0	0	0.026
12	Antibodies	8.0±8.0	35.0±15.2	160±160	0		0.362
9	Immune	1.14±0.1	1.27±0.07	1.24±0.2	1.72±0.05	2.29	0.705***
12	Complexes (CH50 consumed)	0.26±0.26	1.01±0.2	1.54±0.02	0.5		0.351
9	Serum	15.7±0.6	18.2±2.2	15.5±0.8	22.8±3.5	13.0	0.147
12	Complement (CH50/ml)	17.4±1.2	22.1±2.1	16.9±2.1	11.2		-0.347

*** Significant positive correlation with aorta score ($p<0.001$)

λ Product Moment Correlation Coefficient

Table 3.16: Relationship Between Aorta Scores and Calcium Determinations

Time on Diet (months)	PARAMETERS	ATHEROSCLEROTIC SCORE 0-8	8.1-16	16.1-24	24.1-32	32.1-40	r^{Δ}
9	Serum Calcium	3.29±0.05	3.24±0.02	3.19±0.2	3.37±0.05	3.44	0.352
12	(mmol/g)	2.61±0.12	2.52±0.1	2.57±0.12	2.88		0.286
9	Carotid Artery	42.5±10.0	91.5±20.0	179±28.7	111±30.1	890	0.707***
12	(µmol/g)	7.25±2.5	7.75±2.5	8.75±7.5	4.52		-0.015
9	Renal Artery	469±110	446±170	713±589	375±50.0	120	-0.102
12	(µmol/g)	27.0±12.5	66.25±17.5	33.75±10.0	87.0		0.121
9	Kidney	40.0±5.0	44.0±5.0	92.5±52.5	198±±35	835	0.767***
12	(µmol/g)	2.25±0.5	3.25±0.75	2.4±0.25	1.42		-0.123
9	Aorta (µmol/g)	346±128	322±92.5	168±42.5	675±125	1230	0.59*

* Significant positive correlation with aorta score ($p<0.05$)

*** Significant positive correlation with aorta score ($p<0.001$)

Δ Product moment correlation coefficient

Table 3.17: Arterial Histology (nine months)

DIET	HISTOLOGICAL APPEARANCE OF SECTIONS		
	Normal	Minimal Changes	Significant Changes
PsMO	4/5	1/5	0/5
PsCO	3/4	1/4	0/4
PDMO	3/5	1/5	1/5*
PDCO	4/5	0/5	1/5*

* Presence of giant cells

4/5 refers to the number of animals exhibiting the changes shown over the total number of animals examined.

Table 3.18: Summary of Final Individual Data (nine months)

Rabbit No.	Diet	Aorta Score	Antibody Titre	Immune Complexes (CH50 consumed)	Plasma Cholesterol (mmol/l)	Plasma Triglycerides (mg/l)	Presence of giant cells
6		8	0	1.3	0.57	290	-
7		9.5	0	1.24	0.596	270	-
8	PsMO	8.5	0	1.22	2.12	290	-
9		9	0	1.1	2.38	300	-
10		19	0	1.0	1.84	280	-
6		/	10	0.4	8.39	380	-
7		12.5	0	1.09	5.75	570	-
8	PsCO	8	0	1.1	4.56	310	-
9		11	0	1.08	5.02	280	-
10		8.5	0	1.07	3.83	380	-
6		8.5	20	1.32	0.8	320	-
7		9.5	10	1.1	1.04	410	-
8	PDMO	10	40	1.61	3.44	360	+
9		8	0	1.14	2.28	290	-
10		25	40	1.58	5.0	230	+
6		8	0	0.8	2.38	290	-
7		11	80	1.45	5.05	380	-
8	PDCO	36	0	2.29	8.44	1060	+
9		18.5	0	1.44	8.29	270	-
10		30.5	0	1.52	14.63	210	-

- underlined numbers represent high levels

Fig 3.8 The abdominal aorta from rabbit PsM0-10 (x18) stained with Sudan IV, showing an area of normal aorta with slightly depressed firm atherosclerotic areas (*).

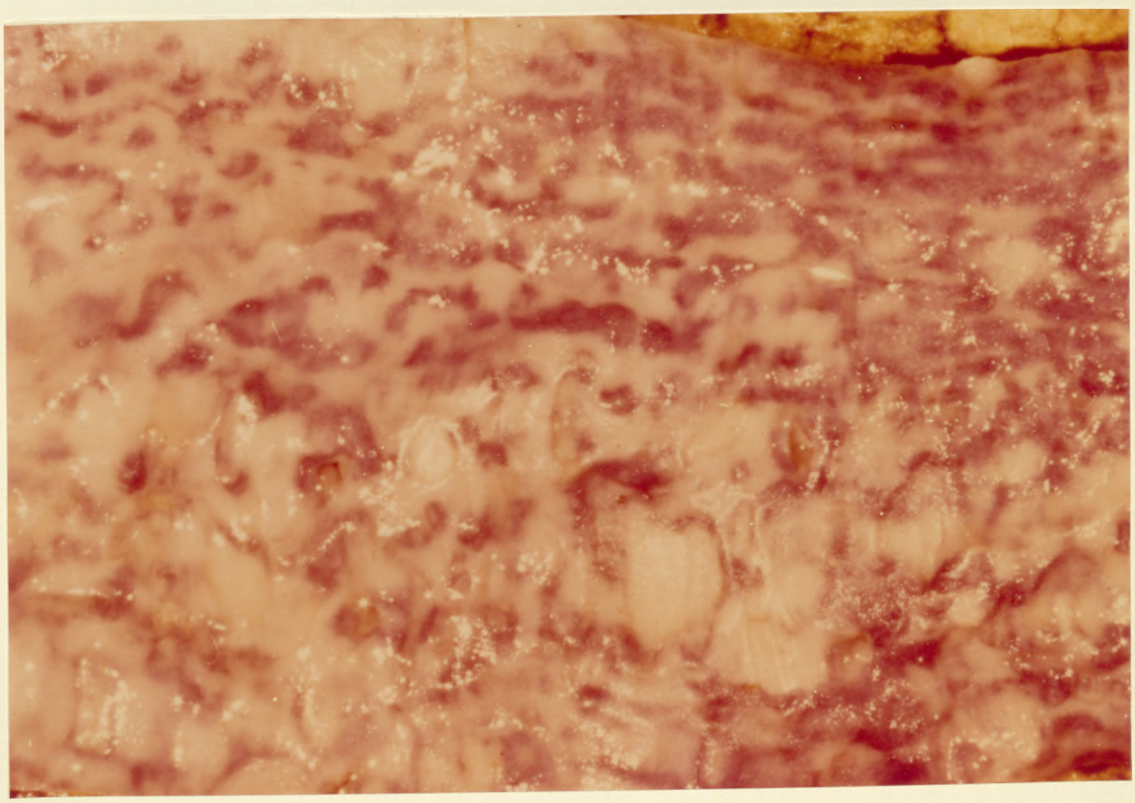


Fig 3.9 The abdominal aorta from rabbit PDC0-8 (x18) stained with Sudan IV, showing diffuse staining with depressed firm atherosclerotic areas.

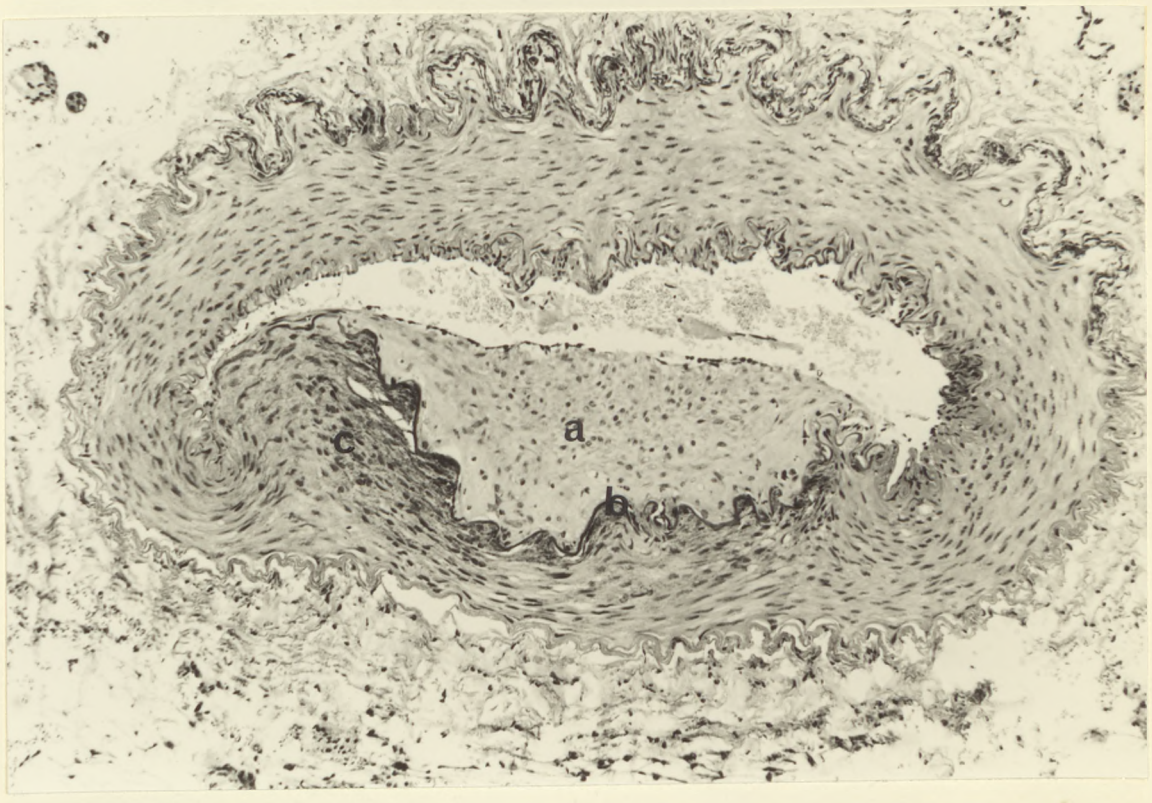


Fig 3.10 Renal artery from rabbit PDMO-10 stained with haematoxylin and eosin (x125). This section shows intimal thickening (a), slight disruption of the internal elastic lamella (b) and calcification in the media (c).

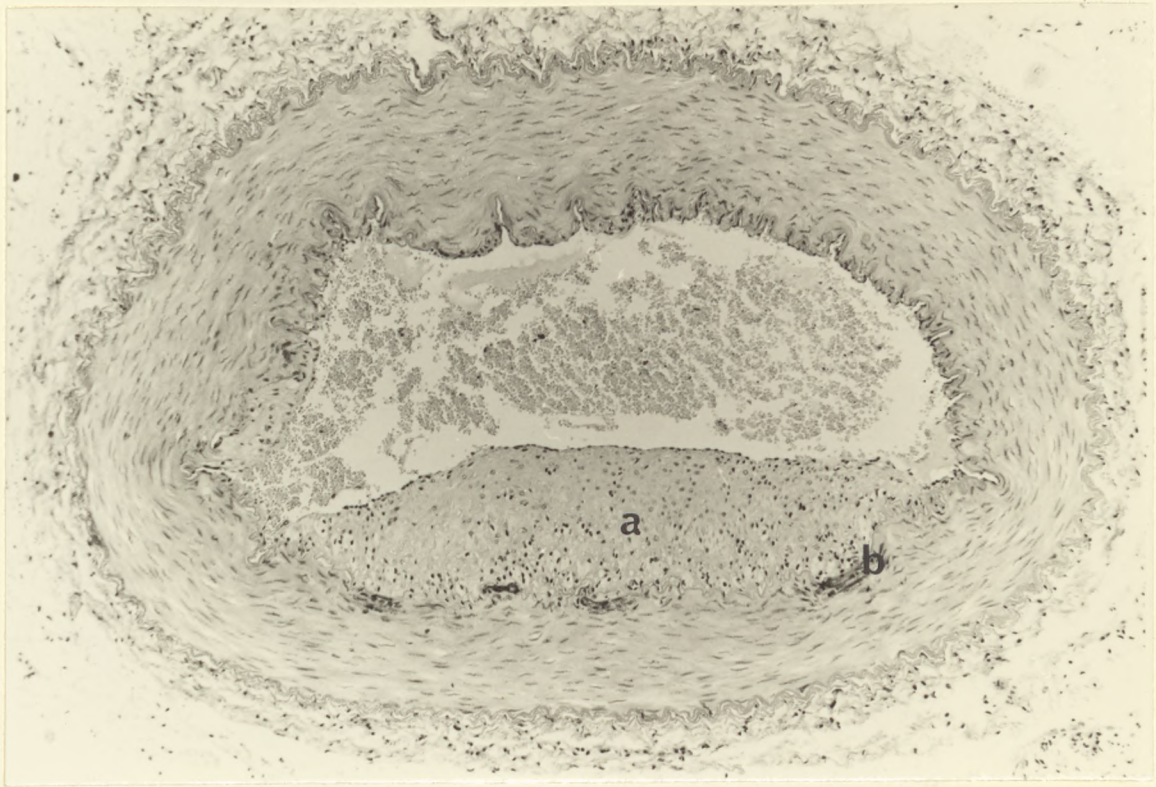


Fig 3.11 Renal artery from rabbit PDC0-8 stained with haematoxylin and eosin (x95). This section shows intimal thickening (a) and calcification around the internal elastic lamella (b).

Fig 3.12 Renal artery from rabbit PDC0-8 stained with haematoxylin and eosin (x310). This section shows intimal thickening (a), the disruption of the internal elastic lamella with an associated inflammatory infiltrate with mononuclear cells (arrowed).

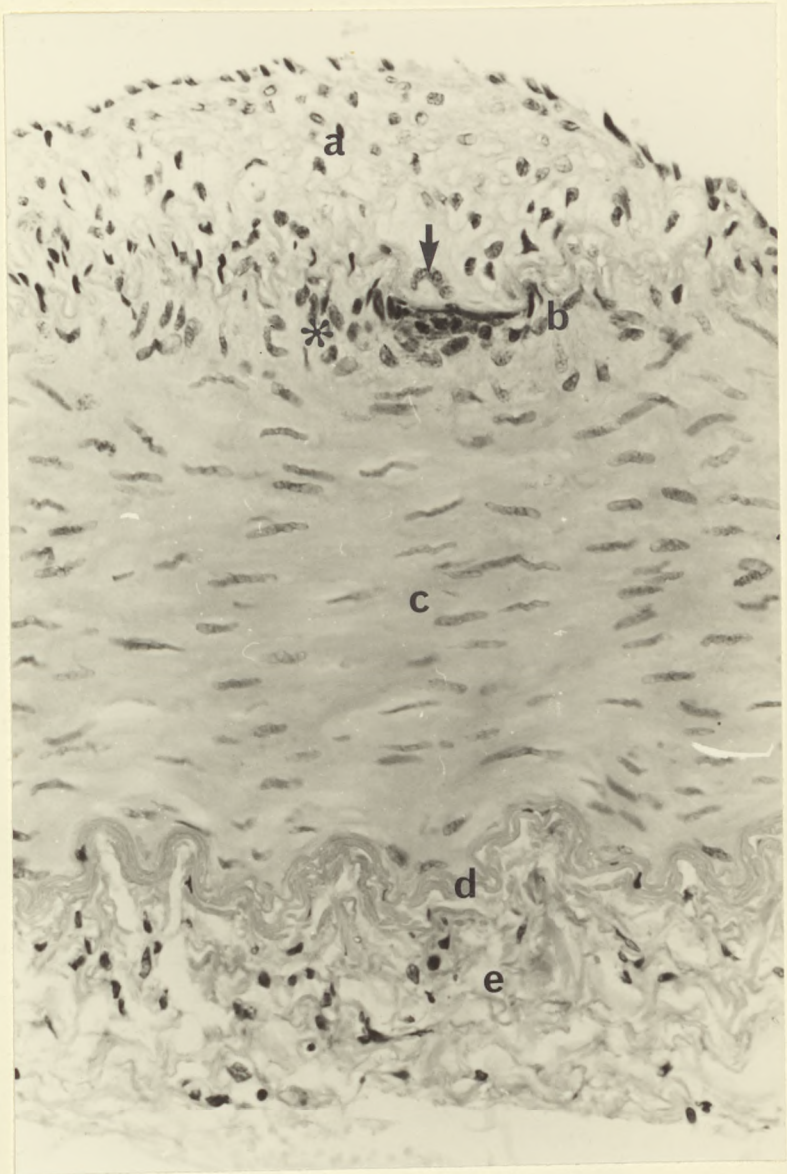


Fig 3.13 Renal artery from rabbit PDC0-8 stained with haematoxylin and eosin (x375). This section shows some intimal thickening (a) with fragmented internal elastic lamella (b), mononuclear cells (*) with a single giant cell (arrowed). The media (c) the external elastic lamella (d) and adventitia (e) appear normal.

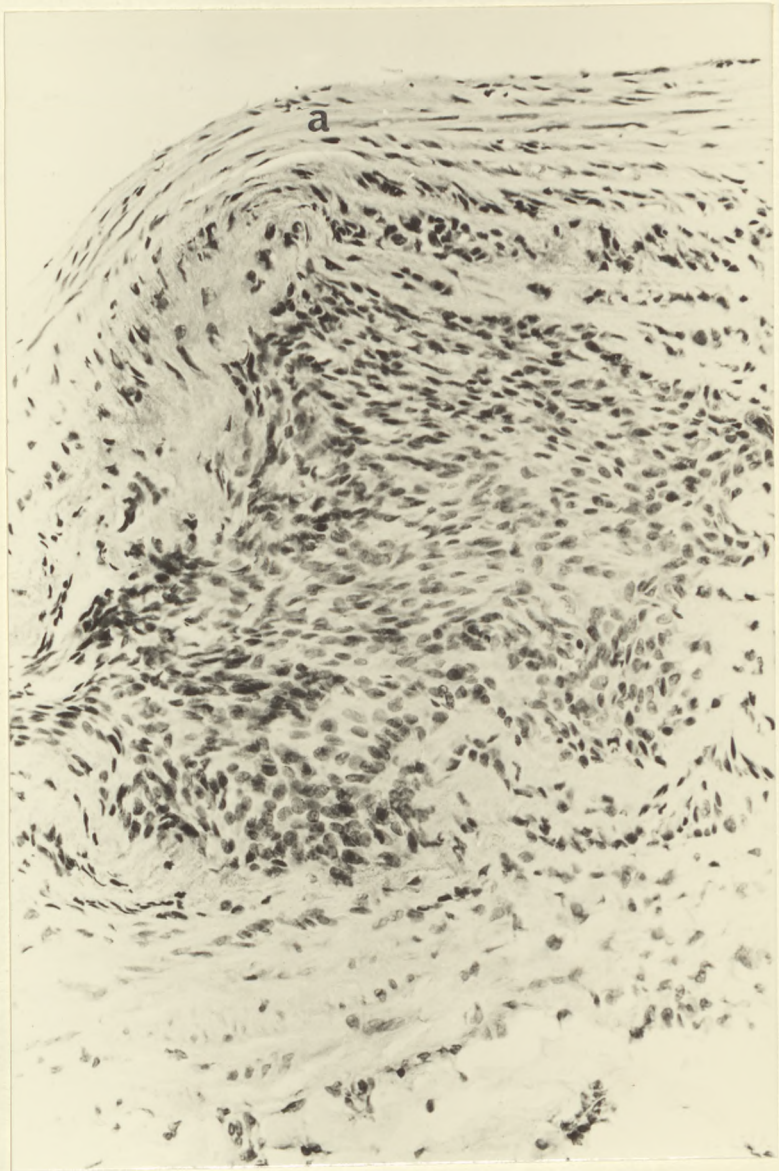


Fig 3.14 Aorta from rabbit PDCO-9 stained with haematoxylin and eosin (x420). This section shows slight intimal thickening (a), and a dense mononuclear inflammatory infiltrate which has obliterated the structure of the media and adventitia.

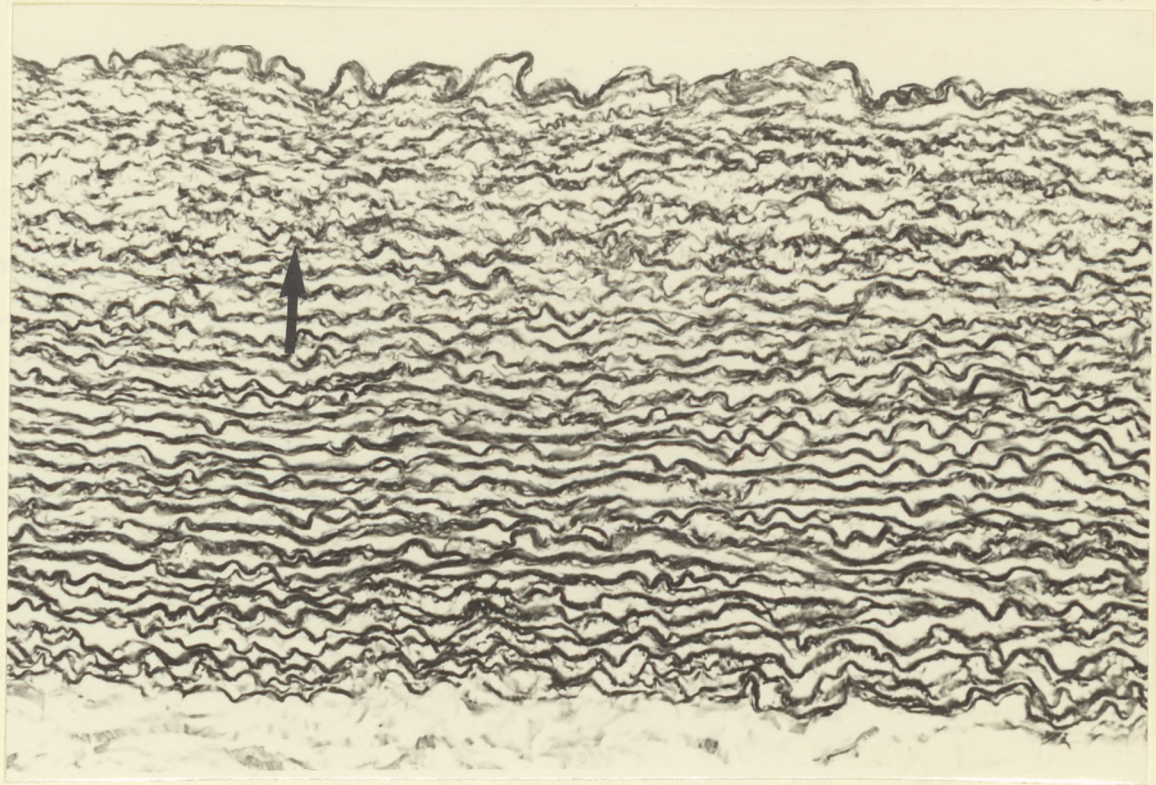


Fig 3.15 Aorta from rabbit PsMO-10 stained with:

A Elastic van Gieson stain (x240). This section was taken from a macroscopically normal area and shows a normal wavy pattern of elastic tissue although some of the superficial elastica is slightly irregular (arrowed).

B Elastic van Gieson stain (x220). This section was taken through an atheromatous plaque. The elastic tissue has lost its wavy appearance, the lamella is compressed and the wall thinned. One giant cell can be seen (arrowed).

CHAPTER 4

EXPERIMENT 3

4.1 Introduction

It could be argued that the arterial disease produced in Experiments 1 and 2 was mainly the result of the high fat content rather than the antigenic protein of the diet. High fat diets have been shown to produce an atherosclerotic disease in rabbits.[57] Experiment 3 designed to examine this possibility.

4.2 Materials and Methods

The materials and methods used in the experiment are the same as in Experiment 2 and are detailed in Appendix 1. The only difference was in the diets. Five weanling rabbits were fed a diet containing either PD or Ps (App.1, Table 5) for ten months. The fat content of the diet was 20g MO/kg which provided the essential fatty acids; the diet was made up to 1 kg with maize starch.

4.3 Results

4.3.1 Food Intake, Live Weight and Serum Calcium

All the rabbits gained weight satisfactorily but the group fed PD gained more weight than those fed Ps as shown in Table 4.1. This was not a result of an increased intake of the PD diet as shown in Table 4.2 because there was no consistent increase in intake of the PD diet. One rabbit was lost in the Promine D fed group, the cause of which was unclear. The serum calcium shown in Table 4.3 stayed within narrow limits (2.34-3.35 mmol/l) and were similar in both groups.

4.3.2 Plasma Cholesterol and Triglycerides

It can be seen from Table 4.4 that the cholesterol levels were not particularly elevated except in the first two months in the rabbits fed Ps. The levels ranged from 0.7 to 2.6 mmol/l.

Differences between treatments in plasma triglyceride concentrations occurred in a particular three month period (Table 4.5). Ps appeared to elevate the plasma triglycerides. However, if these levels are compared to those in the high fat-fed animals (Table 3.5) then it would appear that PD had a lowering effect on the plasma triglycerides.

4.3.3 Immunological Parameters

The PD-fed group did not have significantly higher levels of food antibodies as can be seen in Table 4.6 but the incidence of detectable antibodies was greater in the PD-(80%) than in the Ps-fed group (40%). Immune complexes were similar in both groups (Table 4.7) and the serum total complement varied from month to month with no consistent differences being found (Table 4.8).

4.3.4 Aorta Scores

There was no extensive arterial disease found in any of these rabbits although there was a significant difference between the two groups ($p<0.05$), (Table 3.9).

4.3.5 Organ Weights

Organ weights are shown in Table 4.9. The PD-fed group had higher kidney ($p<0.05$), liver ($p<0.001$), heart ($p<0.05$) and spleen ($p<0.05$) weights. However, when these organ weights were expressed as percentages of final live weights these differences disappeared as PD-fed rabbits had higher final live weights.

4.3.6 Tissue Calcium

No differences were found between the two diets in tissue calcium levels which can be seen in Table 4.10. The levels appeared normal when compared with those from Experiment 2 (Table 3.12).

4.3.7 Histological Observations

All the renal and carotid arteries from these rabbits were examined microscopically. All these vessels were normal in appearance. The aorta from one rabbit fed PD showed inner media calcification.

4.4 Discussion

There was no appreciable arterial disease produced in rabbits fed the low fat, high starch diet. The high fat content of the diet used in Experiments 1 and 2 appears to be necessary for the development of atherosclerosis. Cholesterol feeding was found to be a necessary adjunct to foreign protein injections in rabbits[105] in order to produce fatty-proliferative lesions.[107,108] If the injections of foreign proteins were given without the cholesterol feeding then only proliferative lesions were found.[107] In baboons a high fat diet was necessary in addition to foreign protein injections to speed up the development of arterial lesions.[106]

Atherosclerosis and hypercholesterolaemia (5.28-18.13 mmol/l) has been produced in rabbits using a cholesterol-free, low fat diet (20g MO/kg diet).[182] The plasma cholesterol levels during Experiment 3 were normal (0.7-2.6 mmol/l). The reason for this difference in results might be that the rabbits which produced hypercholesterolaemia were fed casein as their protein source[182] whereas those with normal cholesterol levels had soya proteins as the protein source. Soya proteins have been shown not to affect plasma cholesterol levels in rabbits whereas casein has been shown to be hypercholesterolaemic.[73]

Other workers[29] have shown that high starch (430g wheat starch/kg diet), low fat diets (4.7g MO/kg diet) produce atherosclerosis to a greater extent than a high unsaturated fat diet (200g MO/kg diet). The degree of atherosis in the high starch diet was 9.5 ± 1.6 compared with 0.7 ± 0.62 in the high fat group.

The aorta scores from the rabbits fed a high unsaturated fat diet in the twelve month part of Experiment 2 (10.8 ± 0.5) were significantly greater ($p<0.025$) than those produced using the high starch diet in Experiment 3 (8.7 ± 0.37). The plasma cholesterol levels in rabbits[29] fed the high starch diet (2.11 ± 0.26 mmol/l) were higher than those fed the high fat diet (0.61 ± 0.1 mmol/l). In contrast, the rabbits in Experiment 2 fed the MO diet had significantly higher ($p<0.025$) plasma cholesterol levels (1.53 ± 0.13 mmol/l) than those in Experiment 3 fed the high starch diet (1.05 ± 0.17 mmol/l). Other workers have also shown that a substitution of complex carbohydrates for polyunsaturated fat causes an increase in serum cholesterol.[64] The difference may occur because the dietary constituents are different, hence, the interactions between them are different. In the experiment[29] where an increase in serum cholesterol was noted when the unsaturated fat (MO) was substituted by a high starch diet (wheat starch) the protein source was casein. In Experiment 3 the protein source was either a soya concentrate or a soya isolate and the carbohydrate source was maize starch. The plasma triglyceride levels in the rabbits were normal (210-550 mg/l) throughout most of the experiment, when compared to rabbits fed a commercial diet (420 ± 30 mg/l).[187]

No difference could be found between the two groups fed different proteins in terms of the immunological parameters measured, whereas in Experiment 2 some differences between the proteins were observed during the course of the experiment. It might be concluded that the fat may have had an effect either through the absorption of the proteins or through the actual immune response. If there had been an effect of the fat on the immune response then a difference in the titre of food antibodies between the two experiments would be observed, however, no difference was found between the titres in Experiment 2 (39 ± 18) and those of Experiment 3 (48.9 ± 23). This does not agree with evidence that shows that

hypercholesterolaemia will reduce the immune response to injected foreign proteins.[200]

It must be concluded that high starch diets containing soya proteins and low levels of unsaturated fats do not produce atherosclerosis in rabbits in ten months.

GRAPHS AND TABLES

Legend for graphs

Symbol	Diet
O	Promosoy 100 and maize oil (PsMO)
●	Promosoy 100 and coconut oil (PsCO)
△	Promine D and maize oil (PDMO)
▲	Promine D and coconut oil (PDCO)

Table 4.1: Live Weight (kg)

DIET	TIME (MONTHS) 1	2	3	4	5	6	7	8	9	10	11
Ps2%MO	1.62** ±0.06	2.30 ±0.08	2.89 ±0.1	3.12 ±0.12	3.37 ±0.16	3.41 ±0.18	3.47 ±0.2	3.56 ±0.2	3.61 ±0.2	3.56 ±0.13	3.66 ±0.16
PD2%MO	1.26 ±0.04	2.11 ±0.06	3.16 ±0.02	3.51* ±0.11	3.93* ±0.13	4.27* ±0.15	4.37* ±0.1	4.38* ±0.14	4.46* ±0.2	4.26* ±0.1	4.24* ±0.05

* Significantly greater than corresponding Ps means ($p<0.05$)

** Significantly greater than corresponding PD mean ($p<0.01$)

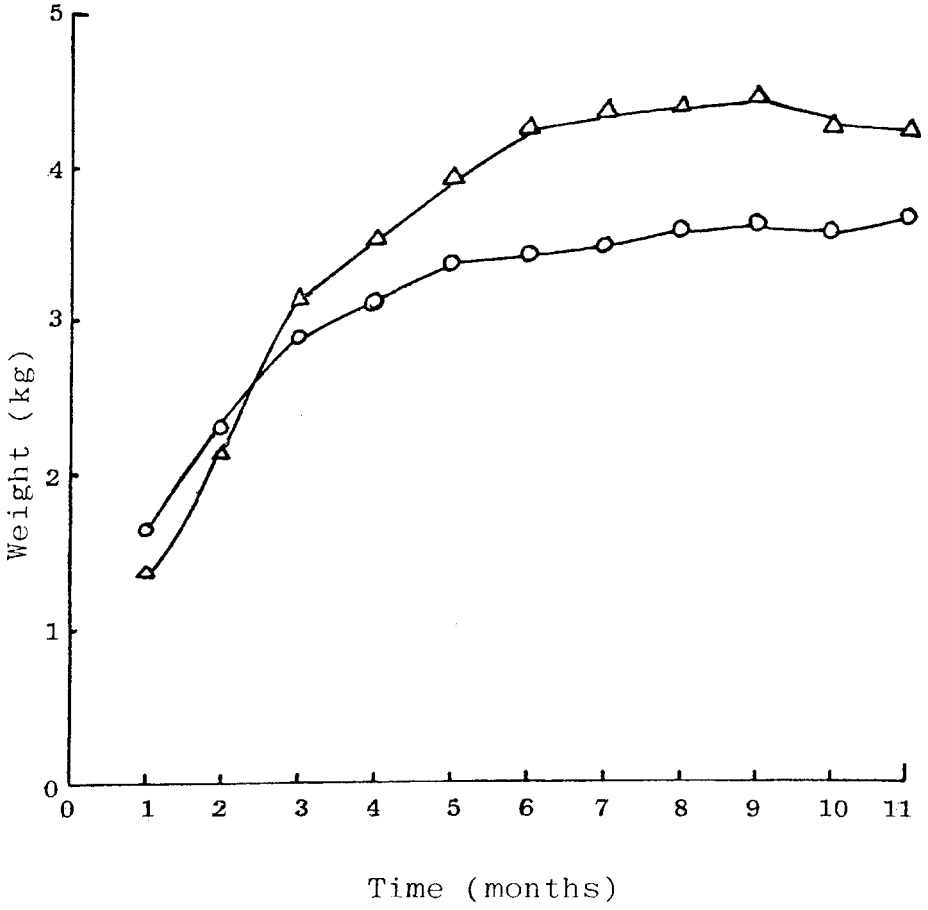


Figure 4.1: Weight Gain

Table 4.2: Food Intake (kcal/$kg^{0.75}$/week)

DIET	TIME (MONTHS)										
	1	2	3	4	5	6	7	8	9	10	11
Ps2%MO	1.36 ±0.6	6.71 ±0.3	4.72 ±0.4	4.74 ±0.4	4.16 ±0.2	3.97 ±0.1	3.84 ±0.6	5.64* ±0.3	1.77 ±0.7	2.99 ±0.6	4.26 ±0.4
PD2%MO	2.67 ±0.4	8.16 ±0.9	6.47* ±0.1	6.75 ±0.9	5.79* ±0.5	4.89* ±0.2	3.71 ±0.4	4.04 ±0.55	3.88* ±0.4	3.89 ±0.5	5.35 ±1.1

* Significantly greater than corresponding means ($p<0.05$)

Table 4.3: Serum Calcium (mmol/l)

DIET	TIME (MONTHS) 1	2	3	4	5	6	7	8	9	10
Ps2%MO	3.30 ±0.05	3.14 ±0.14	3.02 ±0.35	2.89 ±0.1	3.26 ±0.14	3.28 ±0.15	3.05 ±0.1	2.76 ±0.12	2.75 ±0.1	2.6 ±0.02
PD2%MO	3.22 ±0.02	3.05 ±0.02	3.20 ±0.15	3.16 ±0.12	2.87 ±0.2	2.59 ±0.25	2.8 ±0.1	2.73 ±0.1	2.47 ±0.12	2.65 ±0.05

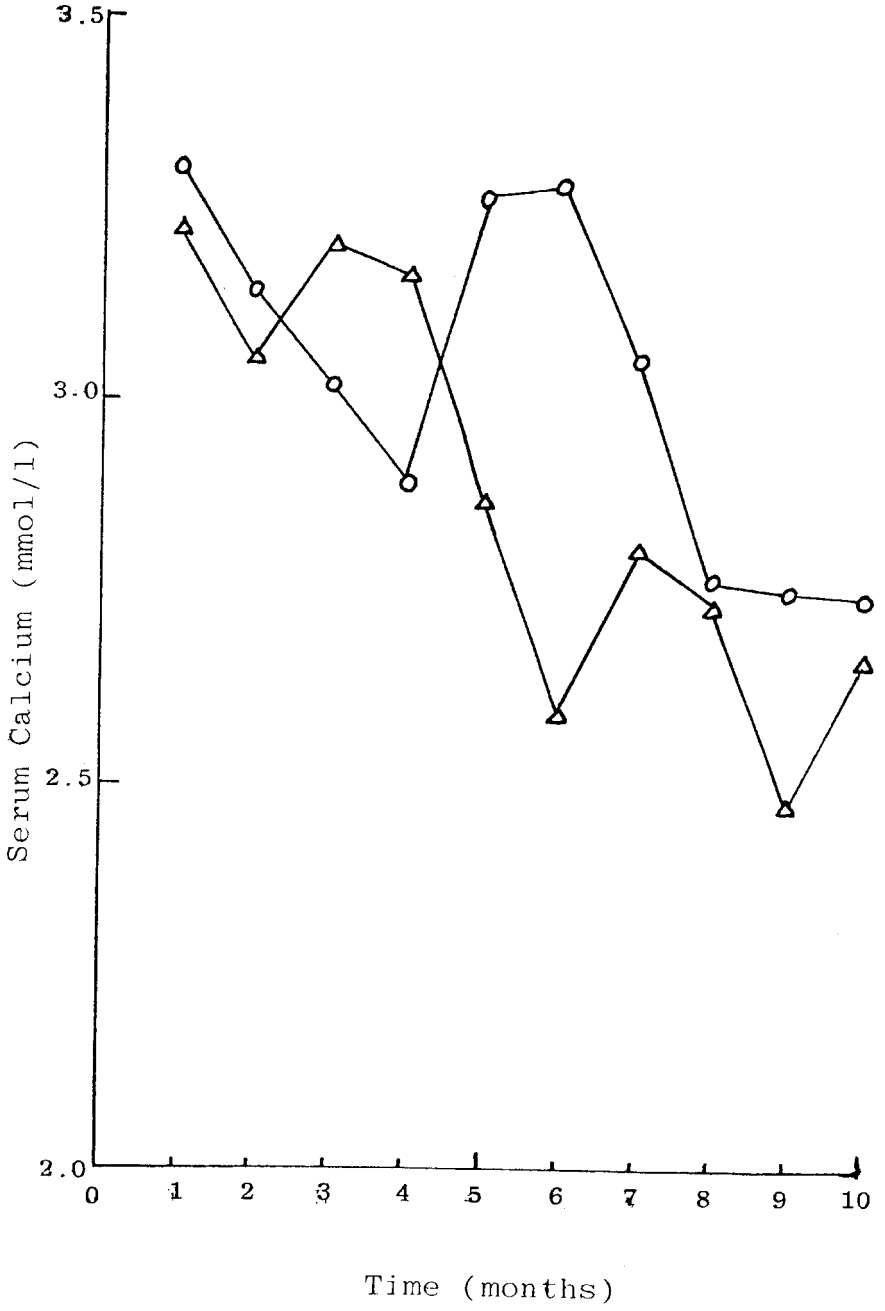


Figure 4.2: Serum Calcium

Table 4.4: Plasma Cholesterol (mmol/l)

DIET	TIME (MONTHS)									
	1	2	3	4	5	6	7	8	9	10
Ps2%MO	3.45 ±0.8	5.34 ±1.7	2.3 ±0.38	1.82 ±0.6	1.82 ±0.36	1.54 ±0.3	1.41 ±0.2	1.03 ±0.1	1.06 ±0.1	0.87 ±0.06
PD2%MO	1.94 ±0.3	2.07 ±0.5	2.14 ±0.6	2.10 ±0.6	1.63 ±0.6	1.50 ±0.5	1.40 ±0.3	1.20 ±0.07	1.19 ±0.4	1.00 ±0.3

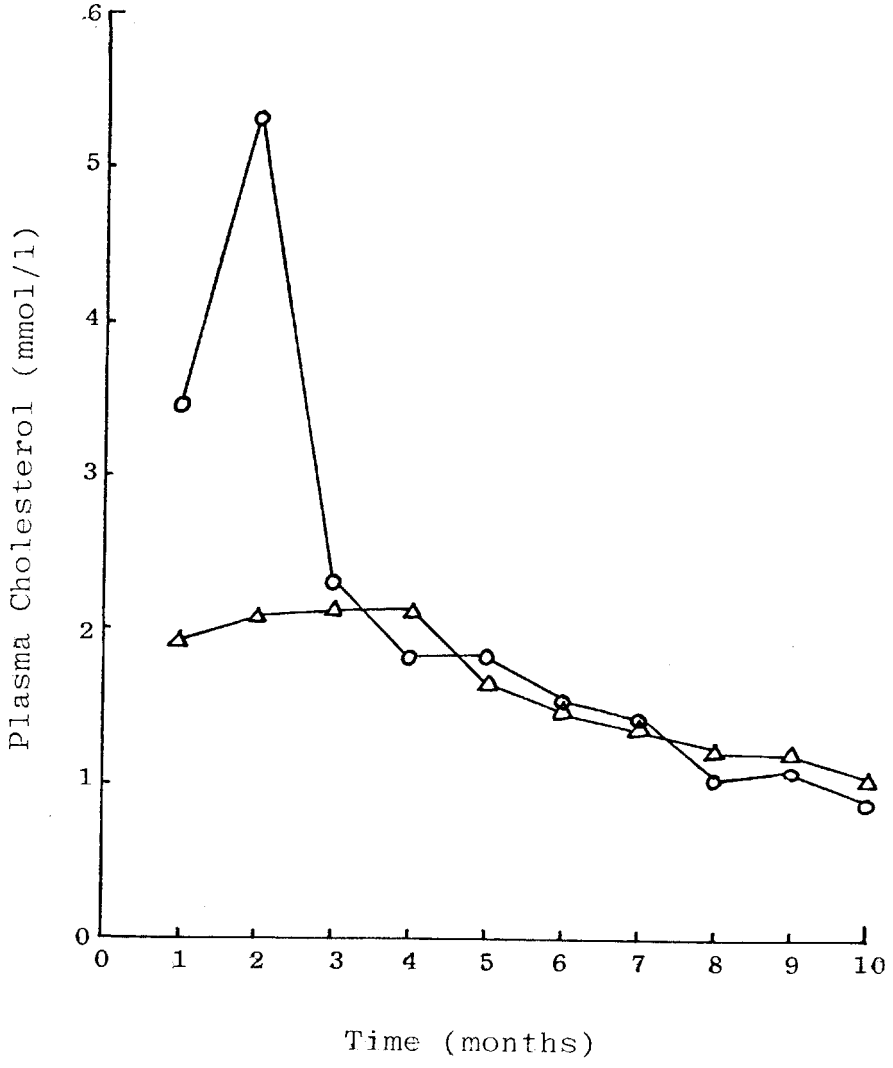


Figure 4.3: Plasma Cholesterol

Table 4.5: Plasma Triglycerides (mg/l)

DIET	TIME (MONTHS)									
	1	2	3	4	5	6	7	8	9	10
Ps2%MO	448.3 ±102	366.2 ±45	340.6* ±22	310.3 ±35	360.4 ±16	400.3* ±31.5	428.4* ±14	422.6* ±12	422.5 ±4	446.8* ±10
PD2%MO	358.1 ±59	259.9 ±45	199.8 ±44	226.3 ±17	304.2 ±24	185.7 ±33	298.1 ±28	363.8 ±20	340.2 ±45	362.3 ±28

* Significantly higher than corresponding PD means ($p<0.05$)

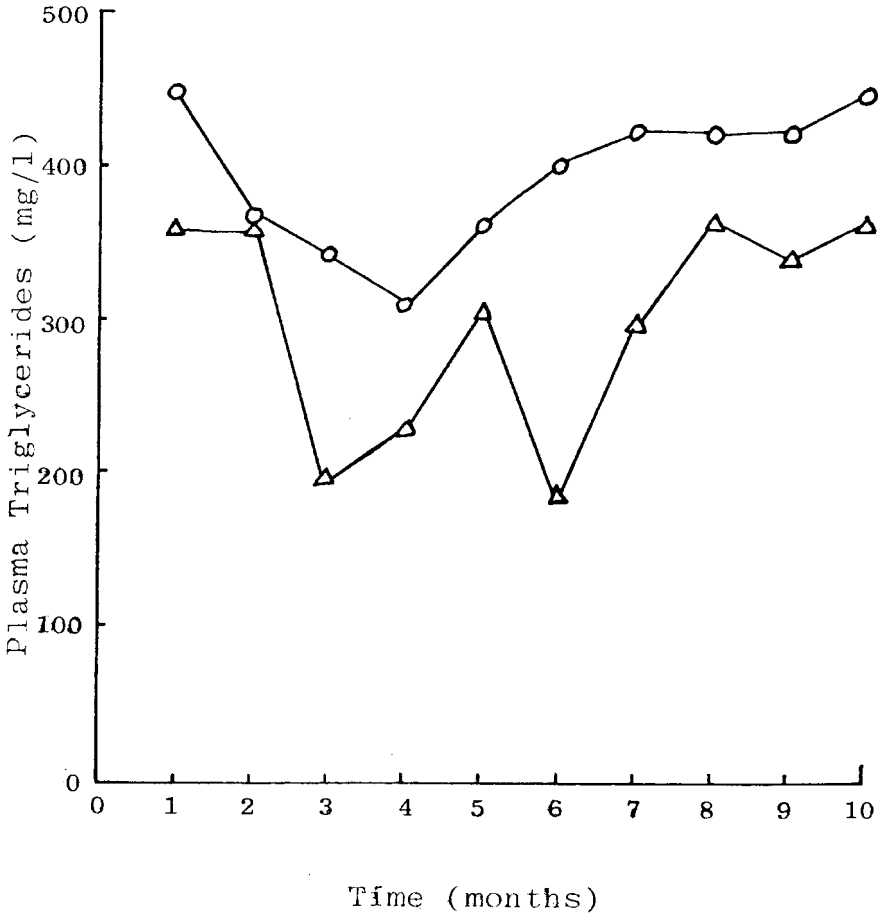


Figure 4.4: Plasma Triglycerides

Table 4.6: Food Antibody Titre

DIET	TIME (MONTHS)									
	1	2	3	4	5	6	7	8	9	10
Ps2%MO	2.0 ±2.0	4.0 ±4.0	8.0 ±8.0	10.0 ±7.7	18.0 ±15.6	36.0 ±31.2	64.0 ±39.2	64.0 ±39.2	128.0 ±78.4	68.0 ±37.7
PD2%MO	0	0	0	4.0 ±4.0	2.0 ±2.0	12.0 ±8.0	12.0 ±8.0	16.0 ±7.5	24.0 ±14.7	35.0 ±17.1

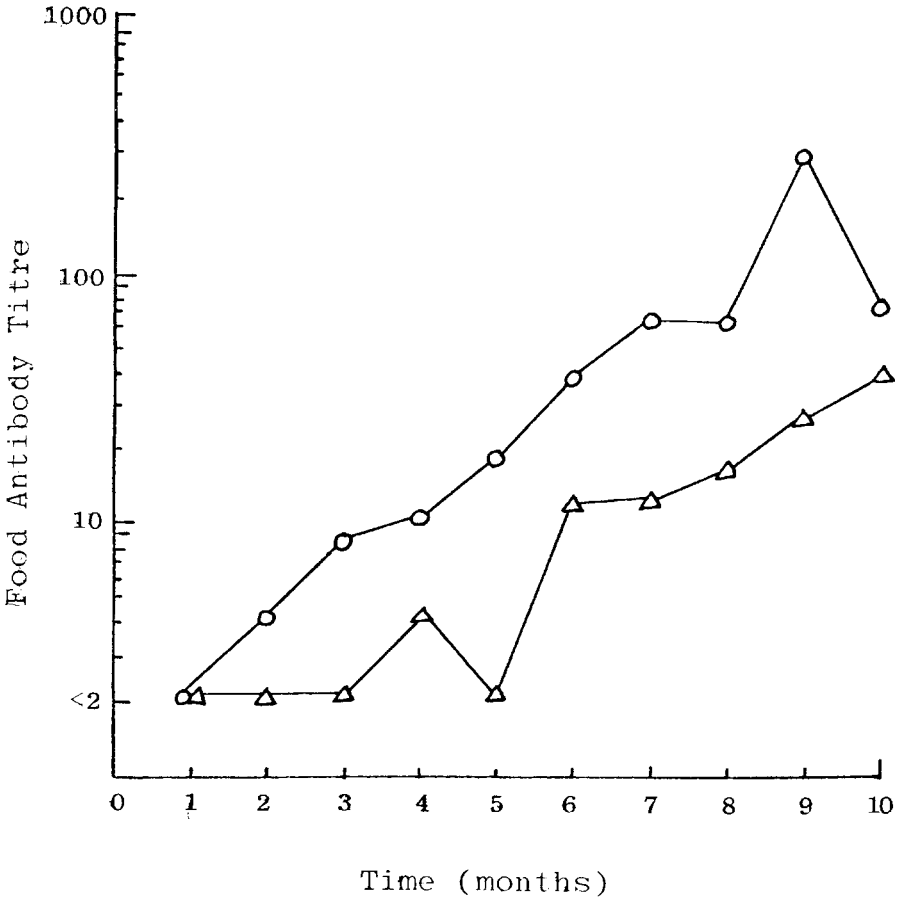


Figure 4.5: Food Antibodies

Table 4.7: Total Serum Complement (CH50/ml)

DIET	TIME (MONTHS)									
	1	2	3	4	5	6	7	8	9	10
Ps2%MO	26.35 ±1.2	21.8 ±0.75	17.8 ±1.4	18.54 ±1.6	21.54 ±4.2	14.28 ±3.6	39.2* ±5.3	28.72* ±4.8	25.26 ±3.2	20.24 ±3.1
PD2%MO	15.3 ±3.9	15.13 ±3.9	17.06 ±6.3	17.76 ±2.9	15.72 ±0.7	16.46 ±0.8	14.82 ±1.2	13.72 ±6.6	39.96* ±5.1	25.05 ±4.6

* Significantly higher than corresponding means ($p<0.05$)

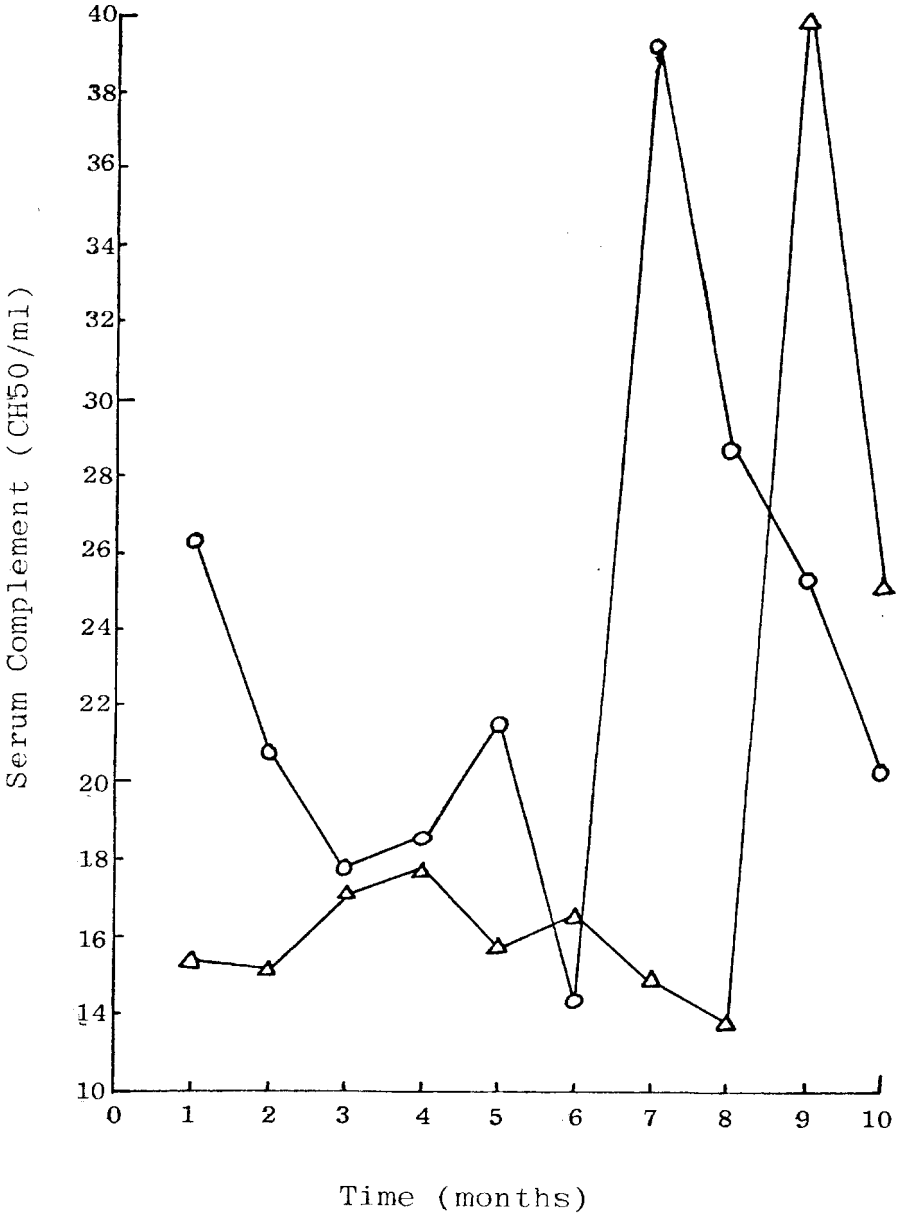


Figure 4.6: Serum Complement

Table 4.8: Immune Complexes (CH50 units consumed)

DIET	TIME (MONTHS) 1	2	3	4	5	6	7	8	9	10
Ps2%MO	0	0.71 ±0.3	0.55 ±0.34	0.85 ±0.35	0.68 ±0.32	0.51 ±0.26	0.54 ±0.25	0.51 ±0.22	0.55 ±0.2	0.57 ±0.28
PD2%MO	0.91* ±0.2	0.93 ±0.2	0.90 ±0.13	0.85 ±0.15	0.79 ±0.12	0.68 ±0.18	0.67 ±0.19	0.68 ±0.29	0.67 ±0.1	0.96 ±0.28

* Significantly higher than corresponding Ps mean ($p<0.05$)

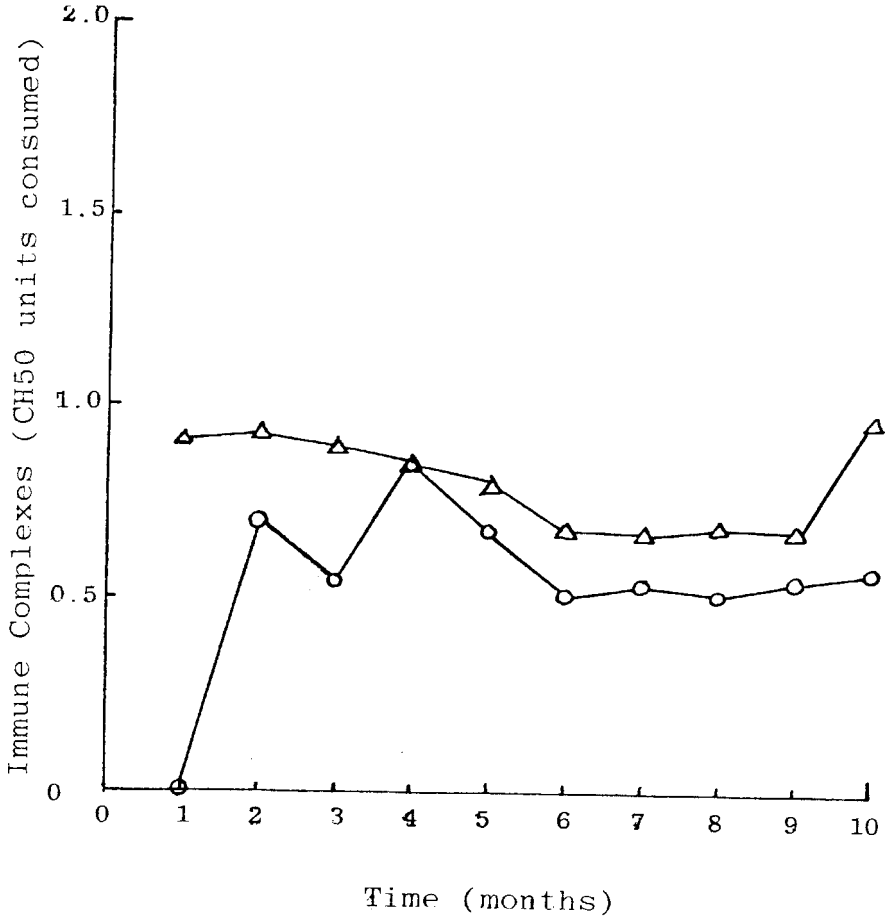


Figure 4.7: Immune Complexes

Table 4.9: Organ Weights (g)

Diet	Kidney	Liver	Heart	Spleen	Adrenals	Thyroid
Ps2%MO	6.81±0.4	66.66±2.8	9.71±0.95	0.98±0.07	0.9±0.1	0.23±0.02
PD2%MO	8.87±0.6*	87.23±1.9**	12.96±0.3*	1.51±0.2*	0.8±0.1	0.26±0.1

* Significantly greater than corresponding Ps means ($p<0.05$)

** Significantly greater than corresponding Ps mean ($p<0.01$)

Table 4.10: Liver Cholesterol and Triglycerides

DIET	Cholesterol (µmol/g)	Triglycerides (mg/g)
Ps2%MO	8.1±0.9	11.05±4.1
PD2%MO	10.0±1.2	8.49±3.1

Table 4.11: Tissue Calcium

CALCIUM CONTENT OF TISSUES (µmol/g tissue)			
DIET	Carotid Artery	Renal Artery	Kidney
Ps2%MO	6.25 1.75	41.5 8.7	4.0 1.0
PD2%MO	4.25 2.5	20.5 10.0	3.5 0.75

CHAPTER 5

MAIN DISCUSSION

It is evident from the results of Experiment 1 that an arterial disease was produced in rabbits by feeding semi-synthetic (cholesterol-free) diets, but the extent of the disease was not reproduced in Experiments 2 and 3. No single cause of the arterial disease was apparent, but rather a combination of causes, including hypercholesterolaemia, hypertriglyceridaemia, the production of food antibodies, the higher levels of dietary vitamin D, and digestive disturbances.

In designing these experiments, PDCO was expected to be the most atherogenic treatment and PsMO the least, with the other two diets falling somewhere in between with equal atherogenicity. This could only occur if the atherogenicity of the saturated fat and the antigenic protein were equivalent. There were no significant differences in aorta scores between treatments in any of the high fat feeding trials. In Experiment 1 PsMO, as expected, had the lowest mean aorta score (2.0 ± 0.4); however, the other three treatments had similar aorta scores (PsCO:4.0 ± 0.4; PDMO:3.5 ± 0.7; PDCO: 3.25 ± 0.3). This pattern was reproduced in the twelve month feeding trial of Experiment 2, (PsMO:9.2 ± 1; PsCO:14.6 ± 3.8; PDMO:12.4 ± 1.2; PDCO:13.2 ± 0.7) but not in the nine month feeding trial where PDCO had the highest mean score (20.8 ± 5.4) and the other three treatments had similar mean aorta scores (PsMO 10.8 ± 2.1; PsCO 10.0 ± 1.1; PDMO 12.2 ± 3.2).

The individual aorta scores of Experiment 1 (Table 2.10) show that although saturated fat is known to be more atherogenic than unsaturated fat,[47,60] two of the rabbits fed PDMO had high aorta scores. In Experiment 2 (Table 3.19) it can be seen that a rabbit fed PDMO had one of the highest aorta scores. Clearly the protein is also having an effect on the development of the disease and the saturated fat is not additive to this effect.

A high fat diet, giving rise to hypercholesterolaemia,[47,60] seems to be necessary for an atherosclerotic lesion to progress into a fatty proliferative lesion.[107] Only 30% of rabbits fed the low fat diet produced any lesions compared to 75% of rabbits on the high fat diet in Experiment 2. Hypercholesterolaemia was not the only factor involved as the mean plasma cholesterol levels in Experiment 1 (4.52 ± 0.9 mmol/l) were not significantly different from those in Experiment 2 (3.18 ± 0.9 mmol/l), whereas the incidence of lesions in Experiment 1 after 11 months (94%) was greater than in Experiment 2 (75%) in which the diets were fed for twelve months. In these experiments only total plasma cholesterol concentrations were measured. Recent evidence published since these experiments were carried out has drawn attention to the fact that different fractions of the total cholesterol have different effects on arterial disease, so that determinations of total cholesterol provide only limited information about the risk of arterial disease.

A factor that has now been shown to be inversely proportional to the risk of coronary heart disease in humans is the level of high density lipoprotein (HDL) cholesterol.[42,203,204] It has also been shown to be an anti-atherogenic factor experimentally.[205,206] HDL-cholesterol levels are higher in females than males[208] and maleness is known to be a risk factor of heart disease.[42]

Patients with myocardial infarction have lower HDL-cholesterol levels than healthy subjects.[207] It has been postulated that the protective effect of HDL may relate to a role in the removal of cholesterol from peripheral tissues, including the arterial wall. The cholesterol in the wall is thought to originate in part from low density lipoproteins (LDL) that filter into the arterial intima from the plasma.[207] As both LDL[208,209] and HDL[42,203,204] are related independently to the risk of coronary heart disease, the cholesterol concentrations in both HDL and LDL must be determined in future if individuals with increased risk of coronary heart disease are to be identified.

Hypertriglyceridaemia has also been implicated in atherogenesis.[209,210] In Experiment 1 and Experiment 2 (nine months) plasma triglycerides are positively correlated with atherosclerosis score. The plasma triglyceride levels in Experiment 1 (2184.4 ± 390.4 mg/l) are significantly higher ($p<0.001$) than those measured in the nine month part of Experiment 2 (358.5 ± 40.83 mg/l). As the arterial disease was more advanced in Experiment 1 the results agree with evidence that shows high plasma triglycerides in heart disease.[210]

During the course of Experiment 1, four rabbits had severe digestive disturbances and several others had minor digestive disturbances. It was suggested that the fibre level in the diet might have been too low in relation to the energy content of the diet. In Experiment 1, the fibre content of the diet was 150g wheat bran/kg; this was raised to 200g wheat bran/kg in Experiment 2. No digestive disturbances were noted in Experiment 2. The extent of the arterial disease was decreased in Experiment 2 and it could be that the increase in fibre content of the diet might have had an effect on the development of the disease. There is evidence to suggest a relationship between the fibre content in the diet and atherosclerosis.[29-31] The effect of fibre is suggested to be through a lowering of cholesterol levels.[29,31] It has been shown[29] that some roughage sources have a hypocholesterolaemic action in rabbits, particularly wheat straw, and that the degree of atherosis is markedly reduced when compared to other fibre sources such as cellophane and Solkafloc. It was suggested[31] that one mechanism of action of the dietary fibre might be to decrease the reabsorption of bile salts in the gut, thus increasing faecal excretion and reducing hyperlipidaemia.

The increase in fibre in the diet from Experiment 1 to Experiment 2 might be expected to have a lowering effect on the plasma cholesterol levels[29] and hence a reduction in arterial disease.[29-31] The plasma cholesterol levels in the nine month part of Experiment 2 (MO diet: 1.53 ± 0.13 mmol/l; CO diet: 3.76 ± 0.52 mmol/l) were significantly lower ($p<0.05$) than those in Experiment 1 (MO diet: 2.76 ± 0.7 mmol/l; CO diet 6.26 ± 1.5 mmol/l). The plasma cholesterol levels of the rabbits in the twelve month part of Experiment 2

(MO diet: 2.01 ± 0.44 mmol/l; CO diet: 6.63 ± 1.1 mmol/l) did not differ significantly from the cholesterol levels in Experiment 1, agreeing with evidence that an increase in wheat bran does not exert an influence on hypercholesterolaemia.[211] If the increase in fibre in Experiment 2 had a significant effect on decreasing the arterial disease, it might not be entirely through its action on plasma cholesterol levels.

The possibility that the high calcification in some of the tissues of the rabbits might be due to hypervitaminosis D induced atherosclerosis was discussed earlier in Section 3.4. Tissue calcification was not reduced when the dietary vitamin D levels were halved. It would appear that the higher level of vitamin D in Experiment 1 did not induce atherosclerosis with extensive calcification, but rather that the calcification was a result of the advanced atherosclerosis.

Food antibodies were not shown to be significantly correlated with arterial disease when the passive haemagglutination assay (App.2, II) for detecting antibodies was used. However, in Experiment 1 (Table 2.7) a significant positive correlation ($p<0.05$) was found when the turbidimetric assay (App.2, I) was used. The haemagglutination assay was only used in the later experiments because it was found to be more sensitive and reliable. The turbidimetric assay was based on the fact that when antigen and antibody are mixed in solution a precipitate is produced. However, the soya proteins used in the diets were found to come out of solution on the addition of some control sera samples and consequently, the results were misleading. The standard curve for this assay is shown in Figure 5.1a which differs from that produced in most antigen-antibody systems (Figure 5.1b). It was therefore decided that as the soya proteins acted unusually in solution a solid phase assay must be used. In the haemagglutination assay the antigen (soya protein) is attached to red blood cells and any precipitate formed by the soya protein coming out of solution can be washed away.

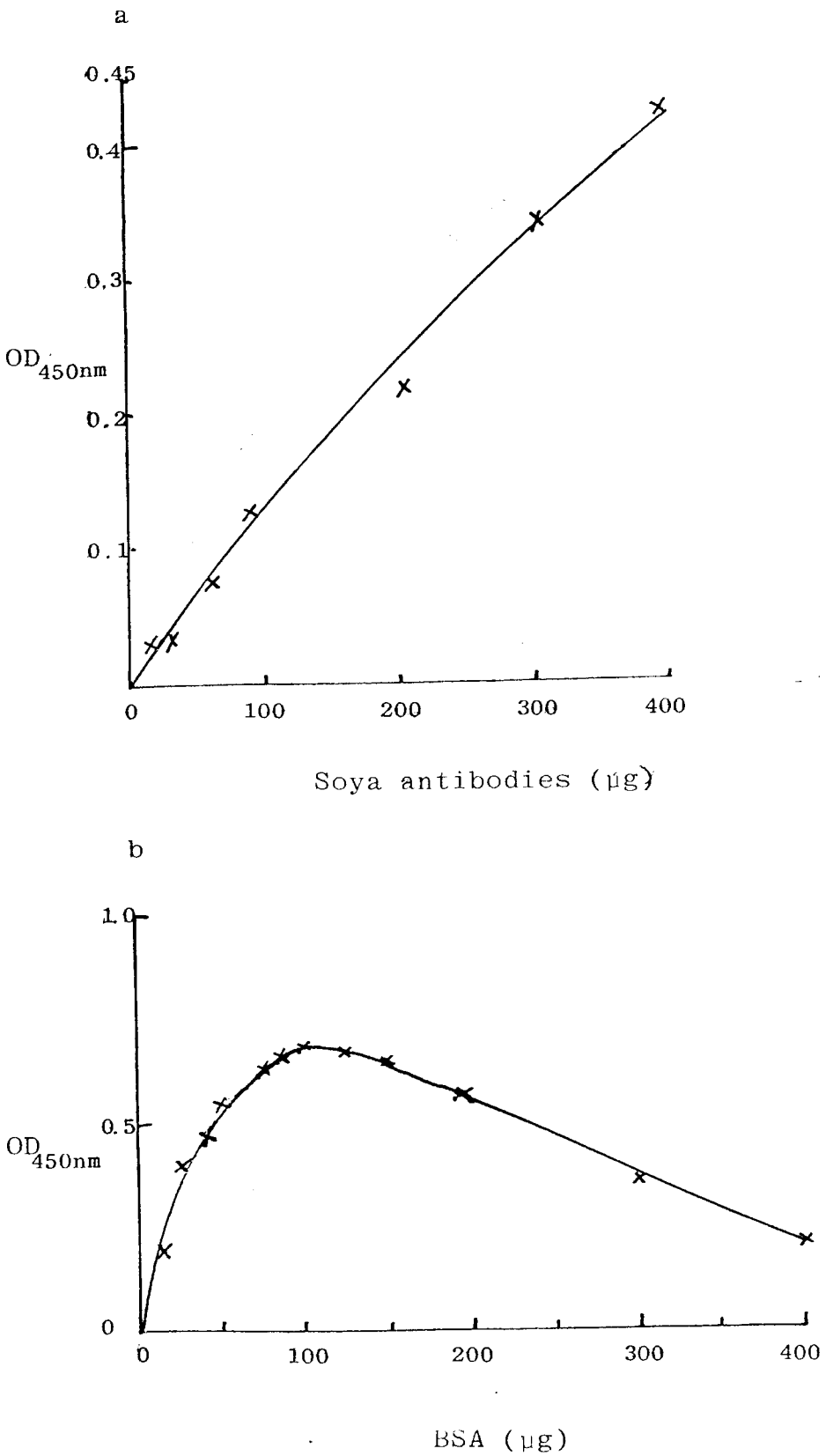


Figure 5.1: Standard Curves

a Turbidimetric Assay

b Precipitation Curve of BSA with a-BSA

When planning these experiments, the two soya proteins used were chosen because PD had been shown to be antigenic[180,181] and Ps had been shown to be of low antigenicity in pre-ruminant calves.[180] The present experiments do not corroborate this evidence as the mean food antibody titres to Ps (Experiment 1: 360.0 ± 205; Experiment 2 (12 months): 6.67 ± 4.7; Experiment 3: 64.0 ± 39.2) did not significantly differ from antibodies raised in response to PD (Experiment 1: 355.0 ± 162; Experiment 2: 72.0 ± 32.6; Experiment 3: 24.0 ± 16) at the end of each experiment. Therefore, in these experiments Ps has been shown to be antigenic in rabbits.

The animals in Experiment 1 had a significantly higher mean food antibody titre ($p<0.01$) of 357.5 ± 126 than those in Experiment 2 which had a mean titre of 39.0 ± 18. The reduction in arterial disease in Experiment 2 might be associated with the lower antibody titres. The reason for this reduction in immune response in the rabbits of Experiment 2 and 3 might be due to a state of immunological tolerance[197] to soya proteins in some of the rabbits.

Immunological tolerance can be produced when a potential antigen reaches the lymphoid cells during their developing phase in the perinatal period, at a time when the lymphoid cells are immunologically immature. If this antigen is later presented to the mature immune system then the normal response to the antigen will be suppressed. It was hypothesised[212] that the mature lymphocytes carry a record of patterns of 'foreign' proteins. When a 'foreign' protein is introduced into the immature immune system, this causes lymphoid cell depletion,[213] and if the 'foreign' protein is reintroduced it will be treated as a 'self' protein. The immune system does not normally raise antibodies to 'self' protein, hence the 'foreign' protein will not stimulate antibody production. It is now suggested that this is a simplistic view of immunological tolerance,[214] 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 the

tolerance is induced is that the antigen or antigen-antibody complex activates suppressor T-cells to release factors which stimulate the macrophages to release non-specific suppressor factors which act on the B-cells, so suppressing the antibody response. It has been shown, in newborn mice, that regeneration of antigen surface receptors on B-cells can be prevented by cross-linking by antigen, or by anti-immunoglobulin, so resulting in a state of tolerance.[215] 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, their products and possibly macrophages.[215]

Immunological tolerance can be induced in rabbits to foreign protein antigens by injections of these proteins during neonatal life.[199,201] The continuing state of tolerance to the antigen depends on the physical state of the antigen that is presented to the immune system.[202] Therefore, it might be that the rabbits in Experiments 2 and 3, which had already come into contact with the native soya protein in their mother's diet, either across the placenta or through the breast milk, would not raise antibodies to Ps or PD because they were tolerant to the soya proteins. However, in some rabbits, an antibody response was detected; this may have been caused by the soya protein crossing the intestine in a different conformation after partial digestion, and so reversing the state of immunological tolerance. Another possibility is that there might be more than one antigenic component of soya, and the rabbit is raising antibodies to a component of soya proteins that it had not previously come into contact with, that is peculiar to Ps or PD but not the soya meal given to the mother. To eliminate any effect that soya proteins present in the mother's diet might have on the immune response to soya antigens, rabbits would have to be raised from conception on a soya-free diet and then given a diet containing soya from weaning, thus repeating exactly the conditions used in Experiment 1.

Due to the occurrence of detectable levels of food antibodies in Experiment 1 coupled with the presence of arteriosclerosis

a mechanism whereby the food antibodies could cause the initiation of the disease has to be worked out. It was suggested[103] that the pathogenic agents mainly concerned in the arterial damage would be circulating soluble immune complexes. One mechanism by which they cause damage can be through the activation of complement. In Experiment 2 these two immunological parameters were measured in addition to food antibodies to try to assess which were the pathogenic agents. It was found that the levels of immune complexes were significantly correlated ($p<0.001$) with aorta score in the nine month part but not in the twelve month part (Table 3.15).

There is therefore some evidence to show that immune complexes might be associated with arteriosclerosis in rabbits. Total serum complement remained fairly constant in Experiment 2 and 3 after the first month. No correlation between serum complement and aorta score was found, this does not confirm the suggestion that complement is a factor involved directly in atherogenesis.[118]

The arterial disease produced in Experiment 1 was characterised by intimal thickening, calcification, disruption of the elastic lamellae and a dense mononuclear infiltrate with giant cells associated with the internal and external elastic lamellae. This differed from the arterial disease produced in a cholesterol-fed rabbit[1] as it was not a cholesterol-storage disease. In fact, there was a distinct lack of fat in most of the lesions except for a few small deposits of fat; this differs from the typical lesions produced in rabbits fed high saturated fat, cholesterol-free diets. In rabbits fed a 25% casein, 14% CO, cholesterol-free diet[60] there was extensive intimal proliferation with extracellular lipid and foam cells. The lipid deposition extended to the superficial media. Rabbits fed 14% MO had minimal intimal proliferation with only focal lipid deposition in the intima and innermost media,[60] clearly a very different histological picture from that described above.

The design of Experiment 2 was to investigate a progressive development of the disease from the initiation of lesions after three months on the diets, progressing to advanced

lesions after twelve months. However, this did not occur; no lesions were found in the rabbits on the three month feeding trial and the most extensive lesions were produced in the nine month part of this experiment. The disease produced in the rabbits in the twelve month part was minimal.

The arterial disease in the renal and carotid arteries of the rabbits in the nine month feeding trial was characterised by intimal thickening, calcification, disruption of the elastic lamellae, a mononumclear infiltrate with occasional giant cells. This was similar to the disease produced in Experiment 1 described above but the lesions were not so advanced.

There was some arterial disease produced in the twelve month part of Experiment 2. Some of the lesions produced here though could have been spontaneous. Spontaneous lesions have been noted in sexually mature rabbits which resemble those produced in the early stages in cholesterol-fed rabbits. The feature of these spontaneous lesions is an accumulation of lipid within the intima.[216] Another type of spontaneous lesion, more similar to the condition found in the twelve month rabbits, is characterised by slight medial degeneration and calcification. These spontaneous lesions are not of an atherosclerotic variety as they do not develop into atherosclerotic lesions.[217] A further group of rabbits (Experiment 3) fed on a low-unsaturated fat, high starch diet did not produce any significant disease.

The occurrence of an infiltration of giant cells in the experiments suggests a possibility either of a connection between giant cell arteritis and atherosclerosis, or of a common initiator of processes which will lead to the two conditions.

Giant cell arteritis,[192] is a condition in which the intima of the arteries becomes grossly thickened. Macrophages, lymphocytes, PMN, giant cells and fibroblasts all infiltrate into the arterial wall; this infiltration is most florid at the site of the internal elastic lamella. The giant cell accumulation is related to the elastic tissue,[189,193] which is markedly fragmented and deficient. There is evidence[192] to show that in humans the presence and severity of arteritic

involvement correlates well with the amount of elastic tissue in the arteries. The maximal inflammatory reaction and the presence of giant cells are both linked to the remains of elastic tissue. It has been suggested[192] that the disease is an allergic arteritis in which the arterial elastic tissue acts as the antigen.

Arteritis is an established component of immune complex disease[211] and, in acute serum sickness produced experimentally in rabbits, the distribution of the lesions is remarkably similar to that of atherosclerotic lesions, affecting the coronary arteries and the branch points of the aorta.[128] This is probably because similar haemodynamic and permeability mechanisms affect the arterial deposition of both immune complexes and lipoproteins. In hypervitaminosis D induced atherosclerosis in rabbits, some inflammatory cell infiltration is also seen in the systemic arteries with fragmentation of the internal elastica with calcified deposits.[218]

Arteritis, produced by inducing immune complex disease in rabbits, begins with a mild proliferation of intimal endothelial cells. This is followed by an infiltration, caused by acute inflammation, of mononuclear macrophages and foreign body giant cells.[219] Neutrophils enter the reaction site and rapidly degrade the underlying membranes, notably the interal elastic lamella, to gain entrance to the media and adventitia, fibrinoid necrosis then develops in the arterial wall. The pathogenic agents, which in this case are immune complexes, are removed extremely rapidly and are not seen under histological examination.[220] Induction of immune complex disease by injection with subsequent fat feeding in rabbits leads rapidly to atherosclerosis.[105-108] It might be that atherosclerosis and giant cell arteritis have a unifying mechanism of initiation in the arterial wall to start a process which then becomes self-perpetuating.

The use of diets for the induction of atherosclerosis by immunological means is necessary so that conditions which could occur in humans may be reproduced in the rabbit. A small, but immunologically significant amount of dietary protein can cross a mature mammalian intestine[94-98] and remain antigenic in experimental animals,[93] leading to the production

of antibodies.[142-160] By injecting foreign proteins into experimental animals, the levels of antigen far exceed those which could normally cross the intestine in humans. Although the absorption of proteins across the intestine in adults is very small, immune complexes containing very little antigen are capable of causing amine release from platelets [221] and arterial damage in animals.[111]

The intestine, though, is supposed to break down proteins so that they can be absorbed and utilised; therefore for antigenic quantities to be absorbed some mechanism must be not functioning properly. In order to find out how proteins can cross the intestine in antigenic quantities and so stimulate a systemic immune reaction, mechanisms by which the intestine keeps these antigens out of the circulation must be understood. Proteins should arrive at the small intestine epithelium as oligopeptides, containing 2-3 or probably more amino acids.[222] Some of these oligopeptides must be antigenic, since the optimal size of antigenic determinants is 3-6 amino acids.[223] On arrival at the epithelial surface it has been suggested [224] that there are intestinal antibodies on the mucous coat which act as receptors to link the antigen to the cell surface proteases,[225] thus immobilising the protein and aiding its breakdown. This process occurs either in the mucous coat of the cell or within digestive vacuoles.[222] Support [226] for this hypothesis is that pre-immunisation of rats with bovine serum albumin (BSA), causes further additions of BSA, the BSA to bind to antibodies at the cell surface and be rapidly broken down. In the absence of antibodies BSA was found intact on the serosal side of the gut.

The antibody at the mucosal surface has been shown to be IgA which is thought to be produced in lymphoblasts populating the lamina propria. These are probably derived from gut-associated lymphoid tissues (GALT) and are precommitted to IgA production before becoming distributed along the mucosal surface.[227] Activation of GALT is dependent on stimulation by antigens. Access to intestinal contents seems to be facilitated by clusters of specialised epithelial cells (M-cells).[228] After uptake into M-cells the antigen is rapidly released into the interstitial space and processed by the lymphoid

cells. Lymphoblasts committed to IgA synthesis then ultimately 'home' in on epithelial surfaces, particularly the lamina propria of the small intestine.[227]

A deficiency of IgA will cause malabsorption.[224] A partial deficiency in intestinal antibodies will give rise to small antigen-antibody complexes. The complexes will activate complement which will opsonise them, attract phagocytes to the site and the result will be an inflammation. This local inflammation will cause an alteration in the permeability of the gut causing an increased uptake of antigens which can then reach the serosal side of the intestine and cause a systemic immune reaction.

A newborn infant is deficient in IgA but when it receives colostrum from its mother it gets antibodies against antigens in the maternal diet,[229,230] small amounts of antigen[231] and immunocompetent cells.[232] Thus the newborn infant is immunised both actively and passively to prepare it for its eventual introduction to antigenic food. If, however, the infant is bottle-fed, the antibodies in the cow's milk are destroyed by the heating and drying process,[233] the antigens in the bottle-feed are given to the infant unapposed. The infant does develop antibodies but until then neonatal tolerance[234] may occur, in that no antibodies will be produced later as the antigen has been introduced to an immature immune system. There is increased permeability at this time and under-degraded antigens may reach the circulation and so stimulate systemic antibodies.[235] In this case, re-exposure at a time later in life when there is much less macromolecular absorption occurring, minute quantities of antigen may be absorbed and result in allergic symptoms.[236]

Therefore, the mechanism by which food antigens can reach the systemic circulation and hence initiate immune reactions might be either through a sensitisation process which occurred in infant life or via a partial deficiency of intestinal antibodies. If during a feeding trial of antigenic protein, a systemic antibody response is noted it might also be important to measure the level of secretary IgA levels.

If the antigen is constantly reapplied to a susceptible individual then the circulating immune complexes will not be cleared from the circulation and so damage to the arterial wall can result. Even if no more of the antigenic protein is applied to the system, once the immunological damage has occurred, other risk factors can aggravate the situation.[23,24] If this additional risk factor is fat then atherosclerosis may develop.

It is clear from these experiments that atherosclerosis can be produced by feeding diets which contain antigenic proteins and are high in fat. On the basis of these experiments, however, it is not possible to attribute the initiation and development of the disease to any single factor.

As dietary constituents are implicated in atherogenesis, there is a requirement for an experimental model which will produce atherosclerosis by the use of diets. The animals used must be started on the treatments under optimum conditions with no previous contact with the dietary protein and with regular monitoring of the serum for all the parameters which are known to affect the development of atherosclerosis.

APPENDIX 1

TABLE 1 Stock Diet RAG*

	Content g/kg diet
Maize	175
Wheatfeed	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 Limited, P O Box 6, Castle Street, Poole, Dorset.

TABLE 2 Composition of Diets (Experiment 1)

	Content g/kg diet		Content g/kg diet
Promine D	332	Prómosoy 100	426
Wheat bran	150	Wheat bran	150
Oil	190	Oil	190
Maize starch	163.8	Maize starch	61.5
Sucrose	100	Sucrose	100
*Minerals	54	*Minerals	54
**Vitamins	10	**Vitamins	10
Methionine	6	Methionine	4.2
KCl	2.2	NaCl	4.3

Metabolizable Energy 18.6 MJ/kg

Crude Protein 312 g/kg

* to provide /kg diet: 11.86 g Ca; 5.89 g P; 0.79 g Mg; 0.5 mg I; 10 mg Cu; 100 mg Fe; 100 mg Mn; 80 mg Zn; 0.1 mg Mo; 0.9 mg Cr; 0.1 mg Se.

** to provide /kg diet: 1500 µg retinol; 50 µg cholecalciferol; 7.75 mg α-tocopheryl acetate; 1.76 g choline chloride; 52.8 mg nicotinic acid; 26.4 mg calcium pantothenate; 8.8 mg pyridoxine hydrochloride; 3.52 mg menapthone; 8.8 mg riboflavin; 3.52 mg thiamin hydrochloride; 0.26 mg biotin; 2.64 mg folic acid; 35 µg cyanocobalamin; 1.76 g myo-inositol.

I

TURBIDIMETRIC ASSAY FOR FOOD ANTIBODIES

This assay utilised the fact that when antigen and antibody are mixed in solution a precipitate is formed. Serum was collected and stored at -20^{o}C. The antigen used for precipitating the antibodies was a solution of Promine D (PD) (Soya Central, Chicago, Ill.) prepared by shaking a suspension of 500 mg of the protein in 100 ml of 0.1 M phosphate buffer (3.468 g/l NaH_2PO_4; 10.096 g/l Na_2HPO_4) at pH 7.4 for 5-6 hours at room temperature. Undissolved material was removed by centrifugation and the supernatant was diluted with an equal volume of 0.1 M phosphate buffer. 1.9 ml of antigen solution containing 2.25 mg protein/ml was added to 0.1 ml serum, incubated for 1 hour at 37^{o}C and then read in a spectrophotometer (Unicam SP600) at 450 nm against a blank containing 1.9 ml buffer and 0.1 ml of the same serum. Precipitation of the solution of PD by serial dilutions of a standard serum; that is the serum of a rabbit that had been immunised against PD by intradermal injection; gave a straight line relationship and the unknown titres were calculated as percentages of the titre obtained with the standard serum (2.34 mg antibody/ml). This assay was only feasible when the antibody titres observed in the experimental animals were high and the concentration of antigen was such that all precipitates were below the equavalence point. Solutions of PD were used as the antigen because preliminary experiments showed that animals fed Promosoy 100 (Ps) (Central Soya, Chicago, Ill.) in the diet had mean titres to PD approximately 8 times greater than to Ps (32.0 and 4.0 respectively). Using rabbits fed on PD the differences were even greater (40.0: 3.2).

II

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 a food protein after first modifying their surface with tannic acid. A modification of an earlier haemagglination assay was used[287].

Preparation of Formalinised Chicken Erythrocytes

Chicken blood was drawn into 4% sodium citrate (1:9 v/v with 0.1 M phosphate buffer pH7.2 (PBS) (8.0 g/l NaCl; 0.2 g/lKCl; 1.15 g/l Na_2HPO_4; 0.2 g/l KH_2PO_4)). The citrated cells were then washed with PBS until the supernatant was clear, the cells were then suspended at a 7% suspension in PBS. An equal volume of 7% formaldehyde solution in PBS was prepared and one quarter of this was added to the cell suspension. The mixture was incubated with gentle shaking for 1 hour at 37°C, then the remaining formalin solution was added and the suspension was further incubated for 24 hours at 37°C with occasional shaking. The cells were then washed ten times in PBS and resuspended as a 10% solution in PBS containing 1:1000 sodium azide as an anti-bacterial agent and stored at 4°C.

Tanning and Antigen Sensitization of Erythrocytes

The formalised cells were washed and resuspended in PBS as a 3.75% solution. Equal volumes of cells and 0.005% tannic acid (Sigma Chemicals, St. Louis, Miss.) in PBS were mixed and incubated for 15 minutes at 37°C. The cells were then washed three times in PBS and resuspended at a 3.75% solution. One volume of 3.75% tanned cells was mixed with one volume of 0.5 mg/ml water-soluble soya protein (see below) in PBS and incubated for 30 minutes 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 minutes at 56°C and absorbed against chicken erythrocytes.

Preparation of Water Soluble Soya-Proteins

A 20% soya protein (either Promine D or Promosoy 100) 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 hour at 18000 r.p.m. on a centrifuge (MSE 18). The supernatant was placed on a freeze-drier until all the liquid has disappeared and the solid was then stored at 4°C in a dissicator.

ASSAY

Serum samples were taken and stored at -20°C until used. The frozen serum was defrosted and then heated in 0.05 ml aliquots to 56°C for ½ hour to remove complement. It was then incubated with formalized chicken erythrocytes for 15 minutes at room temperature to absorb and agglutinating agents. The cells were then spun down and 0.05 ml supernatant was used for one in two serial dilutions on V-shaped microhaemagglutination plates (Flow Labs, Ayrshire) using PBS. To these cells was added 0.05 ml sensitised erythrocytes. A control cell was also set up, this contained 0.05 ml supernatant and 0.05 ml washed non-sensitised erythrocytes, this is to make sure the serum does not agglutinate non-sensitised cells. Two more control wells containing either washed cells or sensitised cells and PBS were made up to check that no haemagglutination occurred in the absence of antibody. The cells were left to descend for a few hours at room temperature and then read. The titre of the serum was given as the highest dilution in which the cells had descended to give a carpet pattern. The cells at the bottom of the control wells should be button-like in appearance.

III

TISSUE AND PLASMA CHOLESTEROL AND TRIGLYCERIDES

Total cholesterol and triglyceride concentrations were determined with an Auto-analyser II (Technicon Instruments Co. Ltd, Basingstoke, Hants.) using Clinical Method AAII No. 24 (1972).

Plasma Cholesterol and Triglycerides

Blood was collected from the marginal ear vein in heparin (BDH Chemicals, Poole, Dorset) and then spun. 0.1 ml of plasma was added to 1.9 ml isopropanol with 2.0 g zeolite (Technicon) and then mixed for ½ hour at room temperature. The samples were spun and the content of the supernatant was then determined with an Auto-analyser II (Technicon Instruments Co. Ltd, Basingstoke, Hants.).

Liver Cholesterol and Triglyceride

A known weight of liver was homogenised in 10 ml of distilled water. 0.1 ml of this homogenate was then added to 1.9 ml isopropanol and 2.0 g zeolite and then processed the same as the plasma samples.

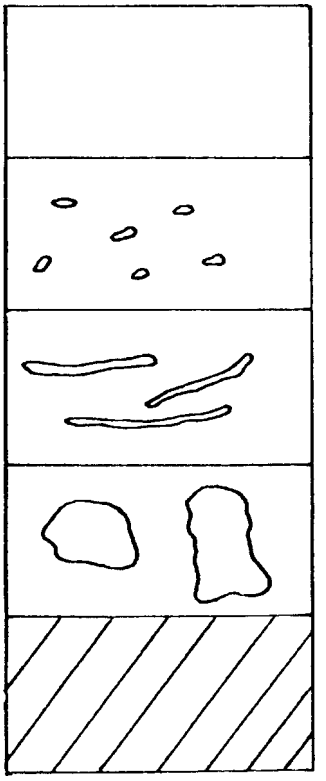
IV

SCORING OF ATHEROSCLEROSIS

The aorta was removed from the rabbit from the aortic valve to the abdominal aorta fixed in 10% buffered (PBS see II) formalin and stained with Sudan IV for fat (George T Gurr Limited, London) and scored with grades 1-5 as follows:[238]

Score	Condition
1	No atheroma
2	Flecks - small raised plaques
3	Fatty streaks
4	Large patches of calcified atheroma
5	Whole area calcified and ulcerated with atheroma

Macroscopic appearance of aorta



V

CALCIUM ESTIMATIONS

Calcium measurements were carried out using atomic absorption spectrophotometry.

Serum Calcium

0.1 ml fresh serum sample was mixed with 5.0 ml of lanthenum chloride buffer (10 mM $LaCl_3$; 50 mM HCl) and the solution was read on a flame spectrophotometer (SP90, Pye Unicam, Cambridge) at 422.7 nm using a Ca^{++} cathode lamp. Standard solutions of inorganic calcium were used to make a standard curve which is linear and from which the test readings can be converted to concentrations.

Tissue Calcium

Known weights of tissue were deposited in 10 ml of concentrated nitric acid until all the tissue was dissolved. 0.1 ml of the mixture was then added to 5.0 ml of lathenum chloride buffer and then treated as the serum samples.

VI

HISTOLOGICAL METHODS - EXPERIMENT 1

Samples of renal and carotid arteries and aortae from each animal were fixed in 10% buffered (PBS see II) formalin for histological examination. The tissues were then routinely processed into paraffin and stained with haematoxylin and eosin stain and with an elastic van Gieson stain. From a small number of rabbits with high aorta scores frozen sections of aortae and renal arteries were stained with oil Red O or Sudan Black (George T Gurr Limited, London) for fat depostion.

TABLE 3 Stock Diet RAG (M)*

	Content g/kg diet
Maize	175
Wheatfeed	322
Oatfeed	100
Apple Pomace	100
Soya	88
Linseed	50
Grass	100
English White Fish	50
RAG Premix etc	15

Metabolizable Energy 7.85 MJ/kg diet

Crude Protein 178.7 g/kg diet

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

TABLE 4 Composition of Diets (Experiment 2)

	Content g/kg diet		Content g/kg diet
Promine D	305	Promosoy 100	405
Wheat bran	200	Wheat bran	200
Oil	180.5	Oil	173
Maize starch	142.3	Maize starch	49.48
Sucrose	100	Sucrose	100
*Minerals	54	*Minerals	54
**Vitamins	10	**Vitamins	10
Methionine	6	Methionine	4.2
KCl	2.2	NaCl	4.32

Metabolizable Energy 16.93 MJ/kg

Crude Protein 306.6 g/kg diet

* to provide /kg diet: 11.86 g Ca; 5.89 g P; 0.79 g Mg; 0.5 mg I; 10 mg Cu; 100 mg Fe; 100 mg Mn; 80 mg Zn; 0.1 mg Mo; 0.9 mg Cr; 0.1 mg Se.

** to provide /kg diet: 1500 µg retinol; 25 µg cholecalciferol; 7.75 mg α-tocopheryl acetate; 1.76 g choline chloride; 52.8 mg nicotinic acid; 26.4 mg calcium pantothenate; 8.8 mg pyridoxine hydrochloride; 3.52 mg menapthone; 8.8 mg riboflavin; 3.52 mg thiamin hydrochloride; 0.26 mg biotin; 2.64 mg folic acid; 35 µg cyanocobalamin; 1.76 g myo-inositol.

VII

TOTAL SERUM COMPLEMENT DETERMINATION

Total serum complement was determined by use of the complement fixation assay [239]. The complement pathway is activated by antigen-antibody complexes which are represented in the assay by antibody-coated erythrocytes. As complement is added to the cells an increasing proportion of them are lysed.

Complement Fixation Test

Fresh or stored serum in 0.05 ml volumes was incubated for 15 minutes at 37°C with 0.1 ml 6-10 MHD (minimal haemolytic dose, see below) haemolytic serum (Wellcome Reagents Limited, Beckenham, Kent) and 0.7 ml complement fixation diluent (CFD) (Oxoid Limited, Basingstoke, Hants). 0.1 ml of a 3% suspension of sheep red blood cells (Oxoid Limited, Basingstoke, Hants) were then added and the mixture further incubated for 15 minutes at 37°C. The remaining cells were spun down and the supernatant was read at 450 nm on a spectrophotometer (SP 600) for haemolysis. By using the reading of complete haemolysis of the cells; percentage haemolysis can be worked out. A standard curve (see below) using known concentrations of complement was constructed and the complement concentrations in the serum read off.

Minimal Haemolytic Dose (MHD) of Antibody

The guinea pig complement (Miles Labs. Limited, Stoke Pages, Bucks.) was diluted to a $\frac{1}{50}$ solution in PBS and 0.1 ml volumes of this was added to 0.1 ml of serial dilutions of haemolytic serum and made up to 1.0 ml with CFD. This mixture was then incubated for 15 minutes at 37°C. 0.1 ml of 3% sheep red blood cells which had been washed in PBS were added to the mixture and this was incubated for a further 15 minutes at 37°C. The remaining cells were spun down and the supernatant read at 450 mm on a spectrophotometer (SP 600) for haemolysis. The MHD of haemolytic serum was the highest dilution that gave maximal haemolysis.

Estimation of 1CH50 Unit of Complement

0.1 ml of 6-10 MHD of haemolytic serum was incubated with 0.1 ml of increasing volumes (0-100 µl) of $\frac{1}{10}$ dilution of guinea pig complement for 15 minutes at 37°C. 0.1 ml of 3% suspension of washed sheep red blood cells were added to this and then incubated for a further 15 minutes at 37°C. The remaining cells were spun and the supernatant read at 450 nm in a spectrophotometer (SP 600) for haemolysis. A tube was included in the protocol with red cells and distilled water which represented 100% haemolysis. A curve was then constructed of percentage haemolysis against volume of complement (Fig. A1). One CH50 unit of complement was the amount which caused 50% lysis of the sheep red blood cells. A curve of percentage haemolysis against concentration of complement (CH50 units/ml) was then constructed by repeating the assay using known concentrations of complement (0-2.5 CH50 units/ml).

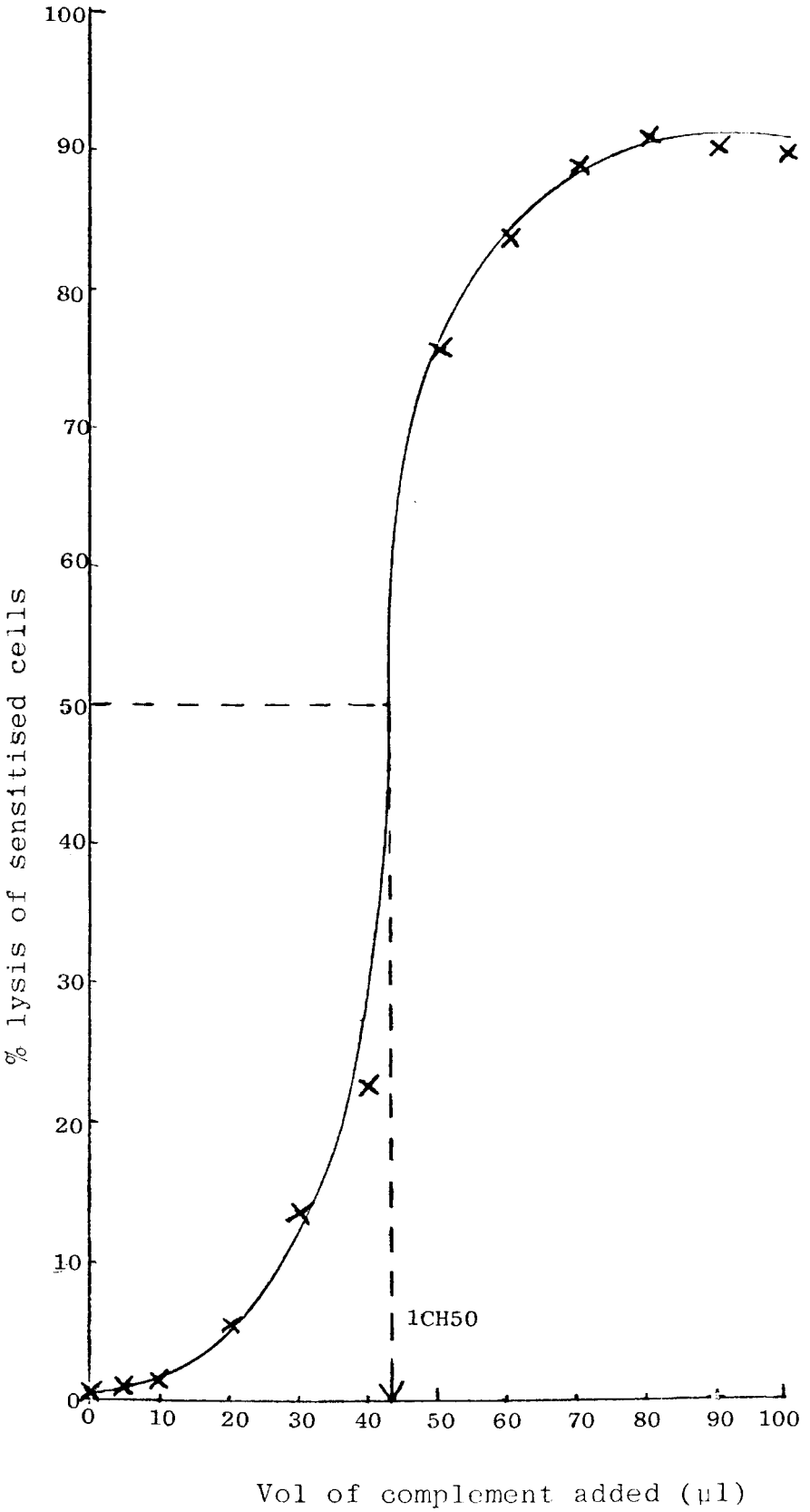


Figure A1: Standard Curve for Complement Fixation

VIII

IMMUNE COMPLEX DETECTION

This assay utilises the fact that immune complexes fix complement: it is called an anticomplementary assay[198]. Serum samples, either fresh or after storage at -70°C, were heated to 56°C for 30 minutes in 0.1 ml volumes. 2.5 CH50 units of guinea pig complement (Miles Labs Limited, Stoke Pages, Bucks) was added to each sample and incubated for 30 minutes at 4°C. 3% red blood cells (Oxoid Limited, Basingstoke, Hants) sensitised with haemolytic serum (Wellcome Reagents Limited, Beckenham, Kent) were added to the mixture and incubated for 15 minutes at 37°C. The samples were then diluted to 1.0 ml with CFD (Oxoid Limited, Basingstoke, Hants) and after centrifugation at 3000 rpm for 5 minutes the haemolysis in the supernatant was measured at 450 nm on a spectrophotometer (SP 600). Using the standard curve construction using 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.

IX

ATHEROSCLEROSIS SCORING SYSTEM

This section is a more detailed procedure than the one described in IV. The whole aorta was removed from the rabbit and the intimal surface was subdivided into 8 sections as follows [47]:(i) the semilunar valves, (ii) the junction of the aorta with the coronary arteries, (iii) the aortic arch, (iv) the junction of the innominate artery with the aorta, (v) the ductus arteriosus, (vi) the thoracic aorta, (vii) the antero-renal abdominal aorta, (viii) the postero-renal abdominal aorta. Each region was then scored individually as in IV and the scores were then totalled.

X

HISTOLOGICAL METHODS - EXPERIMENT 2 AND 3

Samples, from all animals, of renal and carotid arteries, aortae and kidneys were fixed in either 10% buffered (PBS) formalin or 6% cacodylate buffered (PBS) glutaraldehyde. The tissue fixed in formalin was routinely processed into paraffin and stained with haemotoxylin and eosin stain and with an elastic van Gieson stain. Samples of renal artery and kidney were frozen in liquid nitrogen for immunohistochemical study. The brain and samples of heart, ileum and colon were fixed in 10% buffered (PBS) formalin.

TABLE 5 Composition of Diets (Experiment 3)

	Content g/kg diet		Content g/kg diet
Promine D	254.65	Promosoy 100	338.14
Wheat bran	167	Wheat bran	167
Oil	20	Oil	20
Maize starch	398.07	Maize starch	314.26
Sucrose	100	Sucrose	100
*Minerals	43.94	*Minerals	43.94
**Vitamins	8.14	**Vitamins	8.14
Methionine	6.0	Methionine	4.2
KCl	2.2	NaCl	4.32

Metabolizable Energy 13.93 MJ/kg diet

Crude Protein 313.15 g/kg diet

* to provide /kg diet: 9.65 g Ca; 4.79 g P; 0.64 g Mg; 0.407 mg I; 8.14 mg Cu; 81.4 mg Fe; 81.4 mg Mn; 65.12 mg Zn; 0.08 mg Mo; 0.733 mg Cr; 0.08 mg Se.

** to provide /kg diet: 1221 µg retinol; 20.35 µg cholecalciferol; 6.31 mg α-tocopheryl acetate; 1.433 g choline chloride; 42.98 mg nicotinic acid; 21.49 mg calcium pantothenate; 7.16 mg pyridoxine hydrochloride; 2.87 mg menapthone; 7.16 mg riboflavin; 2.87 mg thiamin hydrochloride; 0.212 mg biotin; 2.15 mg folic acid; 28.49 µg cyanocobalamin; 1.433 g myo-inositol.

APPENDIX 2

Table 1a: Serum Calcium (mmol/l) (3 months)

DIET	TIME (WEEKS) 2	4	6	8	10	12
PsMO	3.4 ±0.5	3.8 ±0.28	3.85 ±0.08	3.63 ±0.33	3.03 ±0.13	3.75 ±0.05
PsCO	2.93 ±1.75	3.5 ±0.45	3.93 ±0.05	3.9 ±0.18	3.08 ±0.08	3.48 ±0.1
PDMO	3.15 ±0.5	3.58 ±0.6	3.95 ±0.45	3.7 ±0.35	3.28 ±0.19	3.55 ±0.09
PDCO	3.68 ±1.25	3.8 ±0.35	4.05 ±0.15	4.05 ±0.35	3.23 ±0.09	3.73 ±0.08

Table 1b: Serum Calcium (mmol/l) (9 months)

DIET	TIME (MONTHS) ½	1	1½	2	2½	3	4	5	6	7	8	9
PsMO	3.98 ±0.12	4.1 ±0.11	4.03 ±0.09	3.4 ±0.06	3.4 ±0.06	3.03 ±0.23	3.8 ±0.14	4.05 ±0.08	3.47 ±0.06	3.5 ±0.2	3.2 ±0.08	3.19 ±0.09
PsCO	4.25 ±0.24	4.35 ±0.3	4.28** ±0.03	3.83 ±0.3	3.25 ±0	3.28 ±0.03	3.54 ±0.02	4.23 ±0.24	3.47 ±0.1	3.39 ±0.06	3.29 ±0.05	3.48 ±0.14
PDMO	4.08 ±0.32	4.35 ±0.3	4.0 ±0.08	3.35 ±0.06	3.4 ±0.06	3.38* ±0.06	3.75 ±0.09	3.86 ±0.12	3.44 ±0.1	3.58 ±0.07	3.24 ±0.05	3.43 ±0.13
PDCO	4.1 ±0.19	4.03 ±0.25	4.68** ±0.27	3.83 ±0.31	3.4 ±0.06	3.63* ±0.08	3.81 ±0.04	4.04 ±0.1	3.52 ±0.08	3.66 ±0.13	3.35 ±0.04	3.63 ±0.12

* Significantly greater than corresponding Ps Values ($p<0.05$)
** Significantly greater than corresponding MO Values ($p<0.01$)

Table 1c: Serum Calcium (mmol/l) (12 months)

DIET	TIME (MONTHS) ½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	3.11 ±0.07	3.30 ±0.08	3.62 ±0.09	3.26 ±0.04	2.65 ±0.03	4.71 ±0.21	3.18 ±0.21	2.68 ±0.18	2.35 ±0.16	2.13 ±0.11	2.39 ±0.09	2.50 ±0.16	2.47 ±0.12
PsCO	3.64 ±0.56	3.42 ±0.07	3.67 ±0.11	3.32 ±0.05	2.90 ±0.09	3.47 ±0.18	3.44* ±0.17	3.10 ±0.15	2.77 ±0.21	2.25 ±0.17	2.47 ±0.13	2.87 ±0.04	2.61 ±0.11
PDMO	2.91 ±0.08	3.45 ±0.05	4.45*** ±0.15	3.51 ±0.18	3.02** ±0.07	3.13 ±0.17	3.08 ±0.16	2.90 ±0.16	2.71 ±0.25	2.61 ±0.12	2.48 ±0.16	2.50 ±0.16	2.48 ±0.12
PDCO	2.71 ±0.07	3.39 ±0.07	3.98*** ±0.14	3.36 ±0.02	2.99** ±0.05	3.57 ±0.05	3.54* ±0.06	3.06 ±0.06	2.70 ±0.06	2.37 ±0.2	2.61 ±0.11	2.71 ±0.08	2.71 ±0.05

*** Significantly higher than corresponding Ps levels ($p<0.001$)

** Significantly higher than corresponding Ps levels ($p<0.01$)

* Significantly higher than corresponding MO levels ($p<0.05$)

Table 2a: Plasma Cholesterol (mmol/l) (3 months)

DIET	TIME (WEEKS) 2	4	6	8	10	12
PsMO	2.14 ±0.11	1.96 ±0.27	1.84 ±0.1	1.81 ±0.24	1.95 ±0.38	1.25 ±0.2
PsCO***	4.11 ±0.46	5.07 ±0.36	4.86 ±0.59	6.02 ±0.63	4.61 ±0.55	4.66 ±0.58
PDMO	2.35 ±0.21	2.58 ±0.55	1.84 ±0.3	1.85 ±0.35	1.91 ±0.38	1.43 ±0.27
PDCO***	4.59 ±0.58	5.54 ±0.86	5.35 ±0.55	6.56 ±1.7	4.97 ±0.76	4.63 ±0.76

*** All values in CO groups significantly higher than MO values ($p<0.001$)

Table 2b: Plasma Cholesterol (mmol/l) (6 months)

DIET	TIME (MONTHS) 0	1	2	3	4	5	6
PsMO	2.96 ±1.89	3.42† ±0.56	2.69† ±0.34	2.49† ±1.86	2.23 ±0.2	2.04 ±0.23	1.89 ±0.28
PsCO	3.91 ±0.81	7.58† ±3.1	6.33†* ±2.05	5.44†** ±1.46	5.26* ±2.35	5.07** ±1.59	4.59** ±1.66
PDMO	3.34 ±1.73	1.09 ±0.31	1.25 ±0.26	1.21 ±0.3	1.27 ±0.32	1.21 ±0.28	1.23 ±0.31
PDCO	2.5 ±1.7	0.91 ±0.16	2.29* ±0.27	3.15** ±0.3	3.69* ±0.28	4.09** ±0.37	4.18* ±0.5

† Significantly higher than corresponding PD values ($p<0.05$)

* Significantly higher than corresponding MO values ($p<0.05$)

** Significantly higher than corresponding MO values ($p<0.01$)

Table 2c: Plasma Cholesterol (mmol/l) (12 months)

DIET	TIME (MONTHS) ½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	1.87 ±0.12	1.39 ±0.1	1.85 ±0.16	3.24 ±0.39	4.61 ±0.8	1.85 ±0.08	1.85 ±0.18	1.69 ±0.18	1.36 ±0.28	1.48 ±0.39	1.30 ±0.24	1.32 ±0.85	1.40 ±0.06
PsCO	3.41*** ±0.34	4.59*** ±0.73	5.26*** ±1.0	8.65*** ±1.46	7.4** ±1.81	6.88** ±2.44	7.06*** ±1.97	6.81*** ±2.0	6.27*** ±2.09	5.71** ±2.17	5.67** ±1.96	6.06* ±2.11	4.18*** ±1.02
PDMO	1.99 ±0.16	1.59 ±0.12	2.00 ±0.06	3.85 ±0.35	3.39 ±0.63	2.09 ±0.48	2.05 ±0.33	1.80 ±0.16	1.74 ±0.12	1.74 ±0.34	1.59 ±0.2	1.84 ±0.38	1.67 ±0.24
PDCO	3.57*** ±0.33	4.03*** ±0.59	4.82*** ±0.25	6.41*** ±1.56	8.31** ±1.19	5.59** ±1.07	6.45*** ±1.01	5.72*** ±0.75	5.39*** ±0.56	4.15** ±0.32	3.68** ±0.39	3.39* ±0.61	3.19*** ±0.35

* Significantly greater than corresponding MO values ($p<0.05$)

** Significantly greater than corresponding MO values ($p<0.01$)

*** Significantly greater than corresponding MO values ($p<0.001$)

Table 3a: Plasma Triglycerides (mg/l) (3 months)

DIET	TIME (WEEKS) 2	4	6	8	10	12
PsMO	638 ±62.1	530 ±55.3	486 ±55.1	312 ±96.4	260 ±52.1	160 ±36.3
PsCO	604 ±65.2	586 ±125	600* ±61.6	406* ±42.7	318 ±51.5	310** ±44.3
PDMO	780† ±122	626† ±84	586 ±58	214 ±16.9	326 ±48.7	260† ±26.5
PDCO	810† ±54.6	812† ±96	854* ±134	454* ±73.1	432 ±96.6	400†** ±59.3

† Significantly higher than corresponding Ps values ($p<0.05$)

* Significantly higher than corresponding MO values ($p<0.05$)

** Significantly higher than corresponding MO values ($p<0.01$)

Table 3b: Plasma Triglycerides (mg/l) (6 months)

DIET	TIME (MONTHS) 0	1	2	3	4	5	6
PsMO	420 ±218	410††† ±21.4	367††† ±25.8	316††† ±11.1	265 ±9.7	245 ±12.2	205 ±24.6
PsCO	540 ±76	726†††*** ±36	560†††*** ±33.9	469†††*** ±34.1	365** ±22.8	338** ±19	290* ±16.9
PDMO	512 ±77	290 ±21.7	252 ±18.9	244 ±18.1	232 ±15.7	219 ±19.4	224 ±18.6
PDCO	526 ±97	350*** ±20.7	305*** ±22.3	309*** ±32.6	300** ±36	274** ±33.5	268* ±32.6

†††Significantly higher than corresponding PD values ($p<0.001$)

*Significantly higher than corresponding MO values ($p<0.05$)

**Significantly higher than corresponding MO values ($p<0.01$)

***Significantly higher than corresponding MO values ($p<0.001$)

Table 3c: Plasma Triglycerides (mg/l) (12 months)

	TIME (MONTHS)												
DIET	½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	494 ±144	320 ±86.2	256 ±135	234 ±52.3	414††† ±64.3	264* ±36	386 ±24.6	369 ±20.1	350 ±50.6	348 ±35.7	407 ±26.9	456 ±14.8	488 ±8.6
PsCO	424 ±31.8	356 ±11.4	362 ±87	758 ±469	256 ±75	504* ±257	882 ±395	587** ±79.4	599*** ±30.7	416 ±27.7	433 ±32.1	451 ±32.6	472 ±35.9
PDMO	710 ±137	320 ±69.6	230 ±7.7	364 ±4.1	138 ±23.1	296 ±24.2	380 ±20.7	381 ±26.0	389 ±34.8	416 ±31.6	436 ±17.8	455 ±12.0	486 ±10.8
PDCO	608 ±44.6	630 ±234	314 ±6.8	362 ±55.6	464††† ±86.7	260 ±30.3	428 ±28.3	437** ±36.2	497*** ±69.6	480 ±38.6	488 ±31.8	518 ±20.4	528 ±27.9

††† Significantly greater than corresponding CO value when Ps fed and MO values when PD fed ($p<0.05$)
* Significantly greater than corresponding PD values ($p<0.05$)
** Significantly greater than corresponding MO values ($p<0.01$)
*** Significantly greater than corresponding MO values ($p<0.001$)

Table 4a: Food Antibody Titre (3 months)

	TIME (WEEKS)					
DIET	2	4	6	8	10	12
PsMO	0	0	2.0 ±2.01	0	0	0
PsCO	0	0	0	0	0	0
PDMO	0	4.0 ±2.5	16.0 ±9.8	6.0* ±3.97	44.0 ±29.7	42.0 ±30.3
PDCO	0	10.0 ±7.72	42.0 ±30.3	30.0* ±14.1	40.0 ±30.3	98.0 ±63.2

*Significantly greater than corresponding PD values ($p<0.05$)

Table 4b: Food Antibody Titre (6 months)

DIET	TIME (MONTHS) 0	1	2	3	4	5	6
PsMO	0	2.0 ±2.01	0	3.33 ±3.35	0	0	6.67 ±3.35
PsCO	0	10.0 ±7.7	0	2.0 ±2.01	20.0 ±15.2	0	0
PDMO	0	46.0 ±29.3	30.0* ±14.1	40.0 ±17.9	38.0 ±17.1	128 ±78.4	320 ±185
PDCO	0	6.0 ±3.98	10.0* ±7.75	12.0 ±7.6	32.0 ±19.6	26.7 ±18.5	48.0 ±32.0

* Significantly greater than corresponding Ps values ($p<0.05$)

Table 4c: Food Antibody Titre/9 months

DIET	TIME (MONTHS) ½	1	1½	2	2½	3	4	5	6	7	8	9
PsMO	0	2.0 ±2.0	2.0 ±2.0	2.0 ±2.0	0	6.0 ±3.98	4.0 ±2.50	0	0	0	4.0 ±3.98	8.0 ±7.96
PsCO	0	0	8.0 ±7.96	0	0	0	0	0	0	2.0 ±2.0	2.0 ±2.0	0
PDMO	8.0 ±7.96	6.0 ±3.98	18.0 ±15.6	10.0 ±7.70	10.0 ±7.70	14.0 ±7.47	16.0 ±7.47	18.3 ±8.20	28.0* ±14.6	12.0 ±3.76	18.0 ±6.62	20.0 ±8.94
PDCO	256 ±256	128 ±128	256 ±256	64.0 ±64.0	64.0 ±64.0	2292 ±2287	36.0 ±31.1	16.0 ±16.0	10.0* ±7.74	8.0 ±8.01	8.0 ±8.01	8.0 ±8.01

*Significantly greater than corresponding Ps values ($p<0.05$).

Table 5a: Immune Complexes (CH50 consumed) (3 months)

DIET	TIME (WEEKS) 2	4	6	8	10	12
PsMO	0	0	0	0	0.04±0.02	0.06±0.04
PsCO	0	0	0.02±0.02	0.06±0.04	0.16±0.08	0.26±0.11*
PDMO	0	0	0	0	0.06±0.04	0.1 ±0.06
PDCO	0	0	0	0.08±0.06	0.2 ±0.09	0.8 ±0.13*

* Significantly higher than corresponding MO means ($p<0.05$)

Table 5b: Immune Complexes (CH50 consumed) (6 months)

DIET	TIME (MONTHS) 0	1	2	3	4	5	6
PsMO	0	0	0	0	0	0	0
PsCO	0	0	0	0.622††† ±0.33	0.398† ±0.29	0.44† ±0.28	0.43† ±0.29
PDMO	0	0	0	0.94††† ±0.24	1.25$^{\dagger *}$ ±0.21	1.43$^{\dagger *}$ ±0.23	1.42$^{\dagger *}$ ±0.28
PDCO	0	0	0	0.08 ±0.08	0.358* ±0.33	0.47* ±0.31	0.55* ±0.33

† Significantly greater than corresponding MO means when Ps fed and CO means when PD fed ($p<0.05$)

††† Significantly greater than corresponding MO means when Ps fed and CO means when PD fed ($p<0.001$)

* Significantly greater than Ps means ($p<0.05$)

Table 5c: Immune Complexes (CH50 consumed) (12 months)

DIET	TIME (MONTHS) ½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	0	0	0.77 ±0.08	0.87 ±0.23	0.3 ±0.28	0.32 ±0.27	0.33 ±0.26	0.28 ±0.2	0.37 ±0.25	0.44 ±0.27	0.48 ±0.29	0.46 ±0.28	0.47 ±0.29
PsCO	0	0	1.46 ±0.17	1.05 ±0.02	0.78 ±0.24	0.76 ±0.25	0.77 ±0.29	0.76 ±0.3	0.76 ±0.33	0.54 ±0.32	0.4 ±0.29	0.367 ±0.37	0.38 ±0.38
PDMO	0	0	1.3 ±0.2	1.2 ±0.08	0.91 ±0.28	0.97 ±0.3	0.89 ±0.32	0.87 ±0.33	0.95 ±0.38	1.18** ±0.33	1.08** ±0.31	1.13* ±0.32	1.11* ±0.31
PDCO	0	0	1.02 ±0.11	1.22 ±0.11	1.12 ±0.13	0.97 ±0.18	0.98 ±0.16	1.08 ±0.12	1.08 ±0.16	1.41** ±0.45	1.34** ±0.12	1.37* ±0.11	1.39* ±0.05

* Significantly higher than corresponding Ps means ($p<0.05$)

** Significantly higher than corresponding Ps means ($p<0.01$)

Table 6a: Serum Complement (CH50/ml) (3 months)

DIET	TIME (WEEKS) 2	4	6	8	10	12
PsMO	9.78±1.57	9.8 ±1.52	10.08±1.43	9.02±2.72	12.2 ±2.2	14.12±1.91*
PsCO	7.82±1.2	8.42±1.31	9.4 ±1.44	7.98±2.23	10.42±1.67	9.28±1.32
PDMO	11.4 ±0.86	11.4 ±0.98	12.24±1.03	12.3 ±2.98	11.7 ±2.84	12.8 ±3.17*
PDCO	12.3 ±2.54	11.72±2.58	12.8 ±2.45	13.26±1.11	12.94±1.55	9.08±1.87

* Significantly higher than corresponding CO means ($p<0.05$)

Table 6b: Serum Complement (CH50/ml) (6 months)

DIET	TIME (MONTHS) 0	1	2	3	4	5	6
PsMO	2.2 ±0.62	11.7 ±2.2	17.4 ±9.77	17.76 ±3.62	24.07 ±0.72	23.23 ±0.42	23.93 ±0.93
PsCO	2.85 ±0.57	14.53 ±1.22	25.6 ±11.48	25.18 ±4.56	24.74 ±4.7	24.36 ±4.7	24.54 ±4.43
PDMO	2.11 ±0.47	26.58 ±9.43	29.7 ±15.11	27.2 ±6.86	26.72 ±6.68	26.26 ±6.57	28.65 ±7.32
PDCO	1.95 ±0.63	21.24 ±6.36	28.2 ±10.42	19.3 ±1.03	19.02 ±0.84	19.18 ±0.85	19.58 ±1.14

Table 6c: Serum Complement (CH50/ml) (12 months)

DIET	TIME (MONTHS) ½	1	2	3	4	5	6	7	8	9	10	11	12
PsMO	7.68 ±4.09	8.48 ±4.0	19.56 ±0.81	9.36 ±4.53	17.58 ±1.27	16.52 ±0.98	17.9 ±1.35	16.46 ±0.59	16.14 ±1.08	21.2 ±5.4	21.5 ±5.03	18.12$^{\times}$ ±1.52	19.2 ±2.09
PsCO	17.21† ±2.27	17.8† ±2.25	18.17 ±2.23	21.74† ±3.72	18.4 ±2.33	16.3 ±0.63	17.46 ±0.52	16.62 ±0.8	14.76 ±1.58	17.68 ±2.84	16.8 ±2.27	15.54 ±1.7	14.8 ±0.98
PDMO	20.6† ±0.11	21.04† ±0.17	22.83 ±0.81	23.69† ±4.16	19.36 ±1.77	17.54 ±0.92	18.78 ±1.22	18.3 ±0.95	17.5 ±0.97	26.2 ±2.37	25.64 ±2.42	25.2$^{\times *}$ ±2.54	25.2 ±3.55
PDCO	16.46 ±3.75	17.08 ±3.64	17.71 ±3.77	15.72 ±3.77	14.98 ±3.09	17.22 ±1.41	18.72 ±1.58	16.28 ±1.66	14.36 ±1.58	17.14 ±2.92	17.52 ±1.76	17.85* ±1.86	20.05 ±3.62

† Significantly greater than corresponding MO means when fed Ps, and CO means when fed PD ($p<0.05$)

$^{\times}$ Significantly greater than corresponding CO means ($p<0.05$)

* Significantly greater than corresponding Ps means ($p<0.05$)

REFERENCES

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

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

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

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

5 C.W.M. ADAMS
Arteriosclerosis in man, other mammals and birds, Biological Reviews 39; 372-424 (1964).

6 J C Geer, M.D. HAUST
Smooth muscle cells in atheroma, Monographs on Atherosclerosis No. 2; 1-88 (1972) Karger, Basel.

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

8 C. VON ROKITANSKY
A Manual of Pathological Anatomy 261-272 (1852), Sydenham Society, London.

9 J.B. DUGUID
Thrombosis as a factor in pathogenesis of coronary atherosclerosis, J. Path. and Bact. 58; 207-212 (1946).

10 P. CONSTANTINIDES
Experimental Atherosclerosis, p20 (1965).

11 R. VIRCHOW
Gessamelte Abhandlungen zur wissenshaftlichen Medicin, p458 (1856), Phlogose und Thrombose im Geffesystem.

12 F. MARCHAND
Über Arteriosklerose, Verhandl. d. 21 kongr. f.innere Med. 21; 23-40 (1904).

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

14 A. KEYS
The risk of coronary heart disease, Circulation 23; 805-812 (1961).

15 J.C. KOSEK, C. BIEBER, R.R. LOWER
Heart Graft Arteriosclerosis, Transplant Proc. 3; 512-514 (1971).

16 M.D. ALTSCHULE
The Etiology of Atherosclerosis, Med. Clin. Nth. Am. 58; 397-399 (1974).

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

18 R. ROSS, J.A. GLOMSET, B. KARIYA, L. HARKER
Platelet-dependent serum factor that stimulates the proliferation of smooth muscle cells in vitro, Proc. Nat. Acad. Sci. 71; 1207-1210 (1974).

19 L. JORGENSEN, M.A. PACKHAM, H.C. ROWSELL, J.F. MUSTARD
Deposition of formed elements of blood on the intima and signs of arterial injury in the aorta, Lab. Invest. 27; 341-350 (1972).

20 M.B. STEMERMAN, R. ROSS
Experimental Arteriosclerosis, J. Exp. Med. 136; 769-789 (1972).

21 J. G. MUSTARD, M.A. PACKMAN
Factors influencing platelet function: adhesion, release and aggregation, Pharmac. Rev. 22; 97-187 (1970).

22 H. HOLMSEN, H.J. DAY
The selectivity of the thrombin-induced platelet release reaction, J. Lab. Clin. Med. 75; 840-846 (1970).

23 R. ROSS, J.A. GLOMSET
The pathogenesis of atherosclerosis, New England J. Med. 295; 369-377 (1976).

24 R. ROSS, J. A. GLOMSET
The pathogenesis of atherosclerosis, Pt. 2, New England J. Med. 295; 420-425 (1976).

25 H.J. WEISS
Platelet physiology and abnormalities of platelet function, Pt. 1, New England J. Med. 293; 531-541 (1975).

26 H.J. WEISS
Platelet physiology and abnormalities of platelet function, Pt. 2 New England J. Med. 293; 580-588 (1975).

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

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

29 J.H. MOORE
Effect of type of roughage in diet on plasma cholesterol levels and aortic atherosis in rabbits, J. Brit. Nut. 21; 207-215 (1967).

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

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

32 J. YUDKIN
Diet and coronary thrombosis, Lancet ii; 155-162 (1957).

33 J. YUDKIN
Dietetic aspects of atherosclerosis, Angiology 17; 127-133 (1966).

34 D. KRITCHEVSKY
Diet and atherosclerosis, Am. J. Path. 84; 615-632 (1976).

35 L.N. KATZ, J. STAMLER, L. HORLICK
Cholesterol metabolism in health and disease; its relationship to atherosclerosis, Amer. Practit. 1; 461-468 (1950).

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

37 A.C. ROBERTSON, D.A. KHAIRALLAH
Arterial endothelial permeability and vascular disease, Exp. Mol. Path. 18; 241-260 (1973).

38 P. ASTRUP, K. KJELDSON
Carbon monoxide, smoking and atherosclerosis, Med. Clin. Nth. Am. 58; 323-350 (1974).

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

40 D.L. FRY
Responses of the arterial wall to certain physical factors, Atherogenesis-Initiating Factors 12; p93-121 (1973).

41 R.W. WISSLER, D. VESSELINOVITCH
The effectos of feeding various dietary fats on the development and regression of hypercholesterolaemia and atherosclerosis, Adv. Exp. Med. Biol. 60; 65-76 (1975).

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

43 W.G. MACCALLUM
Acute and chronic infections as etiological factors, Arteriosclerosis - A survey of the problem; Cowdry p355-362 (1933).

44 T.W. MEADE, R. CHAKRABARTI
Arterial disease research: Observation or intervention?, Lancet ii; 913-916 (1972).

45 G.C. MCMILLAN, B.L. WEIGENSBERG, A.C. RITCHIE
Effects of dietary factors in rabbits fed cholesterol, A.M.A. Arch. Path. 70; 220-225 (1960).

46 W.B. KANNEL, W.P. CASTELLI, T. GORDON, P.M. MCNAMARA
Serum cholesterol, lipoproteins and the risk of C.H.D., Ann. Intern. Med. 74; 1-12 (1971).

47 J.H. MOORE
Diet and atherosis in rabbits, Br. J. Nut. 18; 253-273 (1964).

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

49 N. ANITSCHKOW, S. CHALATOW
Über experimentelle cholesterinsteatose und ihre Bedeuhung für die Enstehung eineger pathologischen Prozesse, Zbl. allg. Path. path. Anat. 24; 1-9 (1913).

50 J.P. STRONG, H.G. MCGILL
Diet and experimental atherosclerosis in baboons, Am. J. Path. 50; 669-688 (1967).

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

52 T.B. CLARKSON
Animal models for Atherosclerosis, Nth. Car. Med. J. 32; 88-98 (1971).

53 T. LEARY
The genesis of atherosclerosis, Arch. Path. 32; 507-555 (1941).

54 D. KRITCHEVSKY
Lipid Pharmacology, Experimental Atherosclerosis ed. R. Paoletti, N.Y. Acad. Pr. p63-130 (1964).

55 W.E. STEHBENS
The role of lipid in the pathogenesis of atherosclerosis, Lancet i; 724-727 (1975).

56 G. POPJÁK
Effect of feeding cholesterol without fat on plasma lipids of the rabbit, Biochem. J. 40; 608-621 (1946).

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

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

59 D. KRITCHEVSKY, S.A. TEPPER
Experimental atherosclerosis in rabbits fed cholesterol-free diets, J. Ath. Res. 4; 103-105 (1964).

60 D. KRITCHEVSKY
Comparison of peanut, corn, coconut oils and butter, Exp. and Mol. Path. 24; 375-391 (1976).

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

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

63 E.H. AHRENS Jnr, J. HIRSCH, K. OETTE, J.W. FARQUHAR
Carbohydrate-induced and fat-induced lipaemia, Trans. Ass. Amer. Physicians 74; 134-146 (1961).

64 F. GRANDE
Dietary carbohydrates and serum cholesterol, Am. J. Clin. Nut. 20; 176-184 (1967).

65 D. KRITCHEVSKY
Factors affecting atherosclerosis in rabbits fed cholesterol-free diets, Life Science 4; 1467-1471 (1965).

66 J. YERUSHALMY, H.E. HILLERBOE
Fat in the diet and mortality from heart disease, N.Y. State J. Med. 57; 2343-2354 (1957).

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

68 T.B. CLARKSON
Interactions among dietary fat, protein and cholesterol in atherosclerosis-susceptible pigeons, Circulation Res. 11; 400-404 (1962).

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

70 D. KRITCHEVSKY, S.A. TEPPER, J.A. STORY
Experimental Atherosclerosis in rabbits fed cholesterol-free diets, 6. Skim milk powder, Nut. Rep. Int. 13; 207-211 (1976).

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

72 D. KRITCHEVSKY, S.A. TEPPER, D.E. WILLIAMS, J.A. STORY
Experimental atherosclerosis in cholesterol-free rabbits, 7. Interaction of protein with fibre, Artherosclerosis 26; 397-403 (1977).

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

74 K.K. CARROLL
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 (1971).

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

76 K.B. KELLY, C.B. TAYLOR, G.M. HASS
Experimental atheroarteriosclerosis, localisation of lipids in experimental arterial lesions of rabbits with hypercholesterolaemia, A.M.A. Arch. Path. 53, 419-436 (1952).

77 A.W. WILLIAMS
Relation of atheroma to local trauma, J. Path. Bact. 81; 419-422 (1961).

78 P. CONSTANTINIDES
Ultrastructural Injury of the Arterial Endothelium, Arch. Path. 88; 99-105 (1969).

79 C.G. BECKER, G.E. MURPHY
Demonstration of contractile protein in endothelium, Am. J. Path. 55; 1-37 (1969).

80 N. WOOLF, J.W. BRADLEY, T. CRAWFORD, K.C. CARSTAIRS
Experimental mural thrombi in the pig aorta. The early natural history, Br. J. Exp. Path. 49; 257-264 (1968).

81 L.A. HARKER, S.J. SLICHTER, C.R. SCOTT, R. ROSS
Vascular injury and Arterial Thrombosis, New England J. Med. 241; 537-543 (1974).

82 N. WOOLF
The distribution of fibrin within the aortic intima, Am. J. Path. 39; 521-532 (1961).

83 D.F. DAVIES
The electrophoretic mobility of erythrocytes as a measure of surface activity, Clin. Sci. 17; 563-573 (1958).

84 D.F. DAVIES
An assessment of erythrocyte migration times and cholesterol levels as indices of M.I., Clin. Sci. 20; 279-287 (1961).

85 D.F. DAVIES, V. LLOYD
Relationship between platelet adhesiveness and plasma surface activity in C.H.D., Cardiovasc. Res. 1; 108-110 (1967).

86 D.F. DAVIES
A comparison of erythrocyte and chylomicron migration times and serum cholesterol levels in atheroma, Clin. Sci. 18; 263-268 (1959).

87 D.F. DAVIES
A new theory relating atherogenesis and C.H.D. to impairment of plasma surface activity, Am. Heart J. 70; 720-722 (1965).

88 D.F. DAVIES, A. CLARK
Increase of γG-globulin in coronary heart disease, J. Ath. Res. 8; 724-730 (1968).

89 D.S. NELSON
Immuno-adherence, Adv. in Immunol. 3; 131-180 (1963).

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

91 O.M. SCHLOSS
The intestinal absorption of antigenic protein, Harvey Lectures Ser. 20; 156-187 (1924-25).

92 A.R. RICH
Hypersensitivity in Disease, Harvey Lectures Ser. 42; 106-147 (1946).

93 A.L. WARSHAW, W.A. WALKER
Intestinal absorption of antigenic protein, Surgery 76; 495-499 (1974).

94 I.D. BERNSTEIN, Z. OVARY
Absorption of Antigens from the Gastrointestinal Tract, Int. Arch. Allergy Appl. Imm. 33; 521-527 (1968).

95 R. CORNELL, W.A. WALKER, K.J. ISSELBACHER
Small intestinal absorption of horseradish peroxidase. A cytochemical study, Lab. Invest. 25; 42-48 (1971).

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

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

98 A.L. WARSHAW, W.A. WALKER
Small intestine permeability to macromolecules, Lab. Invest. 25; 675-684 (1971).

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

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

101 W.A. WALKER, A.L. WARSHAW
Intestinal uptake of macromolecules 11. Effect of Parenteral Immunisation, J. Immunol. 111; 221-226 (1973).

102 W.A. WALKER, K.J. ISSELBACHER, K.J. BLOCH
Immunological control of soluble protein absorption from the small intestine, Am. J. Clin. Nut. 27; 1434-1440 (1974).

103 L. LEVY
A form of immunological atherosclerosis, The reticuloendothelial system and atherosclerosis p426, ed N.R. diLuzzio, R. Paoletti (1967).

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

105 M. VAN WINKLE, L. LEVY
Removal of cholesterol-diet upon serum sickness-cholesterol induced atherosclerosis, J. Exp. Med. 128; 497-517 (1968).

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

107 C.R. MINICK, G.E. MURPHY, W.G. CAMPBELL
Experimental induction of athero-arteriosclerosis by the synergy of allergic injury to arteries and lipid rich diet, J. Exp. Med. 124; 635-651 (1966).

108 C.R. MINICK, D.R. ALONSO, L.RANKIN
Immunologic Arterial Injury in Atherogenesis, Prog. in Biochem. Pharmocol. 13; 225-233 (1977).

109 H.V. LAMBERSON Jnr, K.E. FRITZ
Immunological Enhancement of Atherogenesis in rabbits, Arch. Path. 98; 9-16 (1974).

110 H.M. SHARMA J.C. GEER
Experimental aortic lesions of acute serum sickness in rabbits, Am. J. Path. 88; 255-266 (1977).

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

112 A. POMERANCE
Peri-arterial mast cells in coronary atheroma and thrombosis, J. Path. Bact. 76; 55-70 (1958).

113 W.T. KNIKER, C.G. COCHRANE
II Role of vascular amines and other haemodynamic factors, J. Exp. Med. 127; 119-136 (1968).

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

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

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

117 S. WIENER, S. LENDVAI, B. ROGERS, M. URIVETZKY, E. MEILMAN
Non-immune demotaxis in vivo: inhibition by complement depletion with cobra factor, Am. J. Path. 73; 807-816 (1973).

118 P. GEERTINGER, H. SØRENSEN
Complement and Arteriosclerosis, Atherosclerosis 18; 65-71 (1973).

119 E.A. KABAT, M.M. MAYER
Experimental Immunochemistry, 2nd edn. Thomas, Spingfield 1967 p133.

120 I. GIGLI, J.W. MASON, R.W. COLMAN, K.F. AUSTEN, B. ROBERT
Interaction of Kallikrein with the C1 esterase inhibitor, J. Immunol. 101; 814-815 (1968).

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

122 C.G. CARO, J.M. FITZ-GERALD, R.C. SCHROTER
Arterial wall shear and distribution of early atheroma in man, Nature 223; 1159-1161 (1969).

123 G.E. BURCH, C.Y. TSUI, J.M. HARB
Pathologic changes of aorta and coronary arteries of mice infected with Coxsackie B_4 virus, Proc. Soc. Exp. Biol. Med. 137; 657-661 (1971).

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

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

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

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

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

129 T. URIUHARA, H.Z. MOVAT
The role of PMN-leucocyte lysosomes in tissue injury, inflammation and hypersensitivity, Exp. Molec. Path. 5; 539-558 (1966).

130 U.E. NYDEGGER
Biological Properties and Detection of Immune Complexes in Animal and Human Pathology, Rev. Physiol. Biochem. Pharmac. 85; 64-125 (1979).

131 R.C. BAHLER, W.T. BUTLER
Antigenecity of cholesterol-induced chicken atheroma, Proc. Soc. Exp. Biol. 106; 383-387 (1961).

132 B. ROBERT, A.M. ROBERT
Mécanisms immunologiques dans l'athérsclérose, Méd. et Hyg. 27; 822-836 (1969).

133 A.M. ROBERT, Y. GROSGOGEAT, V. REVERDY, B. ROBERT, L. ROBERT
Arterial lesions produced in rabbits by immunisation with elastin and structural glycoproteins of aorta, Atherosclerosis 13; 427-449 (1971).

134 L. SCEBAT, J. RENAIS, N. GROULT, J. LENEGRE
A study of aortic tissue specificity, Adv. in Exp. Med. and Biol. 1; 451-463 (1961).

135 H.W. INTROP, F. MILGROM
Rabbit autoantibodies to aorta, J. Immunol. 103; 544-549 (1969).

136 M. TUSKIEWICZ, E. PLESZCZYŃSKA, J. TOMASZEWSKI
J. LOPATYŃSKI, J. HANZLIK
Antiorgan antibodies in rabbits fed atherogenic diet, Arch. Immun. Ther. Exp. 20; 485-489 (1972).

137 J.D. MATHEWS
Autoimmune mechanisms in human vascular disease, Lancet ii; 1423-1427 (1974).

138 J. POKORNY, Z. JEZKOVA
Significance of immunological studies in peripheral obliterating vascular diseases, Circulation Res. 11; 961-965 (1962).

139 F. STEIN, M.P. PEZESS, L. ROBERT, N. POULLAIN
Anti-elastin antibodies to normal and pathological human sera. Nature 207; 312-313 (1965).

140 H.W. INTROP, F. MILGROM, E. WITEBSKY
Antigens of normal and atherosclerotic intima, J. Immunol. 102; 1404-1410 (1969).

141 N. APOSTOLAKIS, K.O. VOIGT
Preparation of an antiserum against a glycoprotein isolated from sclerotic aortic media, J. Ath. Res. 6; 102-105 (1966).

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

143 P.F. HANSEN
Milk Proteins, Chem. and Mol. Biol. 1; 45-123 (1970).

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

145 K.B. TAYLOR, G.H. SPRAY, R.M. BANNERMAN
Serologic reactions to gluten and cow's milk protein in gastro-intestinal disease, Gastroent. 46; 99-108 (1964).

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

147 I. MACDONALD
Clinical effects of consuming milk and its products, Proc. Nut. Soc. 37; 241 (1978).

148 D.C. HEINER, J.W. SEARS, W.T. KNIKER
Multiple preciptins to cow's milk in chronic respiratory disease, Am. J. Dis. Child. 103; 634-654 (1962).

149 J.W. GERRARD, J.W. MACKENZIE, N. GOLUBOFF, J.Z. GARSON, C.S. MANINGAS
Cow's milk allergy: prevalence and manifestations in an unselected series of newborns, Acta Paediat. Scand. 234; 1-21 (1973).

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

151 M. VON WEST
Nahrungsmittelallergie, ins besondere Kuhmilchallergie bei Säuglingen, Ann. Paediat. 181; 277-294 (1953).

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

153 J.F. SOOTHILL
Allergy and infant feeding, Proc. Nut. Soc. 35; 283-284 (1976).

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

155 S. FREIER
Paediatric gastrointestinal allergy, J. Hum. Nut. 30; 187-192 (1976).

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

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

158 H. WERGELAND
Three fatal cases of probable familial allergy to human milk, Acta Paediat. 35; 321 (1948).

159 E.Z. MORTIMER
Anaphylaxis following ingestion of soybean J. Paediat. 58; 90-92 (1961).

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

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

162 G.R. OSBORN
Incubation Period of Coronary Thrombosis, p177, London (1963).

163 D.F. DAVIES, J.R. DAVIES, M.A. RICHARDS
Antibodies to reconstituted dried cow's milk protein in C.H.D., J. of Ath. Res. 9; 103-107 (1969).

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

165 A. TOIVANEN, M.K. VILJANEN, E. SAVILAHTI
IgM and IgG anti-milk antibodies measured by radio-immunoassay in myocardial infarction, Lancet ii; 205-209 (1975).

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

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

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

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

170 J.G. THOMSON
Production of severe atheroma in a transplanted human heart, Lancet ii; 1088 (1969).

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

172 J.L. BEAUMONT, V. BEAUMONT, M. ANTONUCCI
Presence d'un autoanticorps anti-βlipoprotéines dans le sérum d'un lapin avant une hyperlipdémie par immunisation, C.R. Acad. Sci. 268 D; 1830-1835 (1969).

173 V. JOFFE
K immunologitskeskoj characteritike eksperimental nogo aterosleroza, Bjull. Eksp. Biol. Med. 75; 72-75 (1973).

174 G. FÜST, E. SZONDY, J. SZÉKELY, I. NÁNAI, S. GERÖ
Studies on the occurrence of circulating immune complexes in vascular diseases, Atherosclerosis 29; 181-190 (1978).

175 G.H. GRESHAM, A.N. HOWARD
Is atheroma a reversible lesion?, Atherosclerosis 23; 379-391 (1976).

176 D. KRITCHEVSKY
Dietary Factors other than lipid, Atherosclerosis - is it reversible? p23-32 (1978) ed. G. Scheltler, E. Strange, R.W. Wissler, Spring/ Verlag, Berlin.

177 D. KRITCHEVSKY
Diet and Atherosclerosis, Am. J. Path. 84; 615-632 (1976).

178 N. YU ZABSCHITSKIY, V.A. NAGORNOV
Immunological Aspects of Atherosclerosis, Vestnik. Akad. Med. Navk. SSSR. No. 6 88-94 (1972).

179 D.F. DAVIES
Hypothesis: An immunological view of atherogenesis, J. Ath. Res. 10; 253-259 (1969).

180 R.H. SMITH, J.W. SISSONS
The effects of different feeds on the passage of digesta from the abomasum in the pre-ruminant calf, Br. J. Nut. 33; 329-349 (1974).

181 J.M. VAN LEEUWEN
De Veoderwaarde van Sojabloen mede in Vergelijking met mager melkpoeder, Versl. landbouwk. Onderzoek. Ned. No. 732; 1-19 (1969).

182 A.C. ROSS, C.R. MINICK, D.B. ZILVERSMITT
Equal atherosclerosis in rabbits fed cholesterol-free, low fat diets or cholesterol-supplemented diet, Atherosclerosis 29; 301-315 (1978).

183 C.R. MINICK, G.E. MURPHY
Experimental induction of atheroarteriosclerosis by the synergy of allergic injuries to arteries and lipid-rich diet, Am. J. Path. 73; 265-300 (1973).

184 G. WIGAND
Production of hypercholesterolaemia and atherosclerosis in rabbits by feeding different fats without supplementary cholesterol, Acta Med. Scand. Suppl. 166; 1-91 (1959).

185 J.P. FUNCH, B. KROGH, H. DAM
Effects of butter, some margarines and arachis oil in purified diets on serum lipids and atherosclerosis in rabbits, Br. J. Nut. 14; 355-360 (1960).

186 H.J. THOMASSON
The influence of fats on cholesterol metabolism, Biochemistry of Lipids 194-202 (1960).

187 R. BRATTSAND
Distribution of cholesterol and triglycerides among lipoprotein fractions in fat-fed rabbits at different levels of cholesterol, Atherosclerosis 23; 97-110 (1976).

188 K. HELLSTRÖM, J. SJÖVALL, G. WIGAND
The effect of fat in the diet on serum cholesterol and turnover of bile acids, Biochem. Pharmacol. 8; 87 (1961).

189 F. PARKER, L.A. HEALEY, K.R. WILSKE, G.F. ODLAND
Temporal Artery Alterations, Am. J. Path. 79; 57-80 (1975).

190 F.G. ZAK, M. HELPERN, D. ADLERSBERG
Stenosing coronary arteritis: its possible role in coronary artery disease, Angiology 3; 289-305 (1952).

191 W.G. SPECTOR, N. HEESOM
The production of granulomata by antigen-antibody complexes, J. of Path. 98; 31-39 (1969).

192 I.M.S. WILKINSON
Giant cell arteritis, Br. J. Hosp. Med. 9; 154-161 (1976).

193 W.E. STEHBENS
Pathology of Cerebral Blood Vessels, C.V. Bosby, St. Louis (1972).

194 D. GOMBAL
Vitamin D induced atherosclerosis and calcium metabolism in the arteries and heart, Fed. Proc. 37; 748 (1978).

195 H.E. CLARK, M.E. COATES, J.K. EVA, D.J. FORD, C.K. MILNER, P.N. O'DONAGHUE, P.P. SCOTT, R.J. WARD
Dietary standards for laboratory animals, J. of Lab. Animal Sci. Ass. 11; 1-28 (1977).

196 R.G. STEVENSON, N.C. PALMER, G.G. FINLEY
Hypervitaminosis D in rabbits, Canad. Vet. J. 17; 54-57 (1976).

197 F.M. BURNET
Self and Non-Self, Cambridge University Press (1969).

198 J.W. MOWBRAY
Circulating immune-complexes in dermatitis herpetiformis, Lancet i; 400-401 (1973).

199 R. HANON, J. OYAMA
Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age, J. of Immunol. 73; 49-55 (1954).

200 N. YADAV, M. KUMAR, R.N. CHAKRAVARTI, P.L. WAHI
Development of antibody mediated immune reaction in hyperlipidaemic atherosclerotic rabbits, Indian J. Exp. Biol. 15; 1206-1208 (1977).

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

202 W.O. WEIGLE
Immunologic Unresponsiveness, Textbook of Immunopathology Vol. 1; 60-75 (1968), ed. P.A. Miescher, H.J. Müller-Eberhard.

203 H. MICHELI, D. PAMETTA, C. JORNOT, J-R. SCHERRER
HDL in male relatives of patients with coronary heart disease, Atherosclerosis 32; 269-276 (1979).

204 T. GORDON, W.P. CASTELLI, M.C. HJORTLAND, W.B. KANNEL, T.R. DAWBER
HDL as a protective factor against coronary heart disease - The Framingham Study, Am. J. Med. 62; 707-714 (1977).

205 T.E. CAREW, S.B. HAYES, T. KOSHINSKY, D. STEINBERG
A mechanism by which HDL may slow the atherogenic process, Lancet i; 1315 (1976).

206 J.R. HESSLER, A.L. ROBERTSON Jnr, G.M. CHISOLM
LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture, Atherosclerosis 32; 213-229 (1979).

207 A. NOMA, S. MASUSHITA, T. KOMARI, K. ABE, H. OKABE, K. KURAMOTO, M. MURAKAMI
High and low density lipoprotein cholesterol in myocardial and cerebral infarction, Atherosclerosis 32; 327-331 (1979).

208 E.B. SMITH, R.S. SLATER
The relationship between low density lipoproteins in the aortic intima and serum lipid levels, Lancet i; 463-469 (1972).

209 M.J. ALBRINK, J.W. MEIGS, E.B. MAN
Serum lipids,hypertension and coronary artery disease, Am. J. Med. 31; 4-23 (1961).

210 L.A. CARLSON, L.E. BOTTIGER
Ischaemic heart disease in relation to fasting values of plasma triglycerides and cholesterol, Lancet i; 865-868 (1972).

211 K.K. CARROLL, R.M. HAMILTON, M.W. HUFF, A.D. FALCONER
Dietary fibre and cholesterol metabolism in rabbits and rats, Am. J. Clin. Nut. 31; S203-S207 (1978).

212 M. BURNET
The Clonal Selection Theory of Acquired Immunity? (1959), Cambridge University Press.

213 D.W. DRESSER, N.A. MITCHISON
The mechanism of immunological paralysis, Adv. Immunol. 8; 129-181 (1968).

214 R.B. TAYLOR
Suppressor cells in humoral immunity and tolerance, Br. Med. Bull. 32; No. 2 152-157 (1976).

215 J.H. HUMPHREY
Some reflections on principles and mechanisms of immunological tolerance, Br. Med. Bull. 32; No. 2 182-184 (1976).

216 J.H. BRAGDON
Spontaneous atherosclerosis in the rabbit, Circulation 5; 641-646 (1952).

217 M.D. HAUST, R.H. MORE
Spontaneous lesions of the aorta in the rabbit, Comparative Atherosclerosis 255-275 (1965), Harper & Row U.S.A.

218 G.M. HASS, R.E. TRUEHART, C.B. TAYLOR, M.STUMPE
An experimental histologic study of hypervitaminosis D, Am. J. Path. 34; 395-431 (1958).

219 D.O. ADAMS
The granulomatous inflammatory response, Am. J. Path. 84; 164-191 (1976).

220 C.G. COCHRANE, D. KOFFLER
Immune complex disease in experimental animals and man, Adv. Immunol. 16; 186-264 (1973).

221 D.J. GOCKE, A.G. OSLER
In vitro damage of platelets by an unrelated antigen-antibody reaction, J. Immunol. 94; 236-246, 247-252 (1965).

222 D.M. MATTHEWS
Absorption of amino acids and peptides from the intestine, J. Clin. Endocrinol. Metab. 3; 3-16 (1974).

223 E.A. KABAT
Structural concepts in Immunology and Immunochemistry (1968), Holt, Rinehart & Winston Inc., New York.

224 D.L.J. FREED, F.H.Y. GREEN
Antibody-facilitated digestion and its implications for infant nutrition, Early Human Development 1; 107-112 (1977).

225 A.M. UGOLEV
Progress report: membrane digestion, Gut 13; 735-747 (1972).

226 W.A. WALKER, M. WU, K.J. ISSELBACHER, K.J. BLOCH
Intestinal uptake of macromolecules III, J. Immunol. 115; 854-861 (1975).

227 W.A. WALKER, K.J. ISSELBACHER
Intestinal antibodies, Physiology in Medicine 297; 767-773 (1977).

228 R.L. OWEN
Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine, Gastroenterology 72; 440-451 (1977).

229 EDITORIAL:
Oral Immunisation and antibodies in milk, Lancet i; 77 (1976).

230 D.B.L. MCCLELLAND, T.T. MCDONALD
Antibodies to cow's milk proteins in human colostrum, Lancet ii; 1251-1252 (1976).

231 H. VORHERR
Drug excretion in breast milk, Postgrad Med. 56; 97-104 (1974).

232 J.W.M. LAWTON, K.F. SHORTRIDGE
Protective factors in human breast milk and colostrum, Lancet i; 253 (1977).

233 M. RAPTOPOULOU-GIGI, K. MARWICK, D.B.L. MCCLELLAND
Antimicrobial proteins in sterilised human milk, Br. Med. J. 1; 12-14 (1977).

234 I.M. ROITT
Essential Immunology (1974), Blackwell, Oxon.

235 S.C. TRUELOVE, D.P. JEWELL
The intestine in allergic disease, Clinical Aspects of Immunology (1975), ed. P.G.H. Gell, R.R.A. Coombs, P.J. Lachman, Blackwell, Oxon.

236 W.A. WALKER
Antigen handling by the gut, Arch. Dis. Childhood 53; 527-531 (1978).

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

238 P. KING, J.P. ROYLE
Autogenous vein grafting in atheromatous rabbits, Cardiovasc. Res. 6; 627-633 (1972).

239 P.J. LACHMAN, M.J. HOBART, W.P. ASTON
Complement Technology, Handbook on Experimental Immunochemistry, Vol. 1, Ch. 5.1, ed. D.M. Weir.

240 G.C. LIANG, P.A. SIMKIN, M. MANNIK
Immunoglobulins in temporal arteries: An immunofluorescent study, Ann. Intern. Med. 81; 19-24 (1974).