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Effects Of Dietary Casein And Soy Protein On Lipoprotein Metabolism In Rabbits

Pramod Khosla

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EFFECTS OF DIETARY CASEIN AND SOY PROTEIN ON LIPOPROTEIN METABOLISM IN RABBITS

by

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the requirements for the degree of
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ABSTRACT

Rabbits fed low-fat, cholesterol-free, semi-purified diets containing casein become hypercholesterolemic, whereas rabbits whose dietary casein has been replaced by soy protein, maintain low levels of plasma cholesterol. Experiments were carried out in attempt to further understand this phenomenon.

Time course studies showed that on being transferred from a Chow diet, plasma cholesterol levels increased markedly in casein-fed rabbits. The increase was particularly prominent in the LDL fraction. The increases in LDL cholesterol and protein were appreciable in the first two weeks. Subsequently LDL cholesterol increased more slowly, and LDL protein levels held steady. These increases in LDL preceeded increases in VLDL (and HDL). In contrast rabbits transferred to soy protein maintained low levels of plasma cholesterol throughout the study.

To ascertain if the elevation in LDL in casein-fed rabbits resulted from increased production and/or decreased catabolism of these particles, tracer kinetic studies were carried out. Since LDL is the catabolic product of VLDL, dual-labeled isotope studies were carried out to see if VLDL catabolism contributed to the expanded LDL pool in casein-fed rabbits. These studies showed that the increased LDL pool resulted principally from a decreased efficiency of removal. In addition, the

expanded LDL pool was the result of increased LDL production via VLDL-independent pathways. Precursor-product relationships suggested that LDL was being secreted directly into the circulation.

Further studies were conducted to see if the diminished catabolism of LDL was due to the receptor-mediated or receptor-independent process, and whether this may be due to any changes in the LDL particles themselves. These results clearly showed that decreased LDL catabolism in casein-fed rabbits was due to impaired receptor-dependent catabolism and not to any fuctional abnormalities in the LDL particles themselves. Further experiments on the time-course of impaired receptor-dependent catabolism of LDL in casein-fed rabbits, showed that this down-regulation could be detected within 5 days of casein-feeding and, before, any detectable increase in plasma cholesterol.

Experiments were then conducted to see if the impaired receptor-mediated catabolism in casein-fed rabbits resulted from differences in the digestibility of the dietary proteins, in vivo. The results of these studies collectively suggested that in rabbits fed casein diets, there was decreased digestion in the stomach and increased digestion in the proximal intestine, probably as a result of differing pH-dependent changes which affect the solubility of casein. There was no difference in the

bile acid content in the intestinal contents of casein and soy protein-fed rabbits, suggesting that the difference in plasma cholesterol in the two dietary groups may not be due to differential sequestering of bile acids by the protein digestion products, in vivo. The possibility that casein-induced hypercholesterolemia resulted from impaired release of Cholecystokinin (CCK) was tested by measuring postprandial release of CCK in rabbits fed the two diets. Casein-fed rabbits had significantly lower levels of CCK. Exogenous injections of CCK into casein-fed rabbits tended to slow the development of hypercholesterolemia.

These studies show that elevated LDL pool in rabbits fed casein diets results principally from impaired receptor-dependent LDL catabolism. Furthermore these studies show that the changes are due specifically to the protein component of the diet since they did not occur with soy protein diets. These changes may be hormonally mediated.

For my Mother's love and my Father's sacrifices

I am eternally grateful.

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I am extremely grateful to my supervisor Dr. Ken Carroll for his constant support, guidance and encouragement throughout my project. My sincerest thanks to my Advisory Committee members, Drs. Murray Huff and Stan Dunn for their constructive criticisms, extreme patience and valuable suggestions which have helped me to reach this stage.

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addition to providing chemical analysis of the lipoproteins, yielded a method for their isolation which is routinely used today in most laboratories.

Lipoproteins are essentially spherical particles having an apolar core of neutral lipids, (triacylglycerols and cholesterol esters) which is separated from the external aqueous environment by a surface monolayer consisting of the apolipoproteins and polar lipids (unesterified cholesterol and phospholipids). The outer layer is responsible for the solubility of the lipoprotein particle in plasma.

On the basis of operational definitions such as electrophoretic mobility and ultracentrifugational flotation, lipoproteins are divided into 5 classes; CM, VLDL, IDL, LDL and HDL. Some characteristics of the composition and properties of human plasma lipoproteins are summarized in Table 1.1.

Following the elucidation of the apoprotein components of the lipoproteins, the tendency has been to classify the apolipoproteins into 3 groups; apo A, apo B and apo C. Furthermore the operational definitions of lipoproteins have been slowly superseded by classifications based on the apoprotein classes. Therefore lipoproteins may be defined operationally on the basis of thier physical properties, or by "families" characterized by their chemically-defined protein components. More than

CHAPTER 1

INTRODUCTION AND LITERATURE SURVEY

1.1 Atherosclerosis

In North America alone, almost half a million people die of CHD each year (Roberts, 1987). This is the equivalent of losing 1000 fully loaded 747 Jumbo jets annually!. The principal cause of CHD is atherosclerosis - a disorder of the large arteries which develops over the course of many years.

"Atherosclerosis is a variable combination of changes of the intima of arteries (as distinct from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes". This is the definition of atherosclerosis given by a study group of the World Health Organization, and is printed in the inside cover of every monthly issue of the journal, Atherosclerosis (Elsevier Press).

The disease is characterized by the ocurrence of lesions in the innermost layer of the affected arteries. These lesions consist of plaques of smooth muscle cells which are embedded in a matrix of basement membrane, proteoglycan and connective tissue. The plaques are frequently infiltrated with macrophages. The cells in the lesion are enriched in CE, derived from LDL, and the cytoplasm of these cells has a typical foamy appearance

(Scott, 1987).

As will be apparent already from the above definition, atherosclerosis is a multifactorial disease with heredity, diet, diabetes, cigarette smoking, hypertension and viral injury to the arterial wall being some of the factors known to play a role in its development. According to the "lipid hypothesis", increased levels of plasma lipids, especially cholesterol and LDL-cholesterol, increase the risk of an individual to atherosclerosis.

1.1.1 Diet and Atherosclerosis.

From epidemiological data relating diet, plasma lipid concentration and CHD, it is clear that increased CHD in societies is correlated with high intakes of total fat, saturated fat, animal protein and cholesterol. However since the intake of the latter three dietary ingredients is frequently interrelated, it is difficult to separate the effects of any one of them on CHD.

One of the most comprehensive studies to date, (Keys et al., 1970) has demonstrated a positive correlation between saturated fat intake, CHD and serum cholesterol concentrations. Furthermore this study also showed that total calories derived from dietary fat bears a positive correlation to the incidence of CHD.

The average North American diet contains 40-45% of the total calories as fat (P/S ratio of 0.4),

15-20% of total calories as protein and the rest as carbohydrates. The daily cholesterol intake of a typical North American is 400-800 mg cholesterol. Over the years, the American Heart Association, the National Cholesterol Education Program and various National Institutes of Health consensus conferences have recommended decreasing the fat intake to 35% (P/S ratio of > 1.5), decreasing the daily cholesterol intake to 250 mg or less, with 15% protein and the rest carbohydrate. A blood cholesterol level of < 200 mg/dl is considered desirable. Levels of 200-239 mg/dl as borderline and levels > 240 mg/dl as high. Diet and drug therapy is recommended for the latter group of individuals.

This thesis is concerned primarily with the effects of diet, and specifically protein, on plasma cholesterol concentrations.

1.1.2 Atherosclerosis Research and the Rabbit.

Much of our knowledge of atherosclerosis stems from experiments carried out in various animal species. Several factors have to be considered before deciding on a given animal. These include availability, cost, suitability, animal size, physiological similarity to humans, and the ability to induce lesions similar to those seen in man. The rabbit is perhaps the single most widely used species for atherosclerosis research. The merits and demerits of the rabbit model will not be discussed here. Suffice to say that all the experiments

reported in this thesis have been carried out in rabbits. The suitability of this animal species has been discussed previously (Vesselinovitch, 1979; Huff, 1978).

1.1.3 Dietary protein, serum cholesterol levels and atherosclerosis.

In 1909, Ignatowski suggested that the arterial lesions produced in rabbits by feeding milk, meat and eggs were attributable to the animal protein components of the diet. His suggestion for the role of dietary protein in atherosclerosis was ignored, and his results were attributed to the cholesterol that was also present in the diets he used. Ignatowski's work generated much interest, becuase it now became possible to produce atherosclerosis by dietary manipulations. Even though Meeker and Kesten (1941), some 30 years later, showed that in cholesterol-free diets, dietary casein was more atherogenic than dietary soy protein, their studies also achieved little attention. It was not until the experiments carried out by Carroll and his colleagues (Carroll and Hamilton, 1975: Carroll, 1978) using cholesterol-free, low-fat semipurified diets that the role of dietary protein in modulating serum cholesterol levels was established.

Since the early 1970's there is now a spate of evidence that feeding semipurified diets containing casein results in markedly elevated levels of serum

cholesterol whereas low levels of serum cholesterol are maintained if the casein is replaced by isolated soy protein. (The majority of the studies utilise casein and soy protein as representatives of animal and plant proteins respectively, since these proteins are readily available commercially, in >90% purity.) Much of the work has stemmed from an increased awareness of the available epidemiological data. Though a positive correlation existed between dietary animal protein and CHD in several countries (Carroll and Hamilton, 1975; Carroll, 1978) this was complicated by the fact that a positive correlation also existed between dietary fat and CHD (Carroll and Khor, 1975), which made it difficult to differentiate between the effect of animal protein per se.

An appraisal of data collected by the U.S. Department of Agriculture showed that from 1909 to 1974, the ratio of animal to plant protein in the American diet increased from 1.04:1 in 1910 to 2.36:1 in 1974 (Gortner, 1975; Carroll, 1981; Carroll, 1982) and it was suggested by Stallones (1980) that this may have contributed to the increase in mortality from CHD over this time period. Furthermore it was found that over the same period of time dietary fat and cholesterol content of the diet changed little, with almost no change in the content of saturated fat (Carroll, 1982). In addition, vegetarians in the U.S. had lower levels of plasma cholesterol than the population as a whole (Sacks et al., 1975; Burslem et al., 1978;

Sanders et al., 1978). They were also at a lower risk from CHD than their non-vegetarian counterparts (Phillips et al., 1978). Based on the results of studies with rabbits (Huff et al., 1977) in which it was shown that normocholesterolemic cholesterol levels could be maintained using a 1:1 ratio of animal to plant protein in the diet, with bereficial results even at a ratio of 3:1, Carroll (1981) showed that the ratio of animal to plant protein in the human diet could be changed to give a more beneficial ratio and that this would only involve minor changes in eating habits.

Though the degree of response varies between animal species, and even within a given species subtle differences can be observed due to sex, strain and age, the evidence is unequivocal that dietary protein plays a role in modulating serum cholesterol levels and that long term feeding of casein produces atherosclerotic lesions (Huff et al., 1982). Studies by Hamilton and Carroll (1976) have also shown that in general, dietary animal proteins are more hypercholesterolemic compared to dietary plant proteins.

In some experimental animals, alterations in serum cholesterol produced by dietary protein only become apparent when the diets also contain cholesterol, but this is not so in the rabbit. In this animal model, changes in serum cholesterol can be produced by varying the amount

and type of dietary protein in the absence of dietary cholesterol. Hence, casein, fed in low-fat, cholesterol-free, semipurified diets leads to a 3-4 fold elevation of serum cholesterol compared to isolated soy protein. The excess cholesterol in the serum of casein-fed rabbits accumulates in the atherogenic LDL fraction.

Though numerous studies over the last decade or so have looked at the effects of dietary protein on cholesterol and lipoprotein metabolism, LDL receptor activity, bile acid and neutral steroid excretion - the fundamental question has remained unsolved - what is the mechanism that causes this effect? In order to answer this it is necessary to have a working knowledge of cholesterol and lipoprotein metabolism. Therefore before proceeding further, this will be addressed.

1.2 Lipoproteins.

In fasting human plasma, typical lipid concentrations are of the order of 400-800 mg/dl, despite the fact that lipids are insoluble in water! This problem is resolved by the fact that the presence of lipids in plasma is made possible by its combination in water-soluble macromolecular complexes, the lipoproteins.

The first isolation and identification of a lipoprotein from plasma was reported by Macheboeuf (1929). Subsequent work by Blix et al, (1941), Pedersen (1947), Gofman et al, (1949) and Cohn et al, (1950) paved the way for the classic work of Havel et al (1955) which in

Table 1.1

Human Plasma Lipoproteins

	CM	VLDL	IDL	LDL	HDL
DENSITY (g/ml)	<0.93	0.93-1.006	1.006-1.019	1.019-1.063	1.063-1.21
ELECTROPHORETIC MOBILITY	Origin	prebeta	slow prebeta	beta	alpha
DIAMETER (nm)	75-120	30-80	25-35	18-25	5-12
MOLECULAR WEIGHT (kD)	4,000	10-80	5-10	2	0.18-0.36
APOPROTEINS	A-I, B-48, C-I,C-II, C-III	C-I,C-II, C-III, B-100,E	B-100,C-I, C-II,C-III, E	B-100	A-I,A-II, C-I,C-II, C-III,E
SITE OF SYNTHESIS	Intestine	Liver Intestine	--- Intravascular ---		Intestine Liver

(adapted from Kostner, 1983; Gotto et al., 1986)

addition to providing chemical analysis of the lipoproteins, yielded a method for their isolation which is routinely used today in most laboratories.

Lipoproteins are essentially spherical particles having an apolar core of neutral lipids, (triacylglycerols and cholesterol esters) which is separated from the external aqueous environment by a surface monolayer consisting of the apolipoproteins and polar lipids (unesterified cholesterol and phospholipids). The outer layer is responsible for the solubility of the lipoprotein particle in plasma.

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Table 1.2

Functions of the major apolipoproteins

AI	LCAT activation
A-IV	LCAT activation
B-100	LDL receptor recognition
C-I	Inhibits receptor interaction
C-II	LPL activation; Inhibits receptor interaction
C-III	Inhibits receptor interaction
E	LDL and Chylomicron receptor recognition

(from Gotto et al., 1986)

20 different apolipoproteins have been identified (Kostner, 1983). These differ in their primary, secondary and tertiary structure as well as in their functions and distribution throughout the lipoprotein spectrum. Table 1.2 lists the functions of the major apolipoproteins.

CM and VLDL are the major TG carrying lipoproteins. The former are responsible for the transport of exogenous TG, whereas the latter transport endogenous TG. TG are transported to the peripheral tissues where they provide cells with energy. LDL functions to transport cholesterol to extrahepatic tissues whereas HDL is involved in the transport of cholesterol from peripheral tissues to the liver.

Since the early 1970's, there has been a tremendous increase in our knowledge of LDL metabolism, largely as a result of the pioneering studies from the laboratory of Goldstein and Brown. The discovery of the LDL receptor has gone a long way toward our understanding of the mechanisms that control plasma LDL concentrations.

1.2.1. Lipoprotein synthesis and secretion.

Lipoprotein secretion involves synthesis of the protein and lipid components, assembly of these components to form the lipoprotein particle and finally its secretion into the blood. This secretory pathway involves the endoplasmic reticulum and Golgi apparatus (Alexander et al., 1976)

CM and VLDL are synthesised in the enterocyte

and hepatocyte respectively. The TG of these lipoproteins originates in the SER (Stein and Stein, 1967; Alexander et al., 1976). This is the site of synthesis of the PL and the non polar esters of fatty acids. The apolipoprotein of the CM and VLDL are synthesised on the attached ribosomes of the RER (Bungenberg and Marsh, 1968) along with a signal peptide which is rapidly cleaved off. The nascent particle is then transported to the Golgi within the cisternae of the RER. The particles are then concentrated within secretory vesicles of the Golgi. These vesicles now fuse with either the basolateral membrane of the intestinal cell or with the hepatocyte membrane. The resulting nascent particles are then released into the extracellular space.

An essential requisite for the secretion of the nascent particle is apo B, since inhibitors of protein synthesis abolish the ability of the cell to secrete TG-rich lipoproteins (Siuta-Mangano et al., 1982). In abetalipoproteinemic patients, characterised by high circulating levels of plasma TG concentrations, there is an absence of apo B (Herbert et al., 1978). HDL in these patients is secreted with a normal complement of apo A, C and E.

Following secretion of the nascent particles, apo C and E are obtained by transfer from HDL. In addition to apo B-48, the nascent CM particle contains newly

synthesised apo AI and apo A-IV. These apolipoproteins are partly transferred to HDL in exchange for the C and E apolipoproteins as well as PL immediately following secretion of the CM particles into the blood (Havel, 1978).

Since the liver is able to synthesise apo C, nascent VLDL particles contain some apo C, but additional apo C is acquired from HDL following secretion (Sabesin and Frase, 1977). Nascent VLDL also contains apo E, since the hepatocyte is also able to synthesise this. In humans the apo B of CM is apo B-48 and apo B-100 in VLDL. In the rat nascent VLDL has both apo B-48 and B-100 (Kane, 1978).

The synthesis and secretion of HDL are less well characterised than the secretion of TG-rich lipoproteins. In the absence of LCAT, nascent HDL from rat liver perfusates appears as discoidal particles containing apo AI and E both of which can be synthesised by the liver. Spherical particles appear if LCAT is present, similar to the mature particles seen in the blood. In liver perfusates of rats fed orotic acid, nascent HDL are present but not nascent VLDL particles, indicating independent synthesis of the two lipoprotein particles, (Hamilton, 1984)

Nascent discoidal particles of HDL are also found in rat intestinal lymph (Green and Glickman, 1981; Forester et al., 1983) The major apolipoprotein of HDL, apo AI, can be synthesized in both the liver and

intestine. Latter is a major site of synthesis in the rat (Wu and Windmueller, 1979). A large fraction of the surface components of HDL is transferred to the nascent particle during hydrolysis of CM by LPL. This includes a large proportion of the apo Cs and PL and essentially all of the apo As (Mjos et al., 1975). Similarly apo C and PL are transferred to HDL during hydrolysis of VLDL by LPL (Mjos et al., 1975; Patsch et al., 1978). Apo AI and apo AII, are synthesised initially as proproteins, which undergo extracellular cleavage by a metal-dependent protease to form the mature protein (Edelstein et al., 1983). In animals in which most of the apo A is from the intestine (e.g. rat) it is thought likely that HDL arises from the products of CM metabolism.

In addition to the hepatocyte and enterocyte, certain other cell types are capable of synthesizing apoproteins. Apo E is produced by human kidney and adrenal glands (Blue et al., 1983). Apo E is also secreted by mouse (Basu et al., 1981) and human macrophages, (Werb and Chen, 1983) especially after cholesterol loading (Basu et al., 1981). Apo AI and apo E are also synthesised by kidney and skeletal muscle in birds (Blue et al., 1980; Blue et al., 1982). However little is known about how apo AI and apo E are secreted from these other tissues.

1.2.2. Lipoprotein remodelling and catabolism

Following secretion of the nascent particles,

the lipoproteins are rapidly and extensively remodelled by transfer reactions mediated by lipid transfer proteins and enzymatic reactions mediated by LPL and LCAT. The net result of these processes is the exchange of cholesterol CE, PL and apo A, C and E, between the TG-rich lipoproteins and HDL.

The first lipid transfer protein was characterized by Zilversmit et al (1975). This specific cholesterol ester transfer protein (Barter and Jones, 1979; Dobiasova, 1983) facilitates the transfer of CE from HDL to VLDL and TG from VLDL to HDL in man. It is not found in the rat (Barter and Lally, 1978).

Three distinct and important processes define the circulating lipoprotein pool. These are the exchange and transfer reactions of the lipid and apolipoprotein moieties, the activities of the lipolytic enzymes and finally the activities of the lipoprotein receptors. The first of these has already been alluded to in the preceeding sections.

The TG-rich lipoproteins bind LPL on the endothelial surface of blood capillaries resulting in hydrolysis of the bulk of the core TG. Concomittantly there is an exchange of apo A, C, E, PL and cholesterol between the TG-rich lipoproteins and HDL. LPL is known to be synthesized in the parenchymal cells of the tissues that contain it (e.g. adipose and muscle tissue), from where it is secreted to the capillary endothelium. Here it

is bound to the cell surface by heparin sulphate (Cryer, 1981; Nilsson-Ehle et al., 1980). LPL hydrolyses the sn 1 and sn 3 ester bonds of TG and the sn 1 ester bond of PL and CE (Quinn et al., 1982; Nilsson-Ehle et al., 1980). As mentioned before, apo C-II is a necessary cofactor (Vainio et al., 1983). Binding to sulfated glycosaminoglycans stabilises the enzyme in vitro.

CM TG hydrolysis by LPL is highly regulated, especially by hormones. Insulin appears to play a major role in short term regulation (Cryer, 1981). The hydrolysis products of fatty acids, are taken up by the tissues and reesterified to form cellular TG which are stored in adipose tissue.

Hepatic lipase is involved in the conversion of small VLDL and IDL to LDL possessing both a TG hydrolase and phospholipase activity (Ehnholm et al., 1975; Jansen et al., 1980).

The second stage of CM hydrolysis involves binding of the CM remnants to specific remnant receptors on the surface of hepatocytes. The receptors recognise the ligand, apo E (Havel, 1984). This binding is inhibited by the C apolipoproteins. Hence loss of apo C during remnant formation facilitates binding to the remnant receptor. Following binding the remnant is internalised into the cells through clatharin-coated pits. These pinch off to form a primary endosome. The latter now fuses beneath the

cell surface forming multivesicular bodies. The low pH of the endosomal space now facilitates dissociation of the receptor from its ligand. Primary lysosomes from the Golgi now fuse with the multivesicular bodies resulting in proteolysis and lipolysis of the remnant components.

The initial stage in the hydrolysis of VLDL is similar to that seen for CM. Hence VLDL core TG are hydrolysed by LPL with PL and apo C being transferred to HDL. As hydrolysis proceeds, VLDL remnants are generated, poor in TG, PL and apo C. However the remnants retain apo B and apo E. Small VLDL particles yield small remnant particles which are thought to more likely form LDL than larger remnants, whereas larger VLDL are thought to yield relatively larger remnants, which are more likely to be removed by the liver (Stalenhoef et al., 1984; Packard et al., 1984). It should be stressed that just as VLDL comprises a spectrum of particles, LPL hydrolysis of VLDL produces a spectrum of remnant particles, whose size and density can overlap with precursor VLDL particles. VLDL remnants with a density greater than 1.006 g/ml are frequently referred to as IDL, but as yet there is no evidence that these IDL particles are qualitatively different from VLDL remnants of d<1.006 g/ml.

In the rat newly synthesised VLDL has appreciable quantities of CE due to hepatic synthesis stimulated by ACAT. However little CE are acquired in plasma due to low activity of the CE/TG lipid transfer

protein. In man, hepatic ACAT activity is low, and CE/TG lipid transfer protein activity is high, so that the VLDL acquires CE from HDL. In many species most of the VLDL remnants are taken up by liver hepatocytes via receptor-mediated endocytosis. In man, about half of the VLDL remnants are normally processed further to LDL. This is accompanied by further loss of TG, PL and apo E. Only apo B-100 is retained in LDL. Hydrolysis of VLDL remnants to LDL is believed to be mediated by hepatic lipase.

VLDL remnants and LDL are both removed via LDL receptors (Goldstein et al., 1983). The former are removed almost entirely in liver (Jones et al., 1984) since these contain apo B-100 and apo E. The latter has a marked affinity for the LDL receptor. LDL removal via hepatic receptors is less efficient, since these particles have only B-100. They consequently gain access to various compartments of different extrahepatic organs and tissues which have LDL receptors (Attie et al., 1982; Stange and Dietschy, 1984).

1.2.3. Receptor-mediated endocytosis of LDL

This has been extensively studied in the laboratory of Goldstein and Brown. Using cultured human fibroblasts these workers and their colleagues have meticulously detailed the involvement of the LDL receptor in the regulation of plasma LDL concentrations, an achievement for which they were awarded the Nobel Prize in Physiology or Medicine in 1985. The work has been

extensively reviewed (Goldstein and Brown, 1977; Brown et al., 1981; Brown and Goldstein, 1983; Goldstein and Brown, 1984; Brown and Goldstein, 1986; Brown and Goldstein, 1988) and will therefore only be discussed briefly here.

LDL binds to high affinity receptors in specific regions of the plasma membrane, called coated pits. The ligand recognized is apo B-100 (and apo E). Although coated pits comprise only 2% of the surface of human fibroblasts, they contain 50-80% of the LDL receptors (Goldstein et al., 1983). The pits invaginate into the cells where they pinch off to form coated endocytic vesicles. These then fuse with lysosomes. Here the protein component of LDL is degraded to amino acids and the cholesteryl esters are hydrolysed by an acid lipase. The liberated cholesterol leaves the lysosomes and is used by the cell for membrane synthesis.

The liberated cholesterol regulates three processes; 1) it inhibits HMGCoA reductase, (the rate controlling enzyme in cholesterol synthesis) shutting off cellular cholesterol synthesis, 2) it activates ACAT, the enzyme which reesterifies excess cholesterol, allowing it to be stored in the cytoplasm as CE droplets, and 3) it inhibits the synthesis of LDL receptors, thereby preventing uptake of cholesterol from LDL (see Fig. 1.1). Hence it can be seen that cellular cholesterol levels are under extremely fine control. In normal man, about 60% of

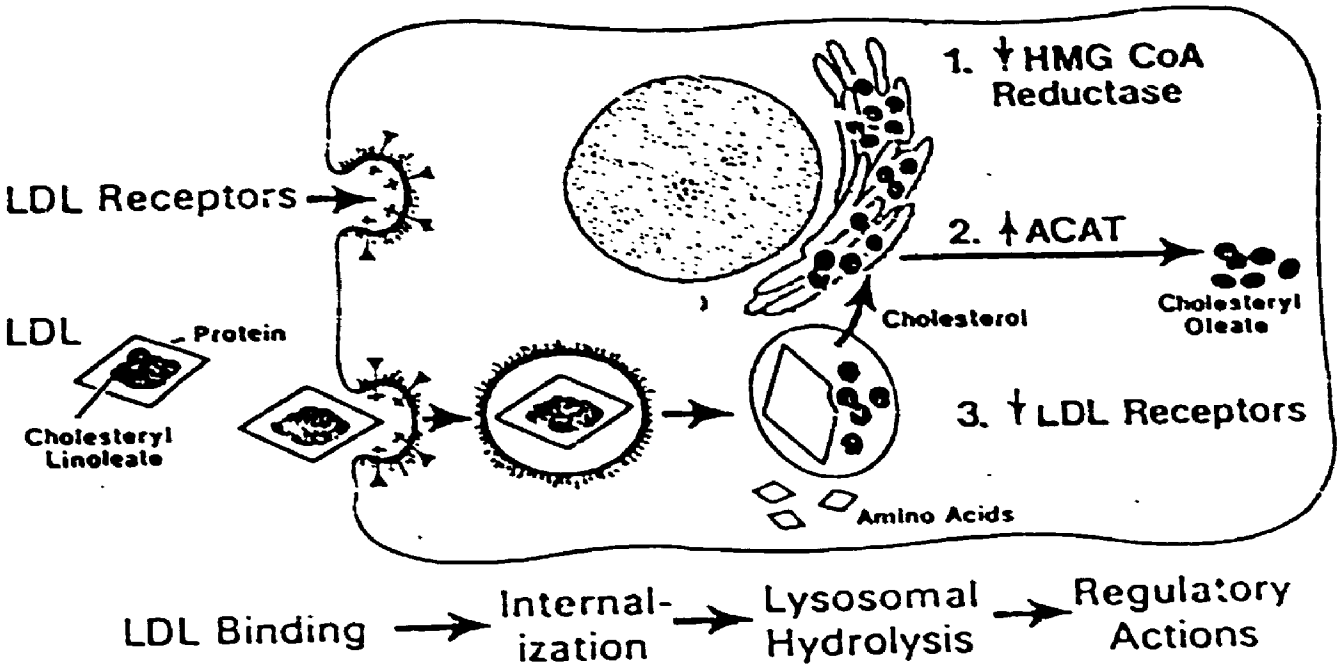

1. ↓HMG CoA Reductase
LDL Receptors
2. ↓ACAT
LDL
Protein
Cholesterol
Cholesteryl Oleate
Cholesteryl Linoleate
3. ↓LDL Receptors
Amino Acids
LDL Binding
Internal-ization
Lysosomal Hydrolysis
Regulatory Actions

Fig. 1.1

Receptor-mediated endocytosis of LDL

(from Brown and Goldstein, 1989)

LDL is metabolized by this receptor-mediated process, the remainder is cleared by receptor-independent routes. Homozygous FH patients having two mutant LDL receptor genes, have an absence of LDL receptors, and exhibit plasma LDL levels 6-10 times higher than those seen in normal man. These patients frequently develop myocardial infarctions before age 20 (Goldstein and Brown, 1980).

1.3 An overview of cholesterol metabolism.

Integrating the information from the preceeding sections, an overview of cholesterol metabolism will now be presented.

Following ingestion, dietary cholesterol reaches the small intestine where it gets mixed with endogenous cholesterol, i.e. cholesterol secreted in bile and from degraded cells in the intestinal epithelium. However in animals on cholesterol-free diets, (the rabbits used for all the experiments outlined in this thesis), all of the intestinal cholesterol is of endogenous origin. Intestinal cholesterol can be either absorbed or excreted in the feces. The process of intestinal cholesterol absorption, synthesis and secretion has been reviewed by Norum et al., (1983) and will not be detailed here.

Once absorbed through the intestinal wall, the cholesterol is incorporated into CM and transported to plasma via the lymph. CM TG are hydrolysed by LPL, whereas the cholesterol remains with the CM remnants that are

generated. CM remnants are now taken up in the liver via the apo E mediated receptor system (remnant receptors).

Within the hepatocytes, the cholesterol expands the intracellular pools initiating homeostatic mechanisms to prevent a cholesterol overload. Therefore HMGCoA reductase is inhibited, and the number of LDL receptors is reduced. Having prevented a cholesterol overload, the liver now removes the excess cholesterol. The latter is either secreted in bile as such or after its conversion to bile acids (the latter process being catalysed by the enzyme cholesterol 7α-hydroxylase). The excess cholesterol is also secreted into the plasma as VLDL. In the plasma pool, VLDL is hydrolysed to IDL and then to LDL. In the rabbit, there is evidence that the liver also secretes LDL directly into the circulation. Once in the plasma additional cholesterol in the form of CE is acquired from HDL in exchange for TG, catalysed by cholesterol ester transfer protein, which is known to be present in the rabbit. Upto 60% of LDL is then removed via LDL receptors, with the remainder thought to be removed by scavenger cells.

A schematic outline of the scheme discussed above is depicted in Fig. 1.2.

1.4 <u>The effects of dietary protein on cholesterol metabolism.</u>

The effects of dietary protein on cholesterol metabolism has been studied extensively over the past 15

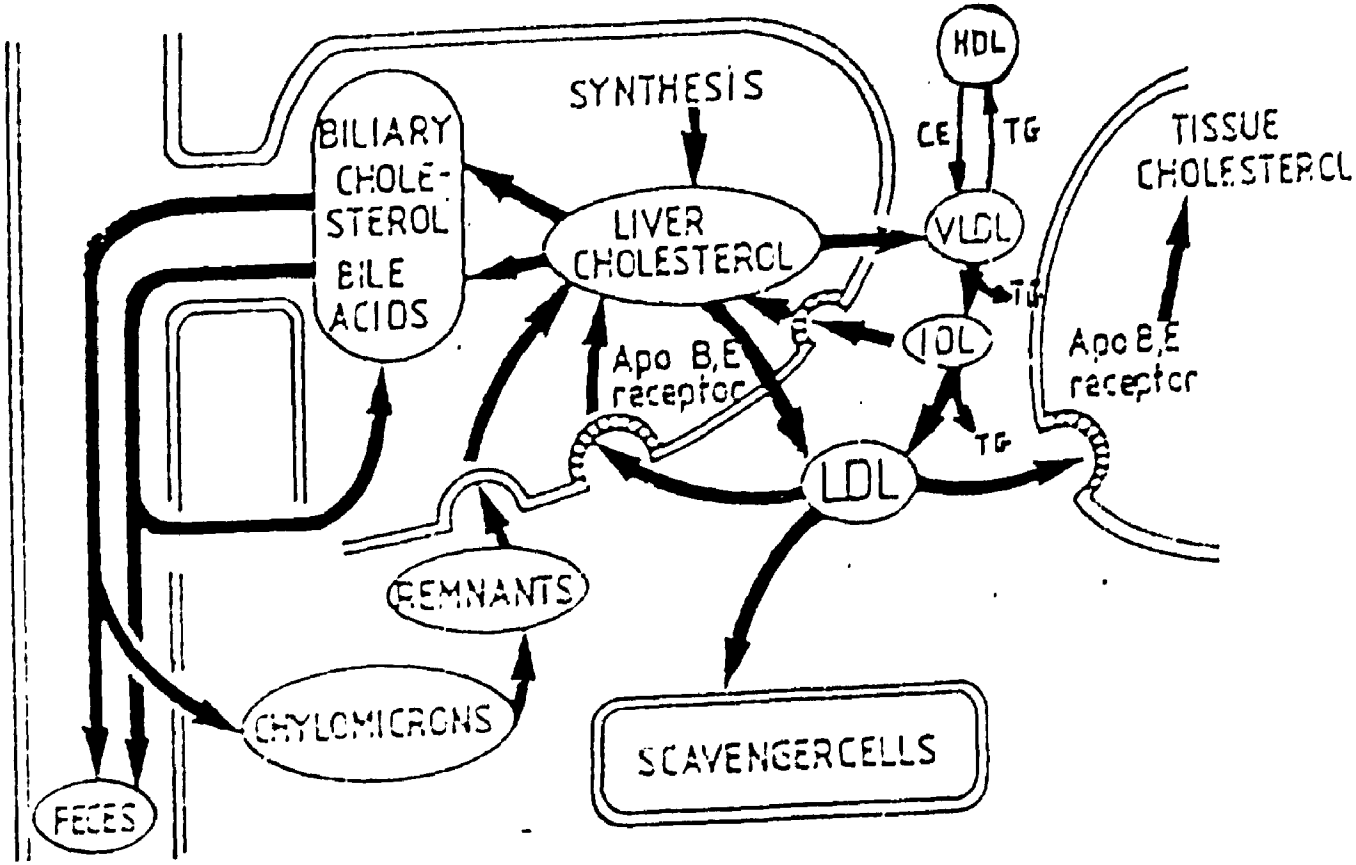

HDL
SYNTHESIS
CE
TG
TISSUE
CHOLESTEROL
BILIARY
CHOLE-
STEROL
BILE
ACIDS
LIVER
CHOLESTEROL
VLDL
TG
Apo B,E
receptor
E
IDL
Apo B,E
receptor
TG
LDL
REMNANTS
CHYLOMICRONS
SCAVENGERCELLS
FECES

Fig. 1.2

Overview of cholesterol metabolism

Schematic representation of cholesterol metabolism
(adapted from Beynen et al., 1986b)

years. Much of the early work was descriptive, detailing the effects of dietary protein and its interaction with other dietary components, on serum cholesterol levels. More recent studies have tended to focus on the physiological/biochemical mechanisms (in line with the scheme for cholesterol metabolism discussed in the preceeding section) in an attempt to explain what causes the effect.

In the following literature survey, some of the early work is discussed briefly, since much of this has been extensively reviewed (Carroll, 1978; Carroll, 1981; Carroll, 1982; Terpstra et al., 1983, Foley et al., 1988). The most recent mechanistic studies are reviewed in more detail (also discussed by Beynen et al., 1986a & b). Most of the work described is from rabbits, but results from other species including rats and humans has been included to a give a more complete account.

1.4.1 Rabbits.

As already discussed, an association between dietary protein and atherosclerosis was first suggested by Ignatowski (1909). Meeker and Kesten (1941) using cholesterol containing diets found soybean protein was hypocholesterolemic compared to casein. Additionally these workers showed that cholesterol-free casein diets induced atherosclerotic lesions identical to those produced by dietary cholesterol. The hypercholesterolemic effects of

casein were subsequently found by Lambert et al., (1958) and Wigand (1959) using cholesterol-free diets. In all of these studies, the role of the dietary protein was not fully appreciated until the classic studies from Carroll's colleagues in the early 1970's.

Though most of the studies have used casein and soy protein as dietary protein sources, previous work from this laboratory (Carroll, 1971; Carroll and Hamilton, 1975, Hamilton and Carroll, 1976; Carroll, 1982) and work done by Kritchevsky (1979) has also shown that generally animal proteins are more hypercholesterolemic than plant proteins.

The ability of dietary casein in cholesterol-free diets to induce atherosclerotic lesions was reported by Huff et al., (1982) and by Katan et al., (1982). In the latter study a significant correlation was found between aortic plaque development and hypercholesterolemia.

The increased serum cholesterol levels in casein-fed rabbits is reflected principally by increased LDL levels (Roberts et al., 1979; Huff and Carroll, 1980; Terpstra et al., 1981; Terpstra and Sanchez-Munis, 1981; Bauer and Cavert, 1984; Hrabek-Smith and Carroll, 1987) although increases in other lipoprotein fractions have also been reported (Terpstra et al., 1981, Kurowska et al., 1989).

Several studies have investigated if the

effect on serum cholesterol levels is due to the amino acid composition of the proteins. Feeding of an amino acid mixture resembling casein produced similar cholesterol levels to that seen with the intact protein whereas the amino acid mixture resembling soy protein produced somewhat higher levels than the intact protein (Huff and Carroll, 1980). Enzymatic hydrolysates of casein and soy protein produced somewhat lower levels of serum cholesterol than the intact proteins but the differential response between the two proteins was still apparent (Huff et al., 1977). Kritchevsky (1979) hypothesized that the ratio of lysine/arginine was responsible for the observed effects, since work from his laboratory had shown that manipulation of the lysine/arginine ratio in a casein diet to match that in the soy protein diet abolished the hypercholesterolemia (Kritchevsky et al., 1978). However the results of these experiments could not be reproduced by Huff and Carroll (1980a) Studies by Huff et al., (1977) designed to elucidate if the effects were attributable to essential amino acids, or whether amino acid supplementation of the intact proteins could abolish casein-induced hypercholesterolemia, were also inconclusive.

Therefore although amino acid composition may be important in determining serum cholesterol concentrations, it cannot exclusively account for the

observed effects. The structure of the intact protein also seems to play a role. Furthermore additional factors, e.g., the rate and extent of protein digestion, the rate of release of the constituent amino acids and their subsequent rate of absorption in the gut, may also be important factors affecting the outcome on serum cholesterol levels (Huff and Carroll, 1980a; Woodward and West, 1984; Woodward and Carroll, 1985; Redgrave, 1984).

It is however clear from all of the studies that serum cholesterol concentration in the rabbit is extremely sensitive to the type and amount of protein in the diet. Furthermore the rabbit is the only species examined to date, in which dietary protein exerts such a marked impact on serum cholesterol levels, even in the absence of dietary cholesterol.

1.4.2 Rats.

Most of the types of studies done in rabbits have also been carried out in rats. However in this species the differential effects of casein and soy protein are only apparent using atherogenic diets (diets containing cholesterol). Furthermore the response observed is also determined by the sex and strain of the rat used. Using atherogenic diets, casein is hypercholesterolemic in female lean Zucker rats but not in males (Terpstra et al., 1982a). The hypercholesterolemia observed is also dependent on the presence of dietary fat. Using cholesterol-supplemented diets, differential effects of

casein and soy protein have been reported by several groups of workers (Moyer et al., 1956; Nath et al., 1959; Yadav and Leiner, 1977; Terpstra et al., 1982a; Cohn et al., 1984; Sirtori et al., 1984, Vahouny et al., 1985).

Using cholesterol-free diets, the effects are greatly diminished or altogether absent. Hevia and Visek (1979a) found casein to be hypercholesterolemic but only using high levels of dietary protein (45%). Sautier et al., (1979) could not find any difference in serum cholesterol concentrations between casein and soy protein. Similar results were reported by Pathirana et al., (1980). Also no trends were found when comparing different plant and animal proteins (Eklund and Sjoblom, 1980; Neves et al., 1980; Jacques et al., 1988).

1.4.3 Humans.

Clinical trials in human subjects comparing the effects of dietary animal and plant proteins on plasma cholesterol levels, have given conflicting results. Studies by Sirtori's group have shown that replacing dietary animal protein by plant protein (soy) in the diets of Type II hypercholesterolemic patients leads to a reduction in plasma cholesterol levels (Sirtori et al., 1977; Sirtori et al., 1979; Descovich et al., 1980). In one study (Sirtori et al., 1979), 42 hypercholesterolemic in-patients, responded with decreased plasma cholesterol levels on being switched from a standard low-lipid diet,

to a soy protein diet. However the response in 18 out-patients was less consistent. A soybean protein diet also decreased plasma cholesterol (by 16%) in 12 severe type II hyperlipoproteinemic subjects compared to an animal protein diet (Lovati et al., 1987). A 32% reduction in plasma cholesterol was obtained in hypercholesterolemic children when their dietary animal protein was replaced by plant protein (Gaddi et al., 1987).

Reductions in plasma cholesterol levels have also been reported by Carroll et al., (1978) in normolipidemic women, and by Wolfe et al., (1981) in type II hypercholesterolemic patients when dietary animal proteins were replaced by plant proteins.

Using mildly hypercholesterolemic patients, Shorey et al., (1981) found no effects on plasma cholesterol levels, when dietary animal protein was replaced by soy protein. Van Raiij et al., (1979) and Holmes et al., (1980) also found no beneficial effects of plant proteins. Similarly Van Raiij et al., (1981) using 69 normocholesterolemic patients and Grundy and Abrams (1983) using 1 mildly hypercholesterolemic and 13 normocholesterolemic subjects, also found no differences between dietary animal protein in comparison to soy protein. Huff et al., (1984) when comparing a mixed protein diet versus a plant protein diet could also not detect any differences in plasma cholesterol levels.

Giovannetti et al., (1986) compared a mixed

protein diet with a plant protein diet with both, a low and medium fat content, in 12 healthy females, and again found no differences in fasting plasma cholesterol levels. Finally Mercer et al., (1987) replaced 500ml of 2% cow's milk with an equal volume of a drink containing similar amounts of soy protein and butterfat in 33 healthy volunteers. Although the group as a whole showed no differences in plasma cholesterol levels, a subset of 5 individuals with the highest plasma cholesterol concentrations, responded with a small but significant reduction (5%) in their plasma cholesterol levels.

1.4.4 Other species.

Roy and Schneeman (1981) have found soy protein to be hypocholesterolemic in mice in comparison to casein, whereas, Raheja and Linscheer (1982) and Weinans and Beynen (1983) found no differences between the two proteins. In hamsters, Beynen and Schouten (1983) found soy protein to be hypocholesterolemic compared to casein, but Mahfouz-Cercone et al., (1984) and Duffy et al., (1985) could find no differences between the two. Richmond et al., (1984) found no difference between cottonseed protein or soy protein on hamster plasma cholesterol levels.

Forsythe (1986) has found casein to be hypercholesterolemic in gerbils in comparison to soy protein, but only in the presence of dietary cholesterol.

Trpstra et al., (1982b) have found casein to be hypercholesterolemic compared to soy protein regardless of whether the gerbils were consuming cholesterol-containing diets.

In rhesus monkeys, Terpstra et al., (1984) using a 0.1% cholesterol supplemented diet, found casein to be hypercholesterolemic. Barth et al., (1984) on the other hand found no difference between casein and soy protein on the plasma cholesterol levels in Macaca fascicularis monkeys.

In chickens fed atherogenic diets casein has been found to be hypercholesterolemic compared to soy protein (Stamler et al. 1958; Kritchevsky et al. 1959; Kenney and Fisher; 1973). With cholesterol-free diets the effect is less pronounced or absent in chickens (Johnson et al. 1958; Hevia and Visek; 1979b) and similarly in pigeons (Lofland et al., 1966)

In pigs the effects of casein or soy protein are dependent on whether the diets included cholesterol and/or fat. Kim et al (1978) using diets with either high or low fat and cholesterol content, found casein to be hypercholesterolemic compared to laboratory chow only in a high fat, high cholesterol diet. With a cholesterol-free diet, casein had no discernible effects on serum cholesterol compared to the chow diet. Forsythe et al (1980) found that a high cholesterol and high fat diet containing a mixture of casein and lactalbumin was

hypercholesterolemic compared to a diet containing a mixture of soy, corn and wheat proteins. The results were not dependent on whether a polyunsaturated or saturated fat was used. Scholz et al., (1985) found no statistical difference in serum cholesterol levels of pigs fed cholesterol-supplemented casein or soy protein diets.

1.4.5 The effects of protein structure and digestibility on plasma cholesterol levels.

From the results of studies with a) feeding amino acid mixtures simulating casein and soy protein, b) enzymatic hydrolysates of the proteins, c) amino acid supplementation experiments, it is clear that although the amino acid composition of the dietary protein may contribute to the plasma cholesterol concentration, there appears to be an overriding effect of the intact protein (i.e. its tertiary structure).

West et al. (1984) found that in rabbits the hypercholesterolemic effect of casein was greatly diminished, if prior to feeding, it had been cross-linked by treatment with formaldehyde. Soy protein gave the same results irrespective of whether it had been treated with formaldehyde or whether the "intact" protein was used. However workers in the same laboratory were unable to reproduce the results in a subsequent study (Roszkowski et al., 1985). Experiments in rats also failed to show any effect of pretreating casein with formaldehyde (Beynen et al., 1985).

Since in vitro studies by Sklan (1980) and Woodward and West (1984) showed that soy protein is able to bind more bile acids than casein, several workers have suggested (Terpstra et al., 1982; West et al., 1984; Woodward and West, 1984; Woodward and Carroll, 1985) that partial digestion products of soy protein, in vivo, may be able to sequester bile acids, consequently preventing their reabsorption. This would result in increased bile acid excretion which would be compensated for by increased bile acid production from liver cholesterol, which may lower plasma cholesterol levels. This hypothesis has yet to be resolved.

Woodward and Carroll (1985) looked at the in vitro digestion properties of casein and soy protein by various enzymes. Soy protein was found to be more soluble at acidic pH and was consequently more readily digested by pepsin, in comparison to casein. Casein, on the other hand, was more soluble at alkaline pH and more readily digested by trypsin. In assessing correlations between the in vitro digestion products of several proteins with their effects on serum cholesterol levels in rats, Jacques et al., (1986) found significant correlations between the tyrosine content or the leucine/isoleucine ratio of protein digestion products and total serum cholesterol levels, but only in rats fed cholesterol-enriched diets as opposed to rats fed cholesterol-free diets.

Sugano's group in Japan compared the in vitro digestion products of casein and soy protein by pepsin to the products isolated from the stomachs of rats fed these diets (Yashiro et al., 1985) The in vitro and in vivo profiles of soy protein were essentially identical as determined by Sepahdex chromatography. Furthermore a peptide produced by peptic digestion of soy protein, was found to have the same hypocholesterolemic effect as the intact protein when fed to mice. The authors suggested that this was responsible for the antihypercholesterolemic action of soy protein, possibly mediated by a hormonal signal generated by this peptide. Redgrave (1984) also suggested that the ability of dietary proteins to affect cholesterol metabolism might reside in their abilities to produce pharmacologically active peptides which can subsequently affect lipid metabolism in vivo.

In the subsequent study from Suagno's laboratory, soy protein was hydrolysed in vitro using proteases, resulting in the production of an undigested high molecular weight fraction and a digested low molecular weight fraction (Sugano et al., 1988). Upon feeding these fractions to rats, hypocholesterolemia was produced by the high molecular weight fraction, whereas in their earlier report (Yashiro et al., 1985) the hypocholesterolemic properties were attributed to the low molecular weight fraction.

1.4.6 Effects of dietary proteins on hormones.

A few reports have been published in which the effects of dietary proteins on various hormones have been investigated, since it is known that several hormones, e.g., insulin, glucagon and thyroxine have the potential to affect the activity of HMGCoA reductase.

Sugano et al., (1982) showed that casein-fed rats had an increase in serum insulin, but not serum glucagon in comparison to rats fed soy protein. Also, fasting insulin levels were found to be lower, and fasting glucagon levels higher, in rats fed vegetable protein (Sugano et al., 1984) in comparison to rats fed animal protein. This resulted in a lower insulin/glucagon ratio in the vegetable protein-fed rats. An increase in post-prandial insulin levels and a decrease in post-prandial glucagon levels was found in human subjects consuming a casein diet supplemented with arginine and glycine, but not in subjects whose casein diet was unsupplemented (Sanchez et al., 1988). In gerbils fed soy protein, plasma insulin, thyroxine and thyroid stimulating hormone levels were higher than in gerbils fed casein (Forsythe, 1986).

In human subjects consuming a liquid soy protein-containing meal, post-prandial release of stomach gastrin was lower than in the same subjects consuming a meat-containing meal (McArthur and Richardson, 1987).

Though some of the above studies have found casual relationships between dietary proteins and circulating hormone concentrations, none of the authors have delineated a pathway to explain how dietary protein-induced changes in plasma cholesterol levels are mediated by the hormone being investigated.

1.4.7 Effects of casein and soy protein on lipoprotein composition.

VLDL.

Cholesterol is increased by casein in rabbits (Huff et al., 1982; Scholz et al., 1982; Terpstra et al., Van der Meer and Beynen, 1987; Hrabek-Smith and Carroll, 1987; Bauer, 1988), and in rats (Sirtori et al., 1984; Lefevre and Schneeman, 1984) but a decrease was reported in the monkey (Barth et al., 1984). Triglycerides are decreased by casein in the rabbit (Bauer, 1988).

Casein increases the apo B concentration in rats (Cohn et al., 1984) and in rabbits (Hrabek-Smith and Carroll, 1987). Casein-fed rabbits also have increased concentrations of apo E (Roberts et al., 1981; Scholz et al., 1982, Hrabek-Smith and Carroll, 1987) and apo C (Roberts et al., 1981; Hrabek-Smith and Carroll, 1987).

IDL.

In rabbits casein increases cholesterol and apo E concentrations (Roberts et al., 1981; Scholz et al., 1982), but causes a decrease in triglyceride concentrations (Bauer, 1988).

LDL

Essentially all of the studies to date in rabbits have found increases in cholesterol and protein induced by feeding a casein diet (Terpstra and Sanchez-Munis, 1981; Terpstra et al., 1982; Huff et al., 1982; Scholz et al., 1982, Hrabek-Smith and Carroll, 1987; Bauer, 1988). In addition to the increase in plasma cholesterol levels, the increase in LDL cholesterol is perhaps the most documented effect of casein. Bauer (1988) also found a decrease in triglycerides.

HDL

Increases in cholesterol have been reported in rats (Sautier et al., 1983; Lefevre and Schneeman, 1984), rabbits (Terpstra and Sanchez-Munis, 1981, Bauer, 1988) and gerbils (Forsyth, 1986) fed casein. These diets have also been reported to cause increases in apo C (Lefevre and Schneeman, 1984) and apo A (Sugano et al., 1982) in rats.

In monkeys soy protein diets led to increases in HDL cholesterol (Barth et al., 1984).

The study by Scholz et al., (1982) in which the sequence of changes in lipoprotein cholesterol was investigated, showed that in rabbits fed casein, cholesterol increased first in LDL, then IDL and finally in VLDL, whereas in rabbits fed soy protein, low levels of cholesterol were maintained in all the lipoprotein fractions for throughout the duration of the study.

Recently Kurowska et al., (1989) reported increases in essentially all the lipoprotein fractions in rabbits fed casein.

1.4.8 Effects of dietary protein on lipoprotein metabolism.

Roberts et al. (1981) studied the effects of feeding semipurified cholesterol-free diets containing casein and soy protein on the decline in plasma radioactivity following an intravenous injection of either ^{125}I-VLDL or ^{125}I-IDL. Both ligands were found to be removed more slowly from the plasma of casein-fed animals. Furthermore it was found that the source of the tracer (i.e. whether it was isolated from a casein or soy protein-fed donor) influenced the results obtained. Generally the tracer isolated from the soy protein-fed donor was cleared faster than the tracer isolated from a casein-fed donor.

Cohn et al (1984) using rats fed an atherogenic diet containing casein or soy protein showed that following an i.v. injection of ^{125}I-VLDL, the removal of radioactivity was faster in the soy protein-fed rats, which was reflected by a higher FCR of VLDL apo B. The production rate of VLDL apo B was however similar between the two dietary groups. A higher FCR of VLDL in rats fed cholesterol-soy protein diets as opposed to cholesterol-casein diets was also found by Lovati et al., (1985).

In endogenously hypercholesterolemic casein-fed rabbits, the FCR of removal of ^{125}I-LDL was slower than the FCR in normocholesterolemic cholestyramine treated rabbits (Chao et al., 1982) or normocholesterolemic mevinolin-treated rabbits (Chao et al., 1983).

In studies by Huff et al., (1984) using hypercholesterolemic control subjects and experimental subjects in which soy protein was partially substituted for the dietary protein component, the FCR of VLDL apo B was increased in the patients receiving soy protein even though the circulating pool was unaltered in the two dietary groups. Furthermore a discernible effect on the production rate of VLDL apo B in four of the five subjects.

Kinetic studies of IDL turnover have shown that the increased pool size of IDL apo B in casein-fed rabbits is due to a significant decrease in IDL apo B FCR and a 2-fold increase in IDL apo B production rate, in comparison to the values obtained in rabbits fed soy protein (Samman, Khosla and Carroll, unpublished observations).

1.4.9 Effects of dietary protein on cholesterol and bile acid dynamics.

Casein fed in cholesterol-free diets leads to a decreased excretion of bile acids and neutral steroids in rabbits (Fumagalli et al., 1978; Huff and Carroll;

1980b, Kuyvenhoven et al., 1986) and rats (Sautier et al., 1979, Nagata et al., 1982), compared to a cholesterol-free soy protein diet. In rabbits the decrease in bile acid excretion in casein-fed animals can be detected within 2-4 days (Beynen et al., 1983, Van der Meer et al., 1985) after switching the animals from a soy protein-diet, and before any detectable increase in serum cholesterol levels (Kuyvenhoven et al., 1986).

Casein stimulates, the absorption of cholesterol in rabbits and rats compared to soy protein (Huff and Carroll, 1980b, Nagata et al., 1982, Vahouny et al., 1984) and increases the absorption of bile acids in pigs (Beynen et al., 1985) in comparison to soy protein. However in rats fed casein or soy protein containing atherogenic diets, cholesterol absorption was similar between the two groups (Cohn et al., 1984)

Cholesterol kinetic studies, which have looked at the decline in plasma cholesterol specific activity, following an i.v. injection of radiolabeled cholesterol, have found a decreased FCR in rats (Nagata et al., 1982; Cohn et al., 1984), and rabbits (Huff and Carroll, 1980b; Kuyvenhoven et al., 1986) fed casein in comparison to those fed soy protein. This decreased FCR resulted in an elevation of the pool size of the most rapidly turning over pool (Huff and Carroll, 1980b; Kuyvenhoven et al., 1986). A decrease in the production rate was detected in

the casein-fed rabbits (Kuyvenhoven et al., 1986) whereas in the casein-fed rats the production rate was not different from the rats fed soy protein (Nagata et al., 1982; Cohn et al., 1984).

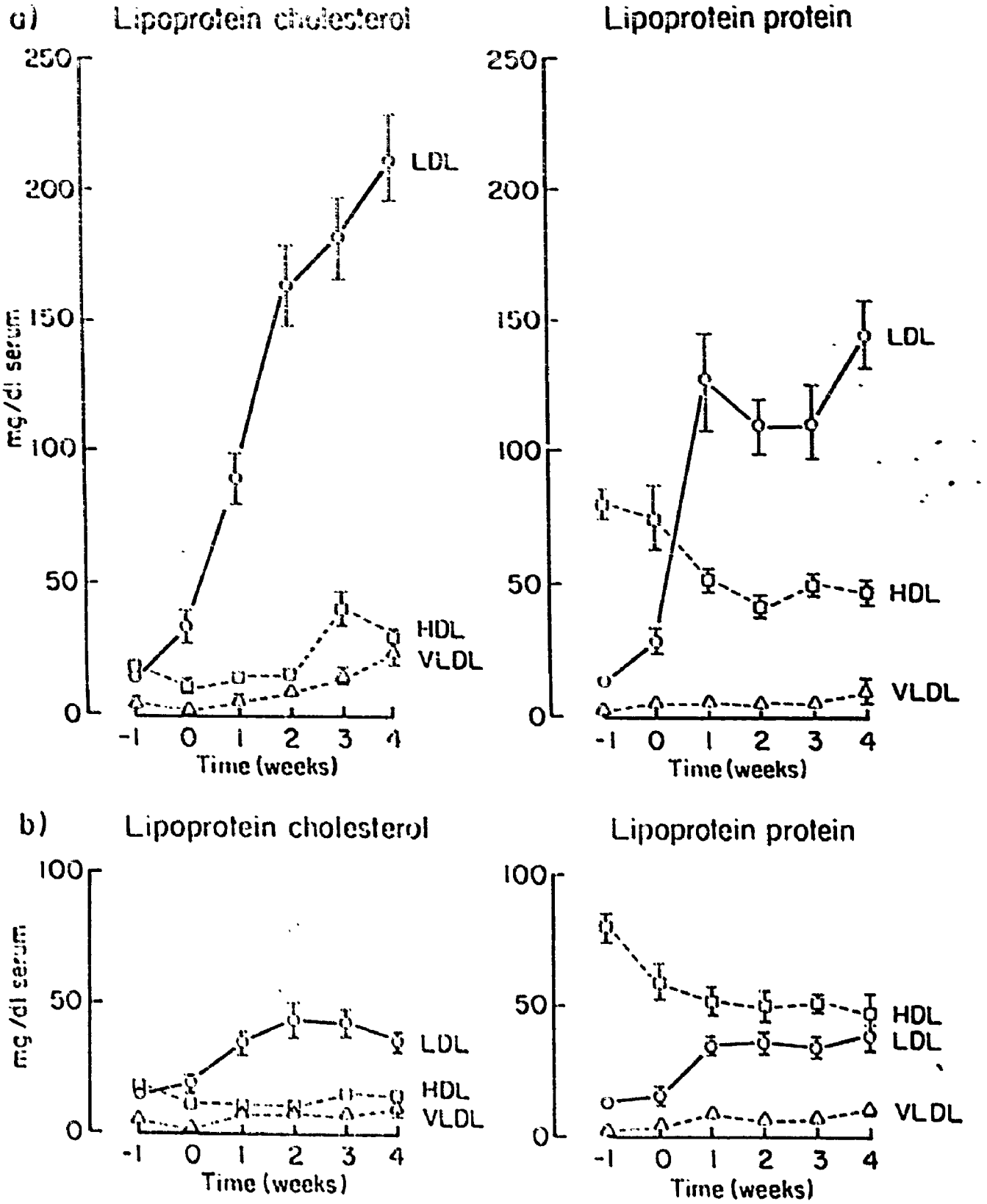
1.4.10 Effects of dietary protein on LDL receptor activity.

Following the pioneering work of Goldstein and Brown (1977) which established that upto 60% of LDL is catabolized by a specific receptor-dependent pathway, a few studies have been carried out to look at the activity of LDL receptors following changes in plasma cholesterol induced by dietary protein, although all the studies to date have been carried out in vitro.

Chao et al, in their studies on the mechanism of action of the hypocholesterolemic drugs, cholestyramine (1982) and mevinolin (1983), used rabbits made hypercholesterolemic by feeding cholesterol-free, wheat-starch casein diets, as their control animals. The in vitro binding of LDL to the liver membranes of these control animals was found to be diminished in comparison to the in vitro binding of LDL to the liver membranes of normocholesterolemic cholestyramine-treated rabbits (1982), normocholesterolemic mevinolin-treated rabbits (1983) or normocholesterolemic rabbits fed laboratory chow diets reported by Kita et al (1981). Furthermore wheat-starch-casein fed rabbits exhibited no EDTA-sensitive binding analagous to the situation observed in hyper-

cholesterolemic WHHL rabbits by Kita et al (1981), or hypercholesteroemic fasting rabbits by Stoudemire et al (1984). These studies by Chao et al. have been frequently quoted as evidence for suppression of hepatic LDL-receptor activity by casein. Though this is probably correct, it should be pointed out that as no comparison was made with rabbits fed a plant protein diet which maintains low levels of plasma cholesterol, the observed effects cannot be attributed to the protein component per se. To date, no study has been published in which LDL receptor activity has been directly compared in casein and soy protein fed rabbits.

A direct comparison of the effects of casein and soy protein on the activity of hepatic receptors was reported in rats (Sirtori et al., 1984; Cohn and Nestel, 1985). In the former study, the binding of β-VLDL to the liver membranes of casein-fed rats was found to be greatly diminished in comparison to the binding seen to the membranes from soy protein-fed rats. Cohn and Nestel (1985) investigated ^{125}I-VLDL uptake using hepatocyte monolayers prepared from rats fed casein or soy protein. The uptake and degradation of the labeled ligand was decreased in the hepatocytes isolated from the casein-fed rats in comparison to those isolated from soy protein-fed rats. However both of the studies used cholesterol supplemented diets. As suggested by Cohn and Nestel (1985), the high cholesterol content may itself have led


a)
Lipoprotein cholesterol
Lipoprotein protein
mg/dl serum
250
200
150
100
50
0
LDL
HDL
VLDL
-1
0
1
2
3
4
Time (weeks)
b)
Lipoprotein cholesterol
Lipoprotein protein
mg/dl serum
100
50
0
LDL
HDL
VLDL
-1
0
1
2
3
4
Time (weeks)

minerals and carbohydrates are known to modulate the effects of dietary protein on plasma cholesterol levels (reviewed in Carroll, 1981; Carroll, 1982; Terpstra et al., 1983; Beynen et al., 1986b, Foley et al., 1988). However as this project was concerned with the effects of dietary protein and since all of the experiments reported in this thesis used two diets (differing only in the protein component - casein or soy protein), the effects of non-protein dietary components will not be discussed further.

1.4.11 Aims of this thesis.

From the preceeding sections it is clear that although a large body of work has been carried out in the last few years, forwarding our understanding of how dietary proteins mediate their effects on plasma cholesterol levels, several important questions still remain. These include

1) Are the elevations in plasma cholesterol due to enhanced production and/or diminished catabolism of the lipoproteins?

2) Is the response to dietary protein mediated by differences in the digestibility of the proteins in vivo?

3) Is the response to dietary protein mediated by a hormonal signal?

4) What are the effects of dietary protein on receptor-mediated LDL catabolism?

5) How quickly are dietary proteins able to regulate LDL-receptor activity?

Most importantly, what is the primary event in dietary protein-induced hypercholesterolemia? It is still teleologically intriguing that a diet devoid of any cholesterol leads to such dramatic effects on plasma cholesterol concentrations.

This thesis describes experiments designed to explore some of these questions and attempts to fit the answers into the mechanistic scheme of events in cholesterol metabolism outlined in earlier sections.

CHAPTER 2

TIME COURSE OF THE EFFECTS OF CASEIN AND SOY PROTEIN ON LIPOPROTEIN CHOLESTEROL AND PROTEIN.

2.1 INTRODUCTION

Numerous studies have shown that the hypercholesterolemia produced in rabbits by feeding low-fat, cholesterol-free, semipurified diets containing casein can be prevented, if this component is replaced by isolated soy protein (Carroll et al. 1979; Carroll, 1982; Gibney and Kritchevsky, 1983; Terpstra et al. 1983). The excess serum cholesterol in casein-fed rabbits is carried mainly in the low density lipoprotein (LDL) fraction, but dietary casein also affects other components in all the lipoprotein fractions (Carroll et al. 1979; Terpstra et al. 1983).

Rabbits fed the soy protein diet maintain low levels of serum cholesterol, comparable to those found in rabbits fed natural ingredient (chow) diets. However, in previous work from this and other laboratories (Terpstra and Sanchez-Munis, 1981; Huff et al., 1982; Hrabek-Smith and Carroll, 1987) it was shown that the rabbits fed soy protein diets had an altered distribution of lipoprotein cholesterol and protein, compared to chow-fed rabbits. This difference in the lipoprotein profiles is probably due to the higher level of protein in the semipurified diets compared to chow diets and to differences in their overall composition.

The studies reported in this chapter are essentially an offshoot of these earlier studies, with some minor modifications which are a consequence of some points that these previous studies raised. Additionally the results of the studies described in this chapter, form the basis of the work subsequently outlined in this thesis.

In order to analyze the time course of the changes in the lipoprotein components associated with the increase in serum cholesterol in casein-fed rabbits, the levels of cholesterol and protein were measured in the three lipoprotein fractions (VLDL, LDL and HDL) before, during and after transferring the rabbits from chow to a semipurified casein diet, and the results were compared with those obtained for rabbits transferred from Chow to a semipurified soy protein diet.

2.2 METHODS

2.2.1 Animals and Diets

Male New Zealand rabbits weighing approximately 1.5kg were obtained from Re' ien's Fur Ranches (Guelph, Ontario). The animals were individually housed in stainless steel cages in a room with temperature controlled around 20°C and a 12-h lighting cycle with lights on from 0700-1900 h. Upon arrival the rabbits were randomly divided into two groups of 4 animals each and fed fed pelle'ed Purina Rabbit Chow (Ralston Purina Co., St.

Table 2.1 Composition of the low-fat, cholesterol-free semipurified diets

Ingredient[a]	g/kg	
Casein (vitamin-free)	270[b]	---
Soy Protein	---	275
Dextrose	600	595
Celluflour	50	50
Salt Mix	40	40
Molasses (50% v/v)	30	30
Vitamin mixture[c]	2	2
Corn Oil	10	10

[a] The vitamins, salt mixture (Philips and Hart salt mixture IV) and "vitamin-free" casein were obtained from ICN Life Sciences Group, Nutritional Biochemicals Division, Cleveland, OH. Dextrose was obtained from Tekland Test Diets, ARS/Sprague-Dawley Division of the Mogul Corporation, Madison, WI. Celluflour was from Chicago Dietetic Supply House, Chicago, IL. Molasses were obtained from a local feed mill, and corn oil from the local supermarket. The soy protein (Supro 910) was from Ralston Purina Co., St. Louis, MO.

[b] Casein and soy protein were added in these amounts to provide 25% (w/w) protein in the diet.

[c] The composition of the vitamin mixture was that published previously (Roberts et al., 1981). The water soluble-vitamins were added at 15 ml/kg of diet. The fat-soluble vitamins were dissolved in corn oil and added at 10 ml/kg of diet.

Louis, MO) for one week. Group 1 was then given a 2:1 (w/w) mixture of powdered Purina Chow and semipurified casein diet and group 2 a similar mixture of powdered Purina Chow and semipurified soy protein diet. (The Purina Chow was powdered by grinding in a kitchen blender). These mixed diets were fed for three days and the proportions were then changed to a 1:2 mixture of powdered Chow and semipurified diet for the remainder of the week. This week served as the adaptation period. Following the week of adaptation rabbits, were fed the casein or soy protein diets alone for a further 4 weeks. The composition of the casein and soy protein diets, used in all the studies described in this thesis, (Table 2.1), was identical to those used previously. The composition of the vitamin mix has been described by Roberts et al., (1981). In all cases the diets were provided ad libitum and the rabbits had free access to water. Body weights were measured weekly.

2.2.2 Study design

Blood samples were collected for analysis at the beginning (week -1) and end of the week of adaptation (week 0) and weekly thereafter. For this purpose rabbits were fasted overnight. They were then anesthetized with a subcutaneous injection of Innovar, 125µl/kg body weight (Tillman and Norman, 1983) and blood was withdrawn from the central ear artery using a 22 gauge needle. The serum was separated by low speed centrifugation (1000 x g, 20

Table 2.2 Body weights and serum cholesterol levels in rabbits fed diets containing casein or soy protein.

Time (weeks)	-1	0	1	2	3	4
Casein						
Body weight	1.6±0.03	1.7±0.1	1.9±0.1	2.0±0.1	2.0^{a}±0.1	2.1^{b}±0.1
Cholesterol	37±5	48±8	111^{c}±12	187^{d}±17	237^{e}±20	267^{f}±18
Soy protein						
Body weight	1.6±0.04	1.7±0.1	1.9±0.1	2.0±0.1	2.3±0.1	2.4±0.1
Cholesterol	34±5	30±7	50±6	66±8	60±7	57±6

Body weight is expressed in kg and serum cholesterol in mg/dl

Each value is the mean±SEM of 9 rabbits except for the serum cholesterol values at week -1. These are the means±SEM of the 4 rabbits per dietary group used in Experiment 1. (In Experiment 2 the cholesterol value in the pooled sera of 10 rabbits was 36mg/dl. From these 10 rabbits, 4 animals were then assigned to each of the two diets).

Values bearing a superscript in the casein group were significantly different from the corresponding values in the soy protein group ($p<0.05$).

min) and the total cholesterol content determined (Zlatkis and Zak, 1969).

Lipoproteins were isolated from serum by sequential ultracentrifugation (Havel et al. 1955) using a Ti80 rotor in a Beckman L8-80 ultracentrifuge. VLDL was separated at a density of d<1.006 g/ml, LDL at 1.006<d<1.063 g/ml and HDL at 1.063<d<1.21 g/ml. Following isolation LDL and HDL were then dialysed against 0.15M NaCl/1mM EDTA adjusted with Tris buffer to pH 8.6 and 8.1 respectively. In some cases the lipoproteins were subjected to SDS-PAGE on 12.6% separating gels (Laemmli and Favre, 1973) and stained with silver stain (Bio-Rad, Mississauga, Ont.) Lipoprotein cholesterol was measured by the method of Zlatkis and Zak (1969), and lipoprotein protein by the method of Lowry et al. (1951) as modified by Markwell et al. (1978).

The results given are means (±SEM) of two different experiments on equal numbers of animals. Statistical analysis was performed using Student's t-test.

2.3 RESULTS

The changes in body weight and serum cholesterol during the 5 weeks of the experiment are shown in Table 2.2. Both groups gained weight throughout the study period. At the end of the study period the weight of the rabbits fed casein was marginally (but statistically) lower than the weight of the rabbits fed soy protein. As in

previous studies the rabbits fed casein became markedly hypercholesterolemic compared to rabbits fed soy protein.

For the rabbits fed casein there was a significant increase in serum cholesterol following the week of adaptation (greater than two-fold). The cholesterol increased steadily upto week three after which there was a smaller increase. Similarly with soy protein fed rabbits, an increase of almost two-fold was seen in the week following the adaptation period. From the second week onwards the serum cholesterol levels in the soy-fed rabbits held steady. Throughout the duration of the study the serum cholesterol levels in the casein-fed rabbits were significantly higher than their soy-fed counterparts.

Fig. 2.1 shows the changes in lipoprotein cholesterol and protein during the course of the experiment. For the rabbits fed casein (Fig.2.1a) the principal increase in cholesterol occurred in the LDL fraction. This more than doubled during the adaptation period (unlike the serum cholesterol level which did not change significantly). Following the adaptation period there was a steep increase in LDL cholesterol, which was somewhat slower from weeks 2-4. VLDL cholesterol rose slowly throughout the course of feeding casein and at the end of the study the VLDL levels were twenty fold higher than at the onset. HDL cholesterol also tended to rise but the percentage increase was much less than for LDL or VLDL.

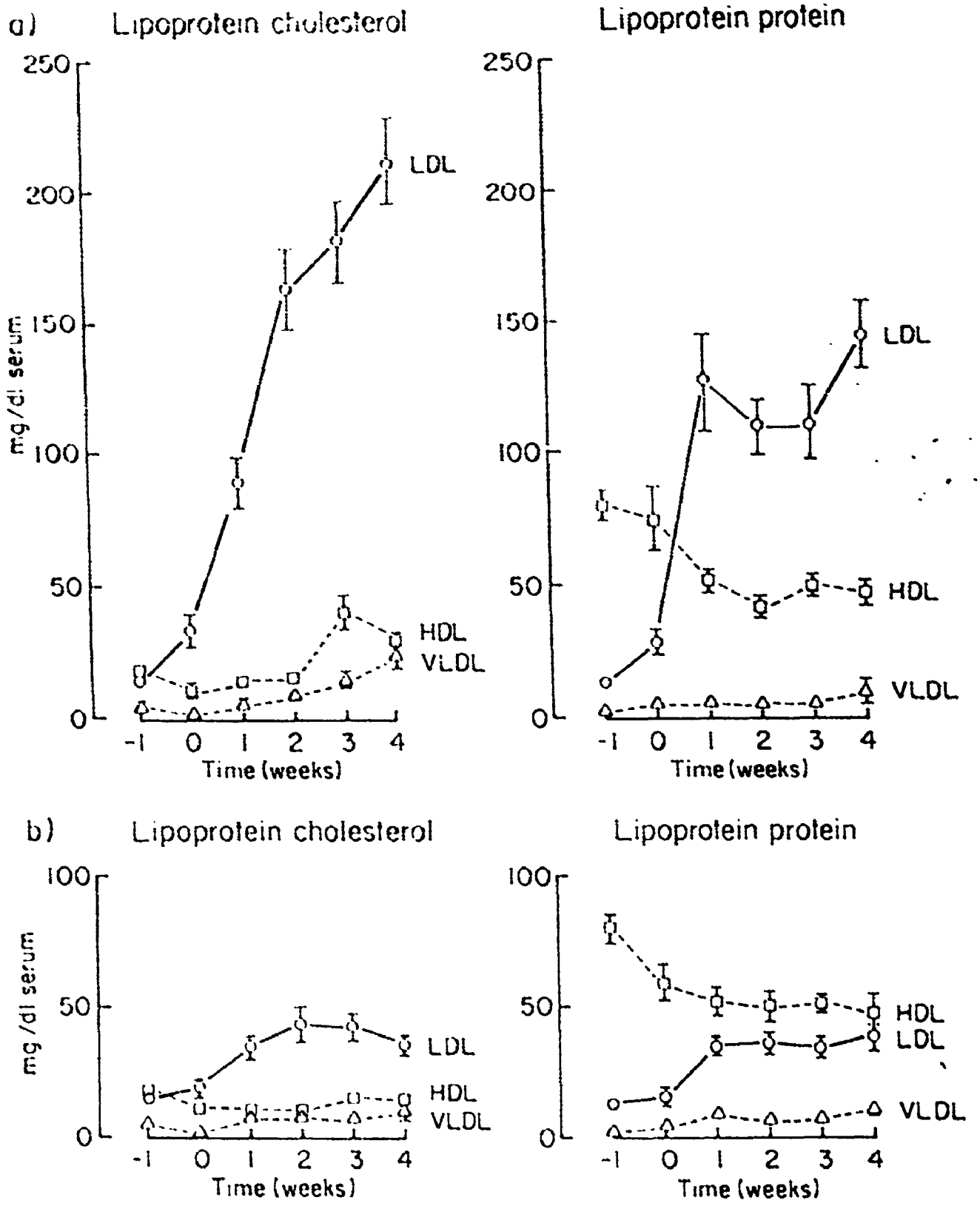

a)
Lipoprotein cholesterol
Lipoprotein protein
250
200
150
100
50
0
mg/dl serum
LDL
HDL
VLDL
-1 0 1 2 3 4
Time (weeks)
b)
Lipoprotein cholesterol
Lipoprotein protein
100
50
0
mg/dl serum
LDL
HDL
VLDL
-1 0 1 2 3 4
Time (weeks)

Fig. 2.1

Time course of the changes in lipoprotein cholesterol and protein in rabbits fed a) casein or b) soy protein diets

Lipoproteins were isolated each week from the number of rabbits indicated in the legend of Table 2.2

VLDL cholesterol and protein levels were significantly higher in the casein group at weeks 3 and 4. LDL cholesterol and protein levels were significantly higherin the casein group from week "0" onwards. HDL cholesterol levels were significantly higher in the casein group at week 3 and 4.

All statistical significance was $p<0.05$.

The rabbits fed soy protein showed similar trends (Fig 2.1b) although the absolute levels of lipoprotein cholesterol were much lower than in the corresponding group fed casein. LDL cholesterol rose two-fold during the first week on diet. Following this time period, LDL cholesterol levels did not change significantly over the remainder of the study. VLDL cholesterol levels rose steadily throughout and were three fold higher at the end of 4 weeks compared to their levels at the beginning of the study. HDL cholesterol levels fluctuated from week to week and showed relatively little change overall.

At the onset of the study HDL was the principal transporter of lipoprotein cholesterol (week -1). However from the earliest time that the semipurified diets were fed (adaptation period) LDL became the principal transporter of lipoprotein cholesterol (week 0) irrespective of the protein source in the diet.

The corresponding changes in lipoprotein protein are also shown in Fig. 2.1a and Fig.2.1b. For rabbits fed casein (Fig.2.1a) LDL protein levels began to rise during the adaptation period and rose steeply in the first week after the adaptation period. From weeks 1-4 LDL protein levels did not change significantly so that the rabbits were in a steady state with respect to serum LDL protein concentrations. These changes in LDL protein were

Table 2.3 **Cholesterol/Protein (C/P) ratios in the lipoprotein fractions**

Time (weeks)	-1	0	1	2	3	4
Casein						
VLDL	0.9±0.2	0.4±0.1	1.1 ±0.2	1.6[a]±0.1	3.3[b]±0.7	2.5[c]±0.2
LDL	1.1±0.1	1.3±0.3	0.8 ±0.1	1.6[d]±0.1	1.7[e]±0.1	1.5[f]±0.1
HDL	0.2±0.02	0.2±0.07	0.3[g]±0.03	0.4[h]±0.05	0.8[i]±0.08	0.7[j]±0.08
Soy protein						
VLDL	0.7±0.1	0.5±0.2	0.8±0.1	1.1±0.1	1.0±0.1	1.0±0.1
LDL	1.0±0.1	1.4±0.4	0.9±0.1	1.2±0.1	1.1±0.1	0.8±0.1
HDL	0.2±0.02	0.2±0.06	0.2±0.02	0.2±0.03	0.3±0.04	0.4±0.09

For number of observations refer to legend of Table 2.2

(In Experiment 2, the C/P ratios for VLDL, LDL and HDL isolated from the pooled sera of 10 rabbits at week -1, were 0.5, 0.7 and 0.2 respectively).

accompanied by opposite changes in HDL protein. At the end of the experimental period HDL protein levels had dropped almost two fold.

For the rabbits fed soy protein (Fig.2.1b), similar trends were observed with HDL and LDL protein levels except that the changes were less pronounced. As a consequence LDL protein levels remained lower than HDL protein levels at all time points. As with their casein counterparts the rabbits fed soy protein also reached a steady concentration of LDL protein since this was unaltered after the first week. In both dietary groups there were modest increases in VLDL protein over the time period that the diets were fed.

As with lipoprotein cholesterol levels, the bulk of the lipoprotein protein was in the HDL fraction at the onset of the study (week -1). However upon introduction of dietary casein, the majority of the lipoprotein protein was found in LDL. In contrast in rabbits fed soy protein, HDL contained the majority of the lipoprotein protein

In rabbits fed casein, the combined effects on lipoprotein cholesterol and protein resulted in the production of particles enriched in cholesterol relative to protein. This occurred in all lipoprotein fractions but the percentage increase was greater in VLDL and HDL (Table 2.3). The changes in rabbits fed soy protein were smaller. As a result the C/P ratio was significantly higher in HDL of casein-fed rabbits at week 1, and in all lipoprotein

fractions from week 2 onwards. However, whereas the increase in the C/P ratio of VLDL and LDL in casein-fed rabbits was due to increased cholesterol in these lipoprotein fractions, the increase in the C/P ratio of HDL was attributable to a decrease in the protein content of this fraction.

2.4 DISCUSSION

These studies confirm the earlier observation (Hrabek-Smith and Carroll, 1987) that HDL contains the highest proportion of cholesterol in chow-fed rabbits, whereas rabbits fed semipurified diets containing either casein or soy protein have most of their cholesterol in LDL (Fig.2.1). Also in the previous report it was shown that rabbits on semipurified diets, for a period of 6-8 weeks, had elevated LDL protein levels and decreased HDL protein in comparison to rabbits maintained on Chow diets. The results from the current studies show that these changes in LDL and HDL protein occurr within the first week that the rabbits are fe the semipurified diets (Fig.2.1), and that over the duration that the diets were fed, LDL and HDL protein changed in opposite directions in both dietary groups. The casein diet produced a greater increase in LDL protein than the soy protein diet so that LDL protein exceeded HDL protein in casein-fed rabbits but remained lower than HDL protein in those fed soy protein.

The rapid change in LDL cholesterol in these

experiments is essentially in agreement with the findings of Terpstra and Sanchez-Munis (1981). However from the results of the experiments described in this Chapter it can be seen that for casein-fed rabbits there was a decrease in HDL protein and a large increase in LDL protein whereas Terpstra and Sanchez-Munis reported an increase in HDL protein and a much smaller increase in LDL protein. It seems probable that these differences are related to differences in the composition of the semipurified diets. Those used by Terpstra and Sanchez-Munis contained a higher content of fat (14.8% v. 1%), and a lower content of protein (20% v. 27%) compared to the diets used in the current studies.

Additionally, there were other differences between the two experimental protocols. Terpstra and Sanchez-Munis used pelleted semipurified diets restricted to 100g/day (which gave similar growth rates in the two dietary groups, whereas in this study the growth rate was marginally higher in the rabbits fed soy protein). The differential in body weights between the casein and soy protein-fed rabbits, in this study, is unlikely to have affected the levels of serum cholesterol since previous studies have shown no correlation between weight gain and serum cholesterol levels in rabbits fed various semipurified diets (Hamilton and Carroll, 1976; Huff et al., 1977). Also, since in this study the feed consumption

of the rabbits was not monitored, it is not possible to decipher whether the differential in body weights was due to less food consumption by the casein group, or whether the rabbits on the casein diet were less feed efficient. (It should also be stated here that of all the studies described in this thesis, the ones described in this chapter are the only ones in which a differential in body weight was observed. In all subsequent results discussed, csein and soy protein fed rabbits had comparable body weights).

In the study by Terpstra and Sanchez-Muniz (1981) rabbits were transferred from Chow to the semipurified diets without an adaptation period, and following the collection of non-fasting blood samples lipoproteins were separated by discontinuous ultracentrifugation which may have resulted in the contamination of HDL with other serum proteins e.g. albumin. In these studies SDS-PAGE of 10µg HDL followed by silver staining showed negligible contamination with albumin.

The elevation in LDL cholesterol and protein in rabbits fed casein may be due to impaired LDL removal and/or increased synthesis. The increase in LDL cholesterol and protein in these experiments occurred earlier and were more pronounced than those in VLDL (Fig.2.1). Scholz et al (1982) also observed that LDL cholesterol and protein increased first, in rabbits transferred from a soy protein semipurified diet to one

containing casein This suggests that the rise in LDL levels is due to impaired removal.

These studies also showed that the cholesterol to protein ratio in the different lipoprotein fractions was consistently higher in casein-fed rabbits compared to those fed soy protein (Table 2.3). An increased ratio was also observed in some lipoprotein fractions of casein-fed rabbits by Terpstra and Sanchez-Munis (1981), and by Scholz et al (1982).

The decrease in HDL protein observed in these experiments when rabbits were switched from chow to semipurified diets is of interest because it has now been established by experiments in rabbits, that HDL plays a key role in reverse cholesterol transport, in vivo, whereby cholesterol is transported from the peripheral tissues to the liver, (via HDL), for degradation (Miller et al, 1985). The fact that HDL protein decreased in these experiments and not those of Terpstra and Sanchez-Munis (1981), may be related to some of the differences in dietary composition and experimental protocol already discussed.

In summary, the results presented in this Chapter show that,

1) Rabbits fed casein develop marked increases in serum cholesterol compared to rabbits fed soy protein

2) This increase is characterized by elevations in LDL

protein and cholesterol

3) The increases are particularly pronounced over the first two week that casein is fed

4) Subsequent to the initial rapid increase in LDL protein levels in casein-fed rabbits, the LDL (VLDL and HDL) protein levels remain stable

5) Casein feeding results in increased C/P ratios of the lipoprotein fractions

6) The increase in C/P ratio of HDL is due to a decrease in HDL protein levels

These results form the platform for the experiments to be described in the following chapters.

CHAPTER 3

THE TURNOVER OF VLDL AND LDL APOLIPOPROTEIN B IN RABBITS FED SEMIPURIFIED DIETS CONTAINING CASEIN OR SOY PROTEIN

3.1 INTRODUCTION

The previous chapter showed the results of studies which looked at the time course of casein-induced hypercholesterolemia. The excess cholesterol in the serum of casein fed rabbits accumulated principally in the LDL fraction. This accumulation of LDL cholesterol could result from either increased production of LDL, decreased clearance of LDL or a combination of both. To address this question turnover studies are reported in this chapter.

In previous studies in this laboratory (Roberts et al, 1981), ^{125}I-labeled VLDL and ^{125}I-labeled IDL were shown to turn over more rapidly in rabbits fed soy protein compared to rabbits fed casein. In these studies the metabolism of whole VLDL and IDL was followed and no attempts were made to characterise the fate of individual lipoprotein apoproteins. It is known that newly synthesised triglyceride (Havel 1961) and cholesterol enter plasma with VLDL particles that contain apolipoprotein B (Hamilton, 1972; Windmueller et al, 1973) Following the action of lipoprotein lipase (Havel et al. 1970) triglyceride is removed along with the small

molecular weight apolipoprotein Cs, which enriches the particles in esterified cholesterol, leading to the formation of smaller IDL and subsequently LDL particles (Eisenberg et al, 1973).

Ghiselli (1982) showed that in rabbits on commercial diet, VLDL apo B is cleared from plasma mainly in IDL. He also showed that more than 80% of the LDL apo B plasma pool was derived from IDL-apo B whereas 20% was synthesized independently.

In order to understand the mechanism underlying the elevation of plasma cholesterol in casein-fed rabbits, the kinetics of VLDL, IDL and LDL apo B metabolism were studied following the simultaneous injection of ^{125}I-VLDL and ^{131}I-LDL. Studies of apo B provide insight into lipoprotein metabolism because apo B stays with the VLDL particle during its sequential delipidation to IDL and then to LDL. The results of these studies are reported in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Diets

Young male New Zealand white rabbits (starting weight 1.5kg) were used. They were fed casein or soy protein diets for 6-9 weeks following a one-week adaptation period.

3.2.2 Isolation and iodination of lipoproteins

Following a 12h overnight fast, blood was

collected from the marginal ear vein of unanesthetised rabbits into EDTA-coated vacutainers (Becton-Dickson, Mississauga, Ontario) that were kept on ice. Plasma was prepared by low speed centrifugation (1500 x g). Sodium azide solution (10% w/v) was added to the plasma (10µl/ml plasma).

Lipoproteins, VLDL ($d < 1.006$ g/ml) and LDL ($1.019<d<1.063$ g/ml) were isolated by sequential ultracentrifugation (Havel et al., 1955) in a Ti 50 rotor at 40,000 rpm, 15°C. Each isolated lipoprotein was recentrifuged once at its optimal density. LDL was dialysed at 4°C against 0.15M NaCl/1mM EDTA, pH 7.4 for 24h.

$Na^{125}I$ and $Na^{131}I$ were obtained from Amersham (Oakville, Ontario). VLDL was radiolabeled with $Na^{125}I$ by a modification (Fidge and Poulis, 1974) of the Mcfarlane method (McFarlane, 1958). Briefly, VLDL was equilibrated to pH 10 with 0.4M glycine-NaOH by passage through a small column of Sephadex G-50 (0.5 x 15cm). Following iodination the VLDL was passed through a second Sephadex G-50 column equilibrated with 0.15M NaCl/1mM EDTA, pH 7.4. The labeled lipoprotein was then exhaustively dialysed against 0.15M NaCl/1mM EDTA pH 7.4, to remove free iodine. The labeled VLDL was sterilised by passage through a 0.45µm Millex-HA filter and gentamycin sulphate (1µg/ml) was added. LDL was treated in identical fashion except that the iodination was carried out using $Na^{131}I$. Within 36h

Table 3.1 Intramolecular distribution of radioactivity in ^{125}I-VLDL and ^{131}I-LDL isolated from rabbits fed casein or soy protein diets

Label	^{125}I-VLDL	^{131}I-LDL
	(Percent of total radioactivity)	
Casein		
Protein	93±1	96±2
Lipid	6±1	3±1
Free iodine	2±1	2±1
Apo B	74±7	91±2
Soy protein		
Protein	89±3	95±2
Lipid	5±1	2±0.01
Free iodine	2±1	1±0.2
Apo B	67±3	95±2

Each value is the mean±SEM (n=3)

of iodination, labeled lipoproteins were reinjected into recipient rabbits.

The intramolecular distribution of radioactivity was determined on small aliquots of the labeled lipoproteins to which 5% (w/v) BSA was added. The free iodine content was determined on the supernatant following precipitation with TCA (5% final concentration) (Fidge and Poulis, 1974). The TCA pellet was then delipidated with diethyl ether/ethanol 3:1 v/v (Scanu and Edelstein, 1971) and radioactivity was determined in the pellet and the supernatant after drying under nitrogen. The supernatant radioactivity was used as a measure of lipid radioactivity, whilst the radioactivity of the delipidated TCA pellet gave the percentage of the label in protein. To determine apo B radioactivity, cold carrier LDL (200µg) was added to aliquots of the labels and the samples adjusted to 1ml with saline. 1ml of isopropanol was added and the samples left overnight (Egusa et al., 1983). Apo B was pelleted by centrifugation, and its content of radioiodine determined. The intramolecular distribution of ^{125}I-VLDL and ^{131}I-LDL, shown in Table 3.1, was similar in the two groups.

3.2.3 Protocol for Kinetic studies

Potassium iodide was added to the drinking water of each rabbit (0.1g/100ml, [Roth et al., 1983]) one day before injection of the tracer, and during the two days

that blood samples were taken following injection of the tracer

Following a 12h overnight fast, unanesthetised rabbits were injected simultaneously with 3-8μCi of ^{125}I-VLDL apo B and 1-2μCi of ^{131}I-LDL apo B into the marginal ear vein using a 23 gauge butterfly needle attached to a 2-way stopcock. Immediately afterwards the cannula was flushed with 2-3 mls of PBS. There were 6 turnover studies, 3 for each group. In each of the first 5 studies (3 soy protein and 2 casein) 3 rabbits were used, whereas in the final study (casein) 2 rabbits were used. Although the amounts injected in the 6 studies were within the range given above, in any one study, each of the rabbits was injected with the same amounts of the tracer.

Following injection of label, 4-5 ml blood samples were obtained at 5min, 20min, 40min, 1, 2, 3, 4, 6, 8, 12, 24, and 48h postinjection from the marginal vein of the opposite ear from each rabbit. To decrease the contribution of hepatically synthesized VLDL apo B, rabbits were not fed until after collection of the 12h blood sample. They were then allowed access to their diets for 4h and fasted for a further 8h prior to collection of the 24h blood sample. Following the 24h sample, rabbits were fed, and were fasted overnight prior to collection of the last blood sample. This procedure resulted in constant concentrations of apo B in the various lipoprotein

fractions. The mean coefficient of variation over the course of the experiment was 11% (range 5% to 16%) for VLDL, 4% (range 2% to 6%) for IDL and 2% (range 1% to 4%) for LDL. There were no consistent differences between dietary groups. Rabbits had access to their drinking water throughout the study.

Blood samples were collected into EDTA-coated vacutainers and kept on ice until isolation of plasma. Following separation of plasma, aliquots of the 5min sample were taken and the radioactivity in apo B determined (Yamada et al., 1986). For this purpose 200µg of cold LDL was added to duplicate 50µl aliquots of the plasma samples. The volumes were adjusted to 750µl with saline. 750µl of isopropanol was added and the samples left for 1h at room temperature. The samples were then centrifuged at 10,000 rpm for 15 min and the pellet radioactivity was measured. The dose of ^{125}I-apo B and ^{131}I-apo B injected was then calculated by multiplying the apo B radioactivity by the plasma volume, taken as 32.8 mls/kg body weight (Roth et al., 1983). The assumption was made that there was similar metabolism of apo B within the first 5 minutes in each dietary group.

For each of the 12 time points, equal volumes of plasma were pooled from 3 animals (5 studies) or 2 animals (1 study). Four to six mls of plasma were used for the isolation of VLDL, IDL ($1.006<d<1.019$ g/ml)

and LDL in a Ti 50 rotor at 40,000 rpm, 15°C. The lipoproteins were recentrifuged once at their optimal densities. IDL and LDL were dialysed against 0.15M NaCl, 1mM EDTA pH 7.4 for 24h.

The protein content of each lipoprotein was determined by Markwell's modification (Markwell et al., 1978) of the Lowry method (Lowry et al., 1951).

3.2.4 Determination of lipoprotein apo B concentrations and specific activities

Duplicate aliquots of each lipoprotein fraction (50-200µg) were adjusted to 1 or 1.5ml with saline. An equal volume of isopropanol was added whilst vortexing. The samples were left overnight at room temperature and apo B was pelleted by centrifugation (1000 x g) for 30min. The supernatant was removed leaving behind the last 500µl above the pellet. The protein concent of the supernatant was determined. The difference between this value and the initial protein was used as a measure of apo B concentration (Egusa et al., 1983).

The pellet from the first centrifugation, above, was then processed for specific activity measurements (Huff et al., 1985). The pellet was washed twice with 2 ml of isopropanol/water (1/1 v/v), once with 3 ml of isopropanol and finally with 3 ml of deionised water. Each time 500µl was left above the pellet. After the final wash, 125µl of 2M NaOH was added. The radioactivity in the pellets was then determined. The pellets were then

left to incubate at 37°C. For VLDL and IDL the incubation period was 24h whereas for LDL it was 48h. After this, the protein content of the pellets was determined (Markwell et al. 1978) except that NaOH was omitted from the Lowry reagent.

3.2.5 Analysis of Kinetic parameters

The specific activity time curves of VLDL and LDL apo B plotted semi-logarithmically were biexponential. They were analysed by the two-pool model of Gurpide et al (1964) [Fig. 3.1]. The kinetic parameters gave values for the mass of apo B in pool A, the irreversible fractional catabolic rate from pool A and the production of apo B in pool A. The latter, by definition, represents input of apo B exclusively into the larger metabolic compartment (pool A) and excludes any apo B that recycles between the two pools.

The use of the Gurpide model for the study of apoprotein B kinetics has been discussed by previous workers (Reardon et al., 1978; Huff et al., 1981; Reardon and Steiner, 1982;) and will not be discussed further at this stage.

The ^{125}I-apo B specific activity curves for VLDL, IDL and LDL were compared and examined for precursor-product relationships between the three lipoprotein fractions. The criteria for precursor-product relationships have been described by Zilversmit (1960).

Fig. 3.1

Calculation of the kinetic parameters for a Two pool system

1. Pool Size (M_A) = $\dfrac{R_A}{C_A + C_B}$

2. Irreversible FCR (k_A) = $\dfrac{\alpha\beta}{k_{BA}}$

where $k_{BA} = -k_{BB}$ and $k_{BB} = -(\alpha + \beta + k_{AA})$

and $k_{AA} = \dfrac{-\alpha M_A C_A - \beta M_A C_B}{R_A}$

3. Production rate (PR_A) = $\dfrac{R_A \alpha\beta}{\alpha C_B + \beta C_A}$

Where R = Dose injected into pool A

C_A = Time zero intercept of fast phase

C_B = Time zero intercept of slow phase

α = Slope of fast phase

β = Slope of slow phase

(from Gurpide et al., 1964; Huff et al., 1981)

Briefly, these state that following injection of a labeled precursor, label must appear in the product and the peak of the product's specific activity time curve must not occur before the time at which the precursor and product specific activity time curves cross each other. Despite the fact that VLDL apo B can be heterogeneous with respect to its lipid and apoprotein content it was assumed that all possible subpopulations were isolated for labeling and that the initial injected radioactivity is proportional to the apo B mass in each subpopulation.

Additionally, the area under the specific activity curve was calculated as described by Goldberg et al (1983). This procedure, which does not require a knowledge of the time at which peak LDL apo B specific activity is reached, involves calculation of a dilution factor (DF) which estimates the extent to which the LDL apo B specific activity curve is diluted by the direct synthesis of unlabeled LDL apo B.

3.2.6 Other analyses

Plasma cholesterol and triglyceride concentrations, lipoprotein cholesterol and triglyceride concentrations were measured enzymatically using kits obtained from Boehringer-Mannheim (Montreal, Quebec); CHOD-PAP and GPO-PAP for cholesterol and triglyceride respectively.

Statistical analyses was performed using Student's t-test.

Table 3.2 Body weights and plasma lipid concentrations in rabbits fed casein or soy protein diets

	Body weight (kg)	Cholesterol (mg/dl plasma)	Triglycerides (mg/dl plasma)
Casein	2.8±0.2	291±31	87±8
Soy protein	2.9±0.2	86±9	83±9
		$p<0.05$	

The values given are the means±SEM of 8 and 9 rabbits fed casein and soy protein diets, respectively.

For each rabbit, the cholesterol and triglyceride values are the means obtained at 5 min, 24h and 48h in the experiment. (The cholesterol values for these time points were 306±40, 279±30, 287±25 and 80±9, 88±8 and 90±11 respectively for the rabbbits fed casein and soy protein)

3.3 RESULTS

3.3.1 Body weights and Plasma lipid concentrations

Body weights, plasma cholesterol and triglyceride concentrations for the two dietary groups, are shown in Table 3.2. Body weights and plasma triglyceride concentrations were similar between the two groups. The casein-fed rabbits had a greater than three-fold elevation in plasma cholesterol compared to rabbits fed soy protein.

3.3.2 Lipoprotein lipid and protein concentrations

The lipoprotein lipid and protein profiles in the two experimental groups are shown in Table 3.3. In the casein-fed rabbits, all three lipoprotein fractions were significantly enriched in cholesterol compared to the group fed soy protein. Most of the increase in plasma cholesterol was associated with LDL. Both groups had comparable lipoprotein triglyceride distributions. The protein content of VLDL and IDL was higher in the casein-fed group but these values were not statistically significant. The LDL protein concentration was 4-fold higher in the casein-fed rabbits. The percentage of LDL protein that was in apo B was also significantly higher in the casein group. Plasma cholesterol and lipoprotein apo B concentrations did not change over the 48h of the experiment consistent with the animals being in a steady state.

Diet	Exp#	VLDL		IDL		LDL	
				Cholesterol[a]			
Casein	1	60		30		136	
	2	42		36		98	
	3	66		62		158	
		56±7[b]		43±10		131±17	
Soy Protein	1	14		6		20	
	2	13		6		19	
	3	11		9		29	
		13±1		7±1		23±3	
		p<0.01		p<0.05		p<0.01	
				Triglycerides[a]			
Casein	1	34		18		38	
	2	30		18		30	
	3	22		10		22	
		29±3[b]		15±3		30±5	
Soy Protein	1	40		10		20	
	2	40		6		10	
	3	24		10		14	
		35±5		9±1		15±3	
				Protein[a] and [Zapo B][c]			
Casein	1	18	[42]	14	[70]	88	[89]
	2	22	[40]	14	[75]	100	[91]
	3	22	[47]	18	[79]	76	[88]
		21±1	43±2	15±1	75±3	88±7	89±4
Soy Protein	1	20	[31]	5	[75]	19	[72]
	2	15	[39]	11	[80]	24	[81]
	3	12	[31]	6	[71]	19	[64]
		16±2	34±3	7±2	75±3	21±2	79±4
						p<0.001	p<0.02

Table 3.3

Lipid and protein profiles of the lipoproteins in rabbits fed diets containing casein or soy protein

[a] mg/dl plasma; [b] Means±SEM; [c] % of lipoprotein protein in apo B.

In each experiment, lipoproteins were isolated from the pooled plasma of 3 rabbits, except for casein experiment #3, in which lipoproteins were isolated from the pooled plasma of 2 rabbits.

For each experiment, the cholesterol and triglyceride values are the mean of the values obtained on the 5min, 24h and 48h lipoprotein samples.

For each experiment, the protein and %apo B values are the means of the values obtained on each of the 12 lipoprotein samples (5min up to 48h).

3.3.3 Precursor-product relationships between lipoproteins following injection of radiolabeled VLDL apo B

The specific activities of ^{125}I-labeled apolipoprotein B in the different lipoprotein fractions over 48h following injection of ^{125}I-VLDL, plotted on a semi-log scale, for the first casein and soy protein experiments are shown in Fig. 3.2. Inspection of Fig. 3.2 for precursor-product relationships between the three lipoprotein fractions, shows that the LDL apo B specific activity peaks before it approaches the IDL apo B curve (its immediate precursor) in both dietary groups. In none of the casein studies did the LDL apo B specific activity curve intersect the IDL apo B specific activity curve over the 48h of the experiment. This is consistent with the interpretation that a large proportion of LDL apo B is synthesized directly, independent of the VLDL/IDL apo B cascade.

By application of the Zilversmit criteria the ratio of peak LDL apo B specific activity/corresponding IDL apo B specific activity, was calculated. This ratio was significantly lower for the casein group compared to the soy protein group (0.045 ± 0.007 vs. 0.31 ± 0.009, $p<0.001$), indicative of the fact that a significantly higher proportion of LDL apo B synthesis in casein-fed rabbits occurs independent of VLDL and IDL catabolism ($95\%\pm2$ vs. $67\%\pm2$, $p<0.001$). The direct input of LDL independent of VLDL was also calculated by the procedure of

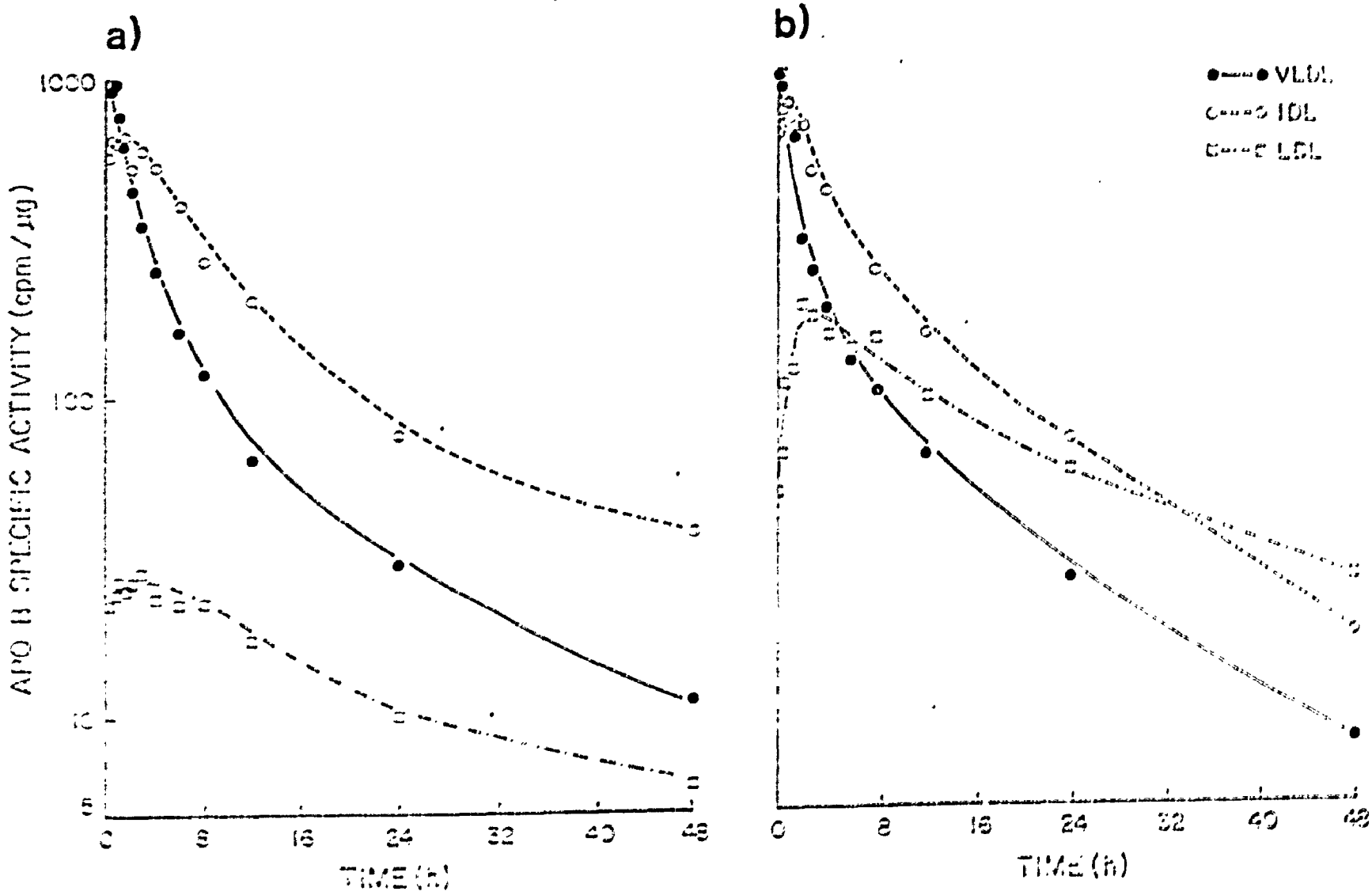

a)
b)
1000
100
10
1
APO B SPECIFIC ACTIVITY (cpm / µg)
0
8
16
24
32
40
48
TIME (h)
VLDL
IDL
LDL

Fig.3.2

Specific activity of apo B in VLDL, IDL and LDL following the intravenous injection of homologous ^{125}I-VLDL into rabbits fed diets containing a) casein or b) soy protein

Table 3.4 Kinetic parameters of ^{125}I-VLDL apo B.

Diet	Exp#	M (mg/kg)	FCR (pools/day)	PR (mg/kg/day)
Casein				
	1	6.5	5.04	32.9
	2	12.6	3.12	39.1
	3	5.2	2.88	14.4
		8.0±2.0[a]	3.68±0.68	28.8±7.4
Soy Protein				
	1	5.5	5.76	31.7
	2	4.1	6.00	24.2
	3	4.9	5.04	24.7
		4.8±0.3	5.60±0.29	26.9±2.4
			0.05<p<0.10	

[a] Mean±SEM

Lipoproteins were isolated from the pooled plasma of 3 rabbits, except for casein experiment #3, in which they were isolated from the pooled plasma of 2 rabbits.

M is the mass in pool A. FCR is the irreversible fractional catabolic rate (K_A) from pool A and PR is the production rate (or flux) in pool A as defined by Gurpide et al., (1964).

Goldberg et al. (1983). This also showed that a higher proportion of LDL apo B is synthesized directly in casein-fed rabbits in comparison to the group fed soy protein (93%±2 vs. 63%±2; $p<0.001$).

3.3.4 Kinetic parameters of ^{125}I-VLDL apo B metabolism

Kinetic parameters of VLDL apo B metabolism calculated from the VLDL apo B specific activity curve are listed in Table 3.4. The mean pool size of VLDL apo B tended to be higher in the casein group but this did not reach statistical significance. The production rate of VLDL apo B was similar for the two dietary groups. The mean fractional catabolic rate of VLDL apo B was lower in the group fed casein compared to the group fed soy protein. This is illustrated in Fig. 3.3 which shows the decline in VLDL apo B specific activity with the 5min specific activity taken as 100% and subsequent values adjusted to this initial value. The decline in VLDL apo B specific activity is more rapid in the soy protein group compared to the group fed casein.

3.3.5 Kinetic parameters of ^{131}I-LDL apo B metabolism

Fig 3.4 shows the curves of ^{131}I-LDL apo B specific activity from the same animals, which show a slower rate of decline in the casein-fed rabbits compared to those fed soy protein. From the absolute values of the ^{131}I-LDL apo B specific activities, the kinetic parameters for LDL apo B metabolism were calculated and are

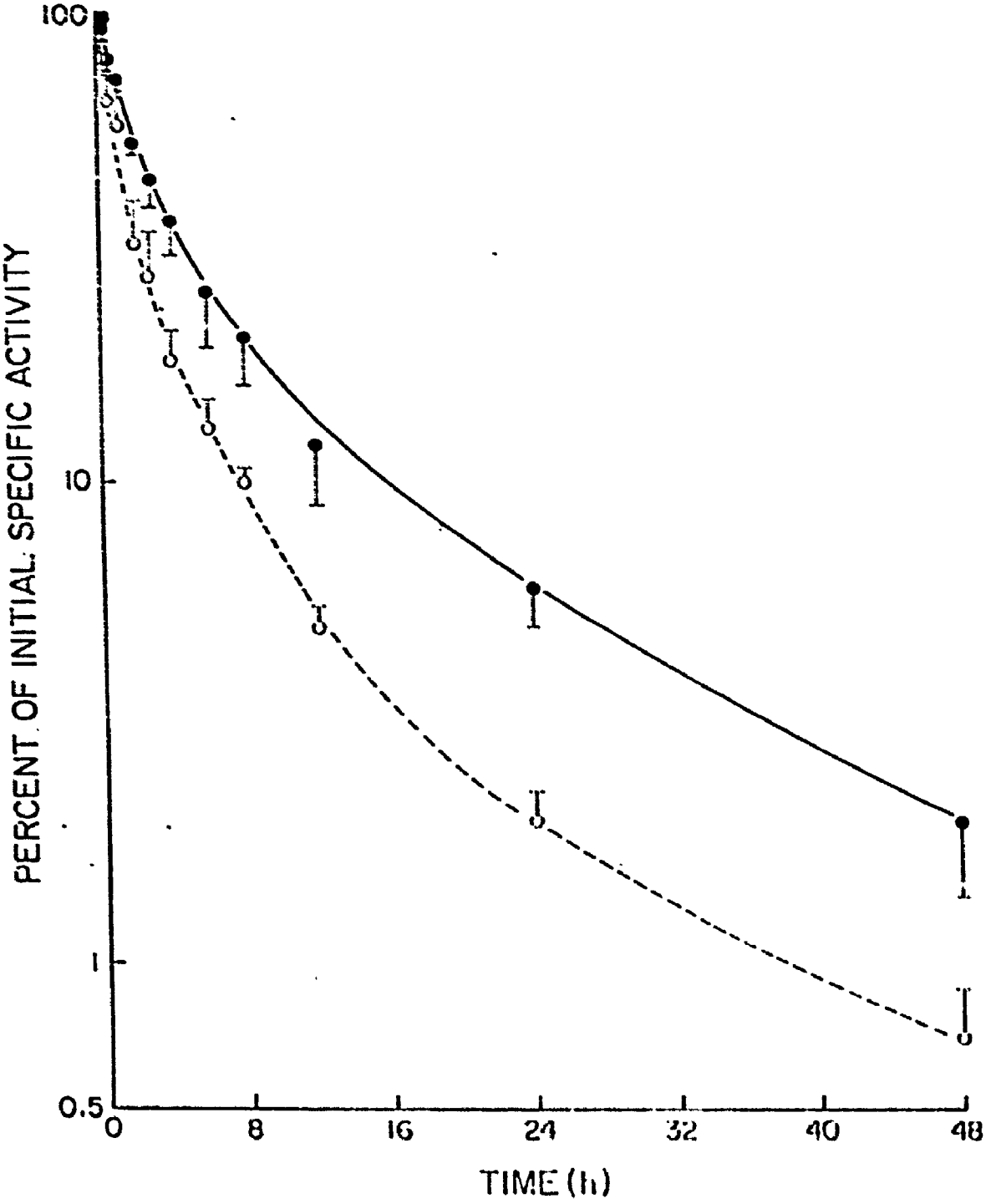

100
10
1
0.5
PERCENT OF INITIAL SPECIFIC ACTIVITY
0
8
16
24
32
40
48
TIME (h)

Fig.3.3

Decline in VLDL apo B specific activity (as a percentage of the 5min value) following the intravenous injection of homologous ^{125}I-VLDL into rabbits fed casein (●——●) or soy protein (O---O).

Each value is the mean±SEM from 3 experiments.

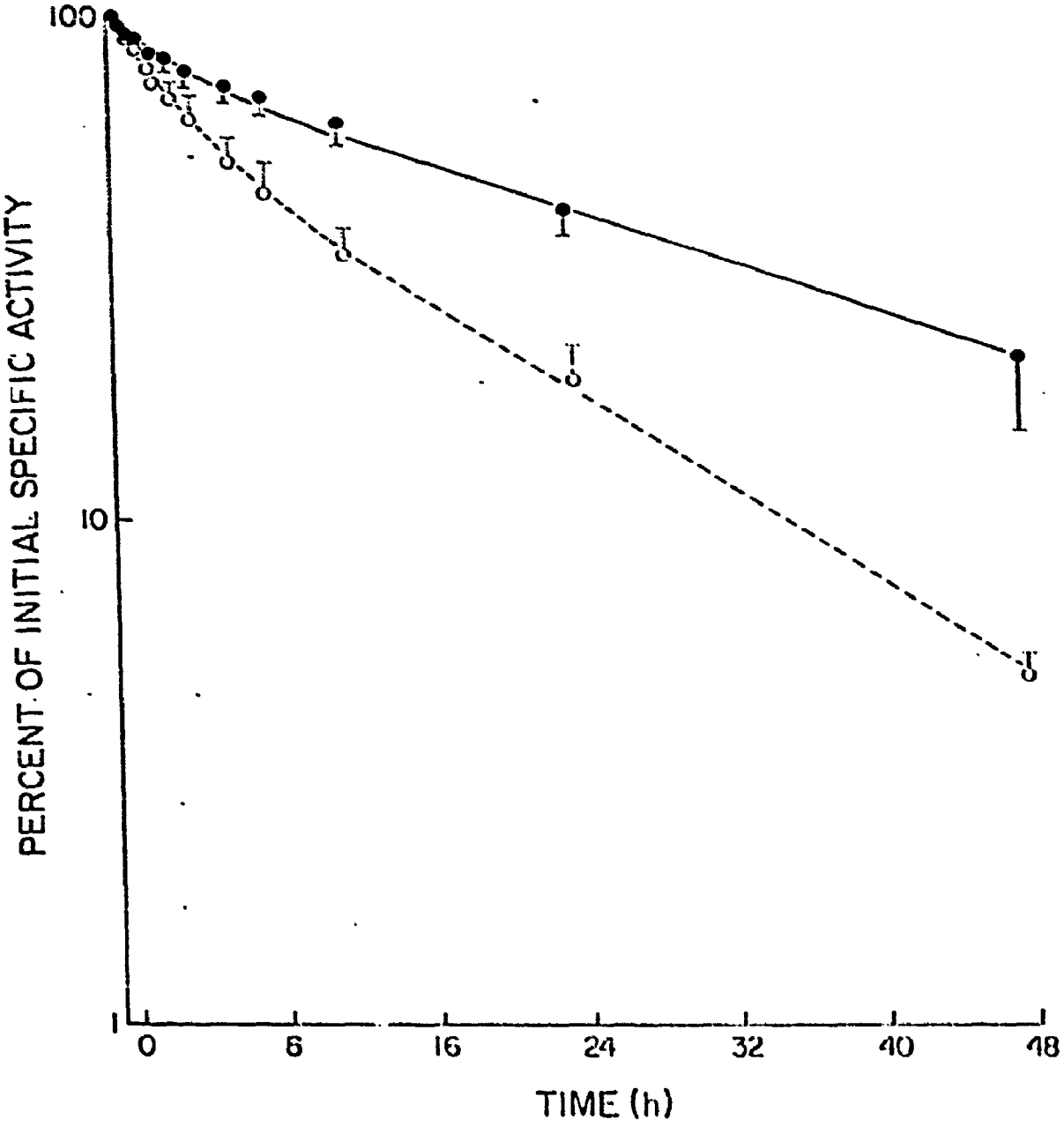

100
10
1
PERCENT OF INITIAL SPECIFIC ACTIVITY
0
8
16
24
32
40
48
TIME (h)

Fig.3.4

Decline in LDL apo B specific activity (as apercentage of the 5min value) following the intravenous injection of homologous ^{131}I-LDL into rabbits fed casein (●——●) or soy protein (O---O).

Each value is the mean±SEM from 3 experiments.

Table 3.5 Kinetic parameters of ^{131}I-LDL apo B.

Diet	Exp#	M (mg/kg)	FCR (pools/day)	PR (mg/kg/day)
Casein				
	1	33	0.84	27.8
	2	35	1.01	35.3
	3	21	0.58	12.0
		30±5[a]	0.81 ±0.13	25.0±6.9
Soy Protein				
	1	3	2.06	6.7
	2	5	2.18	11.0
	3	7	1.54	10.8
		5±1	1.93 ±0.20	9.5 ±1.4
		p<0.01	p<0.01	0.05<p<0.10

[a] Mean±SEM

See legend to Table 3.4

shown in Table 3.5. In agreement with the slower decline in ^{131}I-LDL apo B specific activity in casein-fed rabbits, the mean fractional catabolic rate of LDL apo B was significantly lower in the casein group. The production rate of LDL apo B was increased 2.5-fold in rabbits fed casein although this was significant only at $0.05<p<0.10$. The combination of increased LDL apo B production and decreased FCR resulted in a 6-fold increase in the LDL apo B pool size in casein-fed rabbits compared to the group fed soy protein.

3.4 DISCUSSION

Earlier studies in rabbits (Roberts et al., 1981) showed that the loss of radioactivity from the plasma after injection of ^{125}I-labeled VLDL was faster when the dietary protein was soy protein in comparison to casein. In studies with rats fed soy protein plus cholesterol, the fractional catabolic rate of VLDL apolipoprotein B was increased although its production rate was not affected (Cohn et al., 1984) compared to rats fed casein plus cholesterol. When soy protein was substituted for meat and dairy protein in the diets of hypercholesterolemic men (Huff et al., 1984) the turnover of VLDL apolipoprotein B was increased. Despite similar pool sizes of VLDL apolipoprotein B, the fractional catabolic rate was higher in subjects on the experimental diets, and in four of the five subjects the production rate

of apolipoprotein B was also higher.

The results presented in this section are consistent with the above observations. There was no statistical difference in VLDL pool size but the fractional catabolic rate of VLDL apo B tended to be higher in soy protein-fed rabbits compared to casein-fed rabbits ($0.05<p<0.10$). The results for LDL apo B were more dramatic. LDL apo B synthesis was increased 2.5-fold ($0.05<p<0.10$) and the efficiency with which this was removed, the FCR, was decreased almost 3-fold ($p<0.01$) in casein-fed rabbits compared to the group fed soy protein. This resulted in a 6-fold elevation of the LDL apo B pool size ($p<0.01$) in the casein group. The removal of labeled LDL from the plasma of casein-fed rabbits has also been studied by Chao's group (Chao et al., 1982; 1983), but in their studies the comparisons were made with rabbits fed casein supplemented with cholestyramine or mevinolin respectively, whereas in the studies reported in this section LDL metabolism has been studied with reference to the dietary protein component.

The decrease in the efficiency with which LDL apo B is removed is consistent with down regulation of LDL receptors. However, it should be pointed out that the FCR per se is a measure of both receptor-dependent and receptor-independent catabolism.

The direct production of LDL apo B observed in these studies in both dietary groups has also been

reported in normal rabbits fed chow diets (Kushwaha and Hazzard, 1978; Ghisseli, 1982; La Ville et al., 1984), miniature pigs (Huff and Telford, 1985), Cynomologous monkeys (Goldberg et al., 1983), rats (Faergeman et al., 1975; Fidge and Poulis, 1975) and humans with familial hypercholesterolemia (Janus et al., 1980) or dietary hypercholesterolemia (Kesaniemi et al., 1981). However in rabbits fed chow diets, Ghisseli (1982) found that only 20% of LDL apo B could be accounted for by direct production, whereas in these studies the values were 65% and 95% for the soy protein and casein-fed rabbits respectively.

The physiological significance of the direct production of LDL apo B has been the subject of much debate. It has been argued that some newly synthesized LDL apo B passes through a small VLDL-like precursor pool with a high production rate and is consequently not traced by the injected tracer (Huff and Telford, 1985). Alternatively, newly secreted VLDL may be rapidly converted to LDL such that a large proportion of apo B secreted initially as a component of VLDL, loses the bulk of its triglycerides and appears in the LDL density range prior to entering the circulation (Goldberg et al., 1983; Huff and Telford, 1985). The direct synthesis of LDL particles, rich in cholesterol esters and poor in triglycerides, has been shown to occur in experiments with perfused rabbit livers (Chao et al., 1986). In those studies it was shown that the

perfused livers of casein-fed rabbits had an eight-fold increase in the synthesis and secretion of LDL compared to perfused livers from chow-fed rabbits. The authors concluded that the LDL was directly secreted by the liver and was not the catabolic product of VLDL. Therefore the increased direct synthesis of LDL apo B in casein-fed rabbits compared to soy-protein fed rabbits ($p<0.001$) suggest that in addition to the decreased FCR in casein-fed rabbits, the direct synthesis of LDL apo B maybe a major factor responsible for the elevated pool size of LDL apo B in these animals. It is possible that the increased content of liver cholesterol in casein-fed rabbits (Huff and Carroll, 1980) results in increased secretion of cholesterol-rich LDL like lipoproteins directly into the circulation.

In summary, the results of the studies reported in this chapter show that, casein-induced hypercholesterolemia is associated with

1) Decreased production of LDL apo B from VLDL apo B
2) Decreased efficiency of removal of LDL apo B
3) Increased production of LDL apo B from sources other than VLDL apo B catabolism (i.e. increased direct synthesis of LDL apo B)
4) An elevated pool size of LDL apo B
5) A tendency for decreased efficiency of removal of VLDL apo B
6) A tendency for an elevated pool size of VLDL apo B.

CHAPTER 4

FURTHER STUDIES ON THE METABOLISM OF LDL APO LIPOPROTEIN B

4.1 INTRODUCTION

The previuos chapter presented the results of studies which showed that the hypercholesterolemia in casein-fed rabbits was associated with increased production of LDL apo B from sources other than VLDL catabolism, decreased efficiency of removal of LDL apo B and a tendency for increased production of LDL apo B. Thus it may be that as the amount of lipoproteins synthesised increases the efficiency with which they are catabolised, and subsequently removed from the circulation, decreases. This combination of increased synthesis and decreased removal (FCR) results in the elevation in lipoprotein apo B pools in casein-fed rabbits relative to the pools in rabbits fed soy protein. The results were consistent with impaired LDL-receptor activity.

The studies on lipoprotein turnover were carried out with homologous tracers, and therefore give no information as to whether the differences observed may be due to differences in the lipoprotein particles. In other words are there differences in the LDL of casein and soy-protein fed rabbits which are themselves responsible for the observed differences in turnover rates? If for

example the native LDL in casein-fed rabbits was structurally dissimilar to the native LDL in soy-protein fed rabbits, this itself may be resposible for the altered rates of turnover.

In previous studies by Roberts et al. (1981), it was shown that the turnover of ^{125}I-VLDL and ^{125}I-LDL was influenced by the diet fed to the rabbits that was used for the isolation (and subsequent iodination) of the tracer. In general, VLDL or LDL isolated from soy protein fed rabbits turned over more rapidly than that isolated from casein-fed rabbits, regardless of whether the recipient animals were fed casein or soy protein. Since whole lipoprotein turnover was studied in these experiments the results could have been due to differences in apo B or any of the soluble apoproteins (apo C's or apo E).

In considering LDL metabolism, attention has to be paid to two distinct catabolic pathways. One of these involves a receptor-mediated, high affinity process originally characterized in cultured human fibroblasts by Goldstein and Brown (1977). The other is a low affinity receptor-independent pathway thought to involve scavenger cells, bulk fluid endocytosis and absorptive endocytosis (Goldstein and Brown, 1977; Miller et al., 1978; Goldstein and Brown, 1978). The two processes can be distinguished, in vivo, by using labeled LDL and labeled LDL that has

been reductively methylated. The former is cleared by both processes, whereas the latter, unable to bind the receptor is cleared by the receptor-independent route. The difference, is therefore, a measure of receptor-dependent catabolism.

The present chapter describes experiments carried out to investigate

a) the effect of LDL particle composition on LDL metabolism in rabbits fed the different diets (Study 1),

b) the effects of the different diets on receptor-mediated and receptor-independent catabolism of LDL (Study 2), and

c) the effects of feeding casein for different time period on receptor-mediated catabolism of LDL (Study 3)

4.2 MATERIALS AND METHODS

4.2.1 Animals and Diets

12 rabbits fed casein or soy protein diets for a period of 14-16 weeks were used in Study 1. 8 rabbits fed casein or soy protein for 6-7 months were used in Study 2. In both studies 1 and 2, rabbits were fed the semipurified diets following a one week adaptation period as detailed in Chapter 2.

In Study 3, 16 rabbits were fed soy protein for 2 weeks following a one week adaptation period. They were then fed casein or soy protein for the time period indicated in the text.

4.2.2 Isolation and iodination of LDL

Casein, soy protein or Chow-fed rabbits, were bled from the marginal ear vein following an overnight fast. Plasma was obtained following low-speed centrifugation of the blood. Plasma density was adjusted to 1.019 g/ml by addition of KBr. LDL (1.019 <d< 1.063 g/ml) was isolated by sequential ultracentrifugation (Havel et al., 1955). The isolated LDL was respun once at its optimal density, and then dialysed against 0.15M NaCl,1mM EDTA pH 7.4.

LDL isolated from soy protein fed rabbits was labeled with ^{125}I (hereafter referred to as ^{125}I-LDLs) and LDL isolated from casein-fed donors was labeled with ^{131}I (hereafter referred to as ^{131}I-LDLc) [Study 1]. The intramoecular distribution of the label and the radioactivity associated with apo B was determined. The distribution of radioactivity was similar for both tracers with 92% of the label in apo B and less than 2% as free iodine.

The LDL isolated from Chow-fed rabbits (Study 2 and 3) was divided into two aliquots. One aliquot was labeled with $Na^{125}I$ (which was subsequently methylated) and the other aliquot with $Na^{131}I$.

4.2.3 Methylation of LDL

^{125}I-LDL (5mg) in 0.15M NaCl/1mM EDTA pH 8.6 was methylated using $NaBH_4$ and formaldehyde by

the procedure of Weisgraber et al (1978). Briefly to the ^{125}I-LDL, 2 mg of solid $NaBH_4$ was added. 1ul of 37% (w/v) formaldehyde was now added (time 0). A further 1ul was added at 6 min intervals. After 30 min (6µl formaldehyde added in total) the reaction was terminated by eluting the methylated LDL, through a small column of Sephadex G-50, using 0.15M NaCl/1mM EDTA pH 8.6. The methylated LDL was now dilaysed for 18h against 0.15M NaCl/1 mM EDTA pH 7.4. This procedure is sufficient to modify about 8 of 20 lysine residues per apo B molecule, which abolishes high-affinity LDL binding in cultured fibroblasts (Weisgraber et al. 1978) and delays the clearance of LDL from the plasma of animals (Mahley et al. 1980; Slater et al. 1980) and man (Shephard et al. 1979). The methylated LDL otherwise retains the chemical and physical properties of the native LDL (Weisgraber et al. 1978). The labeled LDL's were found to contain 95-96% of their total radioactivity in apo B, and less than 1% as free iodine.

To check that the LDL had been effectively methylated, aliquots of the ^{131}I-LDL and the methylated ^{125}I-LDL were injected into 2 Chow-fed rabbits and blood samples collected over 12h. From the plasma ^{125}I-apo B and ^{131}I-apo B radioactivities, receptor-dependent removal was estimated to be 60% of the total removal.

4.2.4 Sterilisation of tracers

All tracers were sterilised by passage

through a 0.45μm Millex-HA filter prior to use and gentamycin sulphate added (1μg/ml).

4.2.5 Prevention of thyroidal uptake of radioiodine

Each of the rabbits used in Studies 1, 2 and 3 had KI (0.1g/100ml) added to its drinking water, 2 days before injection of the tracer and for the duration of the kinetic study.

4.2.6 Protocol for kinetic studies

4μCi of ^{125}I-LDLs and 2μCi of ^{131}I-LDLc were simultaneously injected into each of 6 rabbits fed casein and 6 rabbits fed soy protein (Study 1). The amount of labeled LDL apo B injected, was less than 0.01% of the circulating LDL apo B pool. 2ml blood samples were obtained at 3min, 45min, 1.5, 3, 4.5, 6, 12, 24, 36, 48, 60, and 72h post-injection.

In Study 2, each rabbit was injected simultaneously with ^{131}I-LDL (range 8.6-12μCi) and ^{125}I-LDL(CH_3) (range 9.4-13μCi). Blood samples were collected from the marginal vein of the right ear at 3 min, 1.5, 3, 4.5, 6, 10, 25, 36 and 50h post-injection.

In Study 3, rabbits were injected simultaneously with 2-4μCi of ^{131}I-LDL and ^{125}I-LDL(CH_3). Blood samples were collected at the same times as those in Study 2.

4.2.7 Determination of plasma apo B radioactivity

Plasma was separated and plasma apo B radioactivity was determined as described by Yamada et al. (1986).

4.2.8 Isolation of LDL

Equal volumes of plasma were now pooled from each of the time points. LDL was then isolated from 4-6ml of plasma by sequential ultracentrifugation, $1.019<d<1.063$, and washed once at its optimal density (Havel et al., 1955). Protein (Markwell et al., 1978) and apo B concentration of LDL was now determined (Egusa et al., 1984).

LDL was not isolated in Study 3.

4.2.9 Kinetic Analyses

Plasma apo B radioactivity disappearance curves were plotted semilogarithmically against time were biexponential conforming to a two-pool model. The FCR for removal of ^{131}I-LDLc and ^{125}I-LDLs (Study 1) was determined according to the 2-pool model of Matthews (1953) as described by Kushwaha and Hazzard (1977).

$$FCR = \frac{1}{A'/\alpha + B'/\beta}$$

where $A'=A/100$ and $B'=B/100$

and A = Time zero intercept of slow phase

B = Time zero intercept of rapid phase

α = Slope of slow phase

β = Slope of rapid phase

Table 4.1

Body weights and plasma cholesterol concentrations

		Casein	Soy protein
Study 1			
(6)	BW	3.38±0.04	3.36±0.05
	PC	396±49	75±20
Study 2			
(4)	BW	3.77±0.10	3.55±0.07
	PC	279±24	62±14
Study 3			
5 Days	BW	2.59±0.09	ND
(4)	PC	90±10	
10 Days	BW	2.28±0.18	2.31±0.05
(3)	PC	183±1	73±15
25 Days	BW	2.72±0.13	2.49±0.08
(3)	PC	200±31	83±29

Each value is the mean±SEM of the number of rabbits given in parentheses.

BW - Body weight (kg); PC - plasma cholesterol (mg/dl)
ND - not determined

In each study the plasma cholesterol was significantly different between casein and soy protein-fed rabbits ($p<0.001$).

For study 1, plasma cholesterol values are the means of the 3min, 24h, 48h and 72h plasma samples.

For studies 2 and 3 plasma cholesterol values are the means of the 3min, and 24h plasma samples.

Similarly the FCR for removal of ^{131}I-LDL and ^{125}I-LDL(CH_3) [Study 2 and 3] was calculated. The FCR for the former represents the sum of both receptor-dependent and receptor-independent catabolism, whereas the latter represents receptor-independent catabolism. The difference in turn is a measure of receptor-dependent catabolism.

From the LDL apo B concentration and the plasma volume of the rabbit (taken as 32.8 mls/kg body weight) the pool size of apo B was calculated. The production rate of apo B was determined as FCR x apo B pool size.

4.2.10 Other analyses

Plasma cholesterol and triglycerides, LDL cholesterol, triglycerides and phospholipids were measured enzymatically using kits from Boehringer-Manhein. LDL protein was determined by Markwell's modification (Markwell et al., 1977) of the Lowry method (Lowry et al., 1951).

4.3 RESULTS

4.3.1 Body weights and plasma cholesterol concentrations

The plasma cholesterol concentrations and body weights of the rabbits used in the three studies are shown in Table 4.1. In any given study the body weights were not significantly different between the two dietary

Table 4.2

LDL Composition (mg/dl plasma)

Rabbit #	C	TG	PL	P
Casein				
1	209	16	98	103
2	99	14	55	48
3	207	18	95	116
4	158	6	74	80
5	124	5	60	62
6	71	2	34	37
Means±SEM	145±23	10±3	69±10	74±13
Soy Protein				
7	15	3	9	11
8	19	5	9	16
9	15	2	7	8
10	32	4	17	29
11	13	2	7	10
12	20	3	12	19
Means±SEM	19±3	3±0.5	10±2	16±3

The LDL composition for the 12 rabbits used in Study 1 is given. C=Cholesterol; TG= Triglycerides; PL=Phospholipids and P=Protein

Mean values for cholesterol, protein and phospholipids were significantly different between dietary groups ($p<0.001$). Mean triacylglycerol values were significantly different ($p<0.05$).

groups. In any given study the casein-fed rabbits were significantly hypercholesterolemic compared to rabbits fed soy protein ($p<0.05$). The plasma cholesterol values did not change significantly over the duration of a given study consistent with the interpretation that the rabbits maintained a steady-state.

In all of the studies, rabbits fed soy protein had comparable plasma cholesterol concentrations. However for rabbits fed casein (Study 3), the plasma cholesterol concentration increased progressively over the first 10 days.

4.3.2 Composition of LDL

The composition of the ultracentrifugally isolated LDL (Study 2) is shown in Table 4.2. The LDL of rabbits fed casein was enriched 7-fold in cholesterol, 3-fold in triglycerides and 7-fold in phospholipids compared to the LDL from soy protein-fed rabbits. The LDL protein pool was almost 5-fold greater in casein-fed rabbits as compared to soy protein-fed rabbits.

4.3.3 Decline in plasma apo B radioactivity following injection of homologous and heterologous LDL

Fig. 4.1. shows the disappearance of plasma apo B radioactivity (as a percentage of the 3min value), following the simultaneous injection of ^{125}I-LDLs and ^{131}I-LDLc into each of 6 rabbits fed casein and 6 rabbits fed soy protein (Study 2). It can be

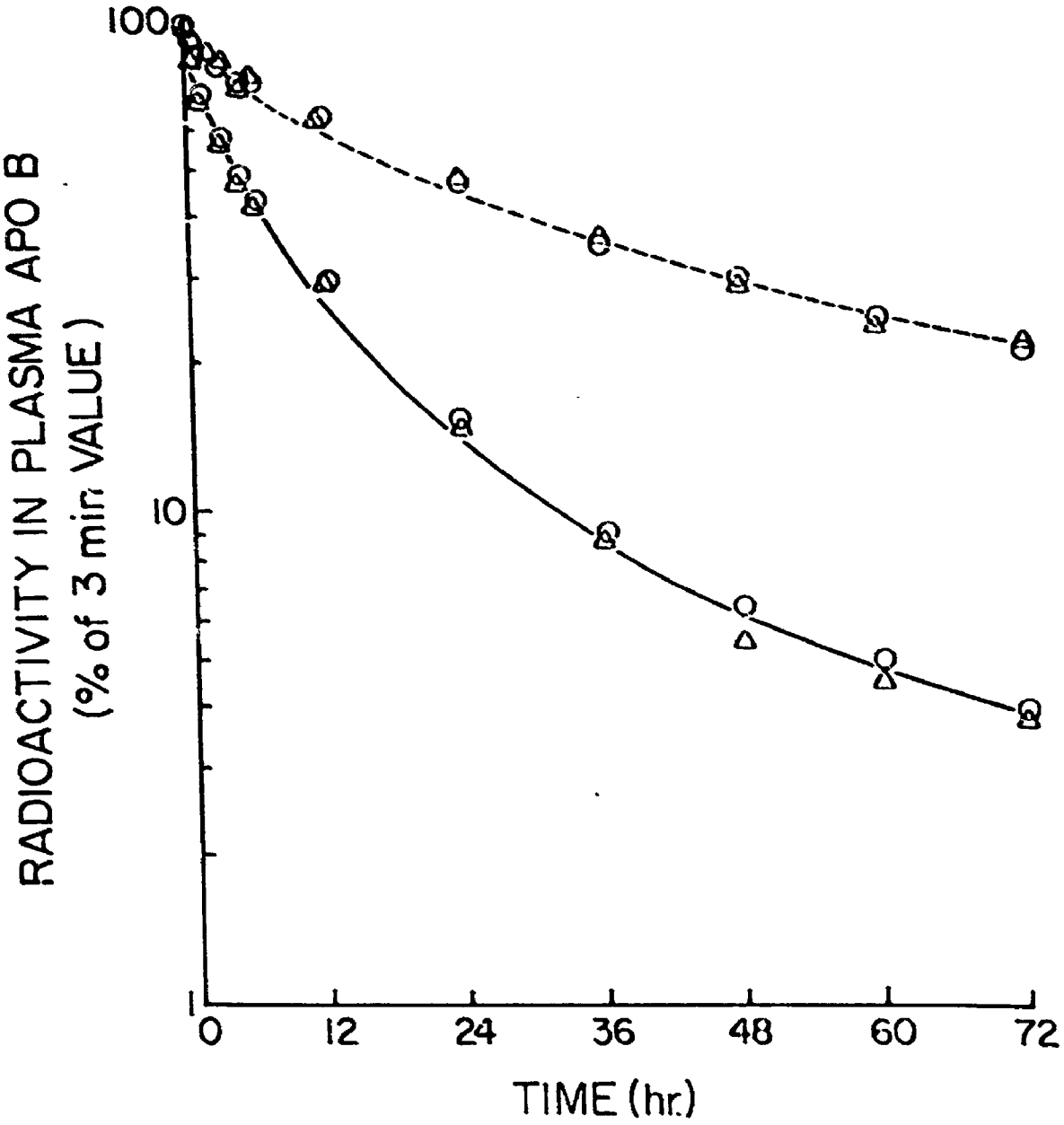

100
10
1
RADIOACTIVITY IN PLASMA APO B
(% of 3 min VALUE)
0
12
24
36
48
60
72
TIME (hr.)

Fig. 4.1

Decline in plasma apo B radioactivity following simultaneous injection of ^{125}I-LDLs and ^{131}I-LDLc.

The decline in plasma apo B radioactivity, as a percentage of the 3 min value following the simultaneous injection of ^{125}I-LDLs (Δ) and ^{131}I-LDLc (O) into rabbits rabbits fed casein (----) or soy protein (——). Each point is the mean of the value from 6 rabbits

Table 4.3

FCR of ^{131}I-LDLc and ^{125}I-LDLs in rabbits fed casein or soy protein diets

	FCR (pools/day)	
	^{131}I-LDLc	^{125}I-LDLs
Casein	0.55 ±0.05*	0.55 ±0.05
Soy Protein	2.14 ±0.43	1.90 ±0.32*

Each value is the mean±SEM (n=6)

Values sharing a common symbol (*) were significantly different between groups.

seen that both tracers were cleared equally effectively in either of the dietary groups. However as can be seen the removal of the tracers was faster in the rabbits fed soy protein. The FCR for the removal of the tracers is listed in Table 4.3. The FCR for the tracers was similarl in a given dietary group. Both tracers were removed faster in the soy protein-fed rabbits compared to the rabbits fed casein. For the homologous tracers, the FCR in soy protein-fed rabbits was 3-fold higher than the FCR in casein-fed rabbits.

Data for homologous tracers was subjected to kinetic analyses. Table 4.4. shows the kinetic parameters for homologous LDL apo B turnover. The increased pool size of LDL apo B in casein-fed rabbits resulted from increased production and slower efficiency of removal in comparison to the pool observed in rabbits fed soy protein.

There was a significant relationship between the FCR (for the homologous tracer) and plasma LDL apo B concentration (Study 2), Fig. 4.2. The FCR decreased with increasing LDL apo B concentrations in rabbits fed soy protein, whereas in rabbits fed casein, there was little or no change in FCR over a 3-fold increase in LDL apo B concentrations.

4.3.4 Decline in plasma apo B radioactivity following simultaneous injection of native and methylated LDL

Fig. 4.3 shows the decline of plasma apo B

Table 4.4

Kinetics of homologous LDL apo B

	Apo B (mg/dl)	PR (mg/dl/day)	FCR (pools/day)
Casein			
1	96.9	69.8	0.72
2	41.8	17.3	0.41
3	90.4	39.1	0.43
4	72.7	34.8	0.48
5	57.3	35.8	0.62
6	33.3	21.6	0.65
	65.4±10.5[a]	36.4±7.6	0.55 ±0.05 [b]
Soy Protein			
7	12.4	31.0	2.50
8	8.2	13.7	1.66
9	5.3	15.4	2.88
10	24.7	17.8	0.72
11	7.8	17.5	2.23
12	16.7	23.8	1.42
	12.5±2.9[a]	19.8±6.4	1.90 ±0.32 [b]

Mean values bearing a common superscript were significantly different between groups using Student's t-test.

P values: a; $p<0.001$: b; $p<0.01$

The P value for the production rates was between 0.05 and 0.10.

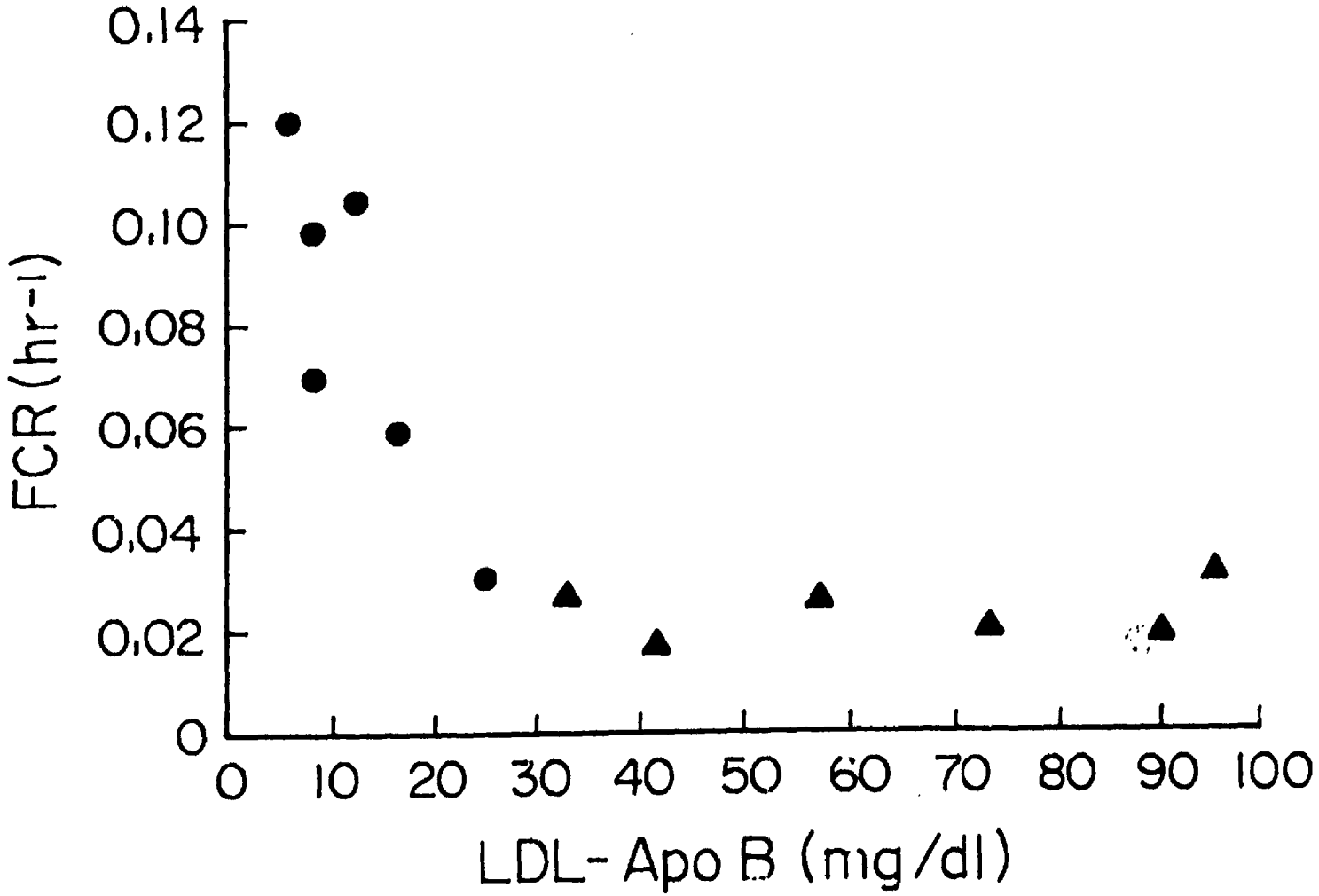

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
FCR (hr-1)
0
10
20
30
40
50
60
70
80
90
100
LDL- Apo B (mg/dl)

Fig. 4.2

Relationship between FCR and LDL apo B

The relationship between FCR and LDL apo B concentration is shown for individual rabbits.

(▲) Casein and (●) Soy Protein

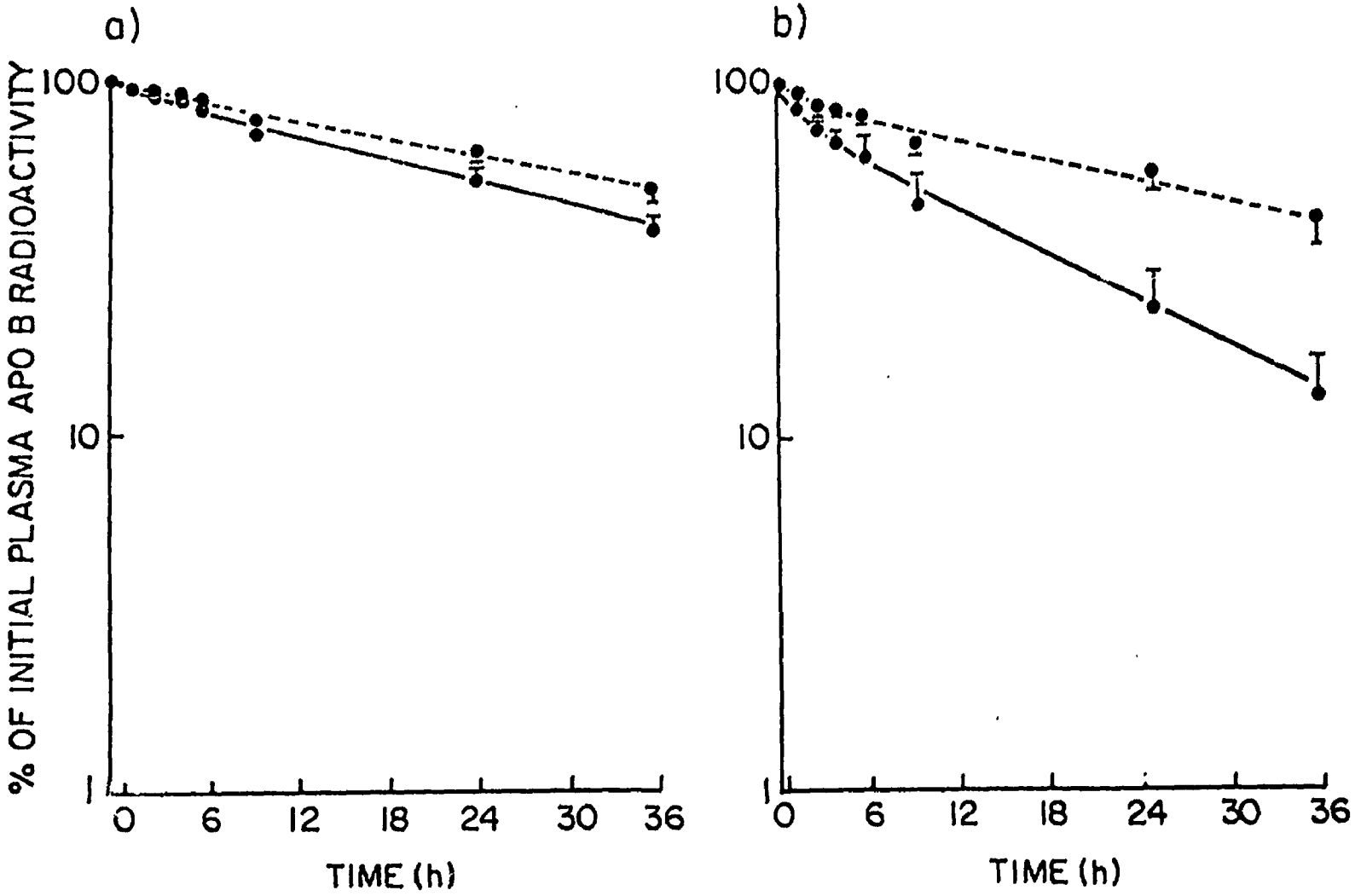

a)
b)
% OF INITIAL PLASMA APO B RADIOACTIVITY
100
10
1
0
6
12
18
24
30
36
TIME (h)

Fig. 4.3

Decline in plasma apo B radioactivity following the simultaneous injection of ^{131}I-LDL and methylated ^{125}I-LDL

The decline in plasma apo B radioactivity, as a percentage of the 3 min value, following the simultaneous injection of ^{131}I-LDL (——) and methylated ^{125}I-LDL (– – –) into rabbits fed a) casein and b) soy protein. Each point is the mean±SEM of 4 rabbits

radioactivity following the simultaneous intravenous injections of ^{131}I-LDL and ^{125}I-LDL(CH_3) into rabbits fed each of the two different diets. The 3min plasma apo B radioactivity value was taken as 100% and all subsequent values adjusted accordingly. For the soy protein-fed animals, radioactivity in the unmodified LDL (^{131}I-LDL) decayed from the plasma faster than the radioactivity from the unmodified LDL in the casein-fed animals. Additionally the methylated tracer was removed less efficiently in both dietary groups, compared to the unmodified tracer. The kinetic parameters for the two tracers, are listed in Table 4.5.

From Table 4.5 it can be seen that the decreased efficiency of removal in the casein group is principally due to a 4-fold reduction in the receptor-dependent pathway since removal via the receptor-independent pathway was essentially similar between the two groups.

4.3.5 Effects of feeding casein for different time periods on receptor-dependent catabolism of LDL

Table 4.6 shows the FCR of LDL via the receptor-dependent pathway in groups of rabbits transferred from the soy protein diet to the casein diet. It can be seen that compared to rabbits fed soy protein, casein-fed rabbits had diminished receptor-dependent catabolism even after 5 days (the

kinetics in man. They found that CCK influenced bile acid kinetics by alterations in gallbladder emptying.

With the above ideas in mind, the following was formulated as a working hypothesis.

Increased digestion of soy protein in the stomach would produce peptides, which once in the duodenum, would stimulate the release of CCK. The latter would cause the gallbladder to contract, resulting in emptying of bile acids from the gallbladder. This would now result in the liver producing more bile acids from its cholesterol pool. The cholesterol would be replaced by cholesterol derived from LDL via receptor-mediated endocytosis, which would lower plasma cholesterol concentrations. For casein the converse would be true, i e., impaired CCK release, decreased gallbladder emptying, decreased conversion of liver cholesterol to bile acids, increased liver cholesterol, decreased uptake of LDL cholesterol resulting in increased concentrations of plasma cholesterol.

The above hypothesis still allows for possible effects at the intestinal level which may result from bile acid sequesteration by the digestion products of dietary protein. Additionally, CCK provides a link between protein and cholesterol metabolism, and suggests a method by which dietary proteins can exert their effects on the LDL receptor. One of the fudamental

Table 4.6

The effect of time on diet on receptor-mediated catabolism of LDL apoB

Time on diet (days)	Casein	Soy Protein
5 days	0.45	
	0.70	
	0.48	
	0.61	
Means±SEM	0.56±0.06	
10 days	0.40	1.08
	0.29	0.80
	0.33	0.58
Means±SEM	0.34±0.03	0.82±0.15
25 days	0.63	1.15
	0.69	1.59
	0.41	0.98
Means±SEM	0.58±0.09	1.24±0.18

Rabbits were fed soy protein for 2 weeks before being transferred to the casein diet. The time on diet is therefore a measure of the time that casein was fed. Values are listed for individual rabbits.

earliest time period after which receptor-mediated catabolism was assessed). Receptor-independent catabolism was similar in all of the rabbits regardless of diet or time on diet.

Since it was shown in Table 4.1 that rabbits fed soy protein had comparable plasma cholesterol concentrations regardless of the time period for which the diet was fed the values for the plasma cholesterol concentrations and receptor-dependent FCR have been combined for the soy-protein fed rabbits used in Studies 2 and 3 to represent "control" data. This data together with the plasma cholesterol concentrations and receptor-dependent FCR for the casein-fed rabbits in Studies 2 and 3 are represented pictorially in Fig. 4.4.

This figure shows that the plasma cholesterol concentrations of casein-fed rabbits were inversely correlated with the FCR for receptor-dependent catabolism. The latter decreased, as plasma cholesterol concentration increased. From Fig 4.4 it can also be seen that even though the plasma cholesterol concentrations in rabbits fed casein for 5 days was not significantly different from "control" rabbits fed soy protein, the FCR for receptor-dependent catabolism in these casein-fed rabbits was decreased by almost 40% compared to the control rabbits.

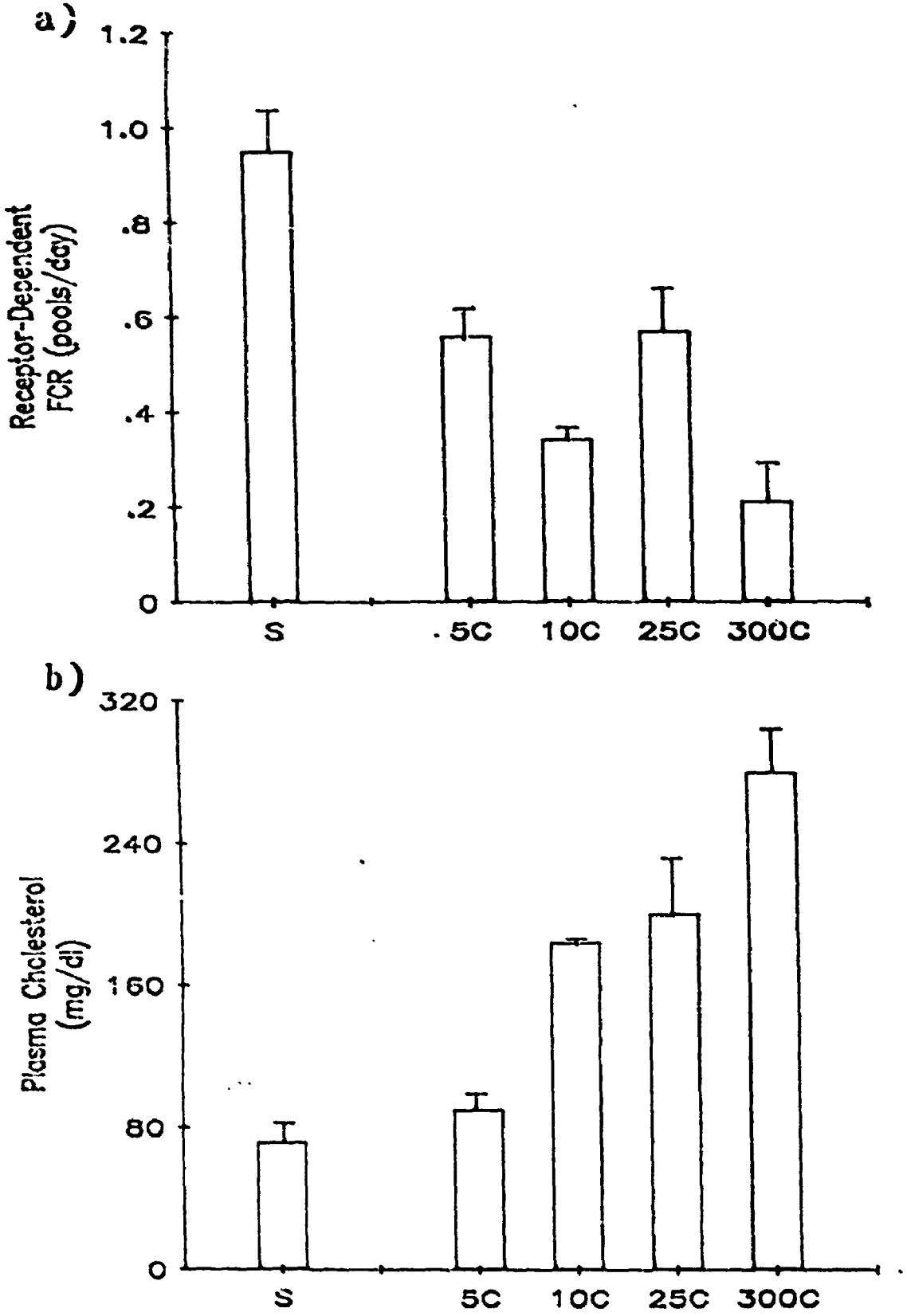

a)
Receptor-Dependent FCR (pools/day)
1.2
1.0
.8
.6
.4
.2
0
S
.5C
10C
25C
300C
b)
Plasma Cholesterol (mg/dl)
320
240
160
80
0
S
5C
10C
25C
300C

Fig. 4.4

Receptor-dependent LDL apo B FCR and plasma cholesterol

a) Receptor-dependent LDL apo B FCR and b) plasma cholesterol concentrations in rabbits fed soy protein (S) or casein (C) diets. The number of days the rabbits were fed casein is indicated.

Values shown are mean±SEM for 10 rabbits fed soy protein; and 4, 3, 3 and 4 rabbits fed casein for 5, 10, 25 and 300 days respectively.

FCR in casein-fed rabbits was significantly different from that observed in the soy protein-fed rabbits.

Plasma cholesterol values in the casein groups were significantly different from the value for the soy protein group, with the exception of the 5 day value.

The soy protein group includes the 4 rabbits used in Study 2.

4.4 DISCUSSION

In Study 1 the use of dual radiolabeled LDL provided a tool for assessing LDL metabolism, separately from any compositional effects. These studies confirmed the results presented in Chapter 3, that casein-feeding is associated with decreased LDL FCR. Additionally the results showed that the source of injected LDL (whether it was isolated from a normocholesterolemic soy protein-fed rabbit or a hypercholesterolemic casein-fed rabbit) was not a factor in determining its subsequent removal. Despite the increased mass of the LDL particles in casein-fed rabbits, the composition of the LDL from the two dietary groups was similar (Table 4.2). Hence the similar FCR observed for the two tracers, in a given dietary group, shows that the removal of the injected LDL is dependent solely on the physiological status of the recipient rabbits. The decreased FCR in casein-fed rabbits is consistent with impaired LDL receptor activity.

Since the FCR per se is not a measure of LDL recptor activity, the second study was carried out. In this study, the use of dual radiolabeled native LDL and methylated LDL, allowed for the discrimination between receptor-dependent and receptor-independent LDL catabolism. The results of this study showed that the impaired removal of LDL in casein-fed rabbits, was the result of diminished receptor-mediated catabolism, since receptor-independent catabolism was similar between the two dietary groups.

This result is in support of the observation of Chao et al. (1981) that the in vitro binding of LDL to liver membranes of casein-fed rabbits was decreased in comparison to the binding to membranes isolated from rabbits fed a chow diet (Kita et al., 1981). Kita et al., (1981) also observed that liver membranes of hypercholesterolemic WHHL rabbits had decreased binding compared to chow-fed rabbits. Stoudemire et al., (1984) showed hypercholesterolemic fasting rabbits had impaired receptor-dependent LDL catabolism, in vivo, and that this was related to decreased LDL binding to liver membranes, in vitro, in comparison to normocholesterolemic rabbits. The decreased binding of LDL in vitro observed by Chao et al (1983), Kita et al (1981) and Stoudemire et al (1984) was the result of the abolition of the EDTA-sensitive binding site.

A down-regulation of hepatic LDL receptors was also observed by Sirtori et al., (1984), and Cohn and Nestel (1985), using liver membranes from rats fed casein or soy protein, but in these studies both diets contained cholesterol. Recently, in type II hyperlipoproteinemic patients, it was shown that a soybean protein diet increased the activity of LDL receptors in mononuclear cells compared to an animal protein diet (Lovati et al, 1987). Although the in vitro activity of LDL receptors was not assessed in these studies, the results of defective clearance of LDL in casein-fed rabbits is consistent with

Table 4.7

Comparison of LDL apoB FCRs obtained in various studies

Time on diet	Casein	Soy Protein
a) 5 days	1.03±0.09	ND
b) 10 days	0.60±0.12	1.63±0.05
c) 25 days	1.19±0.09	2.04±0.21
d) 6-9 weeks	0.81±0.13	1.93±0.20
e) 14-16 weeks	0.55±0.05	1.90±0.32
f) 6-7 months	0.64±0.11	1.42±0.20

Table shows total LDL apo B FCR obtained in the various studies discussed

a, b & c) Study 3, Chapter 4.
d) Study in Chapter 3;
e) Study 1, Chapter 4;
f) Study 2, Chapter 4;

All of the values are determined for individual rabbits except d) in which pooled plasma was used for LDL isolation. All casein values were significantly different from their respective soy protein values.

decreased LDL receptors. Furthermore the results of Study 2 show that it is the protein component of the diet which is responsible for the observed effects, since this was the sole variable in the two diets. (In the studies of Chao et al [1981] only a casein diet was assessed).

Having established that casein-feeding resulted in defective receptor-mediated LDL catabolism, Study 3 was carried out to ascertain how quickly this effect ocurred when rabbits were transferred from soy protein to casein. This study showed that receptor-mediated LDL catabolism was reduced by 40% in casein-fed rabbits, before an increase in plasma cholesterol was apparent. This suggests that the increase in plasma cholesterol in casein-fed rabbits is secondary to effects on LDL receptors. From these studies it is not possible to ascertain whether the effect of casein on LDL receptors is direct, or whether it operates via an intermediate factor(s).

A general point from the studies in this and the previous chapter, is the clear-cut differential in LDL FCR between casein and soy protein fed rabbits which was measured in rabbits fed the different diets for 10 and 25 days, 6-9 weeks, 14-16 weeks and 6-7 months, following the injection of radiolabeled LDL. (These FCR values are summarized in Table 4.7) For all of the studies in this chapter the results were analysed by the two-pool model of Matthews (1951) for which only plasma apo B radioactivity

was needed. The analysis in Chapter 3 utilised the two-pool model of Gurpide et al. (1963), for which a knowledge of LDL specific activity data was required. Table 4.7 shows that the FCR decreased somewhat in both dietary groups with increasing time on diet. This may be due to the age of the rabbits, since down-regulation of LDL receptors is known to occur with age.

In summary, the results of the studies showed that

1) Decreased FCR of LDL in casein-fed rabbits was not due to differences in the LDL particles

2) Decreased removal of LDL was due to impaired receptor-dependent catabolism

3) After 5 days of feeding casein, there was a 40% reduction in receptor-dependent catabolism despite any significant increase in plasma cholesterol concentrations.

CHAPTER 5

THE DIGESTIBILITY OF DIETARY CASEIN AND SOY PROTEIN AND THEIR EFFECTS ON PLASMA CCK.

5.1.1 INTRODUCTION

The fundamental question raised by the results described in earlier chapters is, what is the cause of impaired receptor-dependent catabolism of LDL in casein-fed rabbits? This chapter describes the results of studies (some preliminary) designed to address this question.

The primary event when rabbits are fed casein or soy protein, will likely occur in the digestive tract (this excludes any hormonal or neural effects that the sight, smell or the taste of the diets will induce). The first site of protein digestion is the stomach, where the presence of HCl and the protease pepsin, will result in the digestion of the protein to smaller peptides. In vitro studies by Woodward and Carroll (1985) have shown that soy protein is more readily digested by pepsin owing to its greater solubility at acidic pH in comparison to casein. The digestibility of casein and soy protein, in vivo, have not been assessed in the rabbit. These studies have however been carried out in rats, in which it was found that soy protein was more readily digested in the stomach than casein (Yashiro et al., 1985).

The studies of Woodward and Carroll

(1985) also showed that in vitro, casein is more readily digested by trypsin owing to its greater solubility in an alkaline environment, in comparison to soy protein. The preferrential digestion of casein in the proximal intestine of the rat (where an alkaline environment exists) has also been demonstrated by Yashiro et al., (1985).

As a consequence of the differing solubilities of the two proteins at acidic and alkaline pH, the net result will be that, the two proteins, will in all probability, release their constituent amino acids at different rates. These released amino acids will then be absorbed at differing rates, and may subsequently affect cholesterol metabolism.

Alternatively, the digestion products of the proteins may interfere with the absorption of endogenous bile acids, preventing the latter's reabsorption. This "digestibility hypothesis", discussed in Chapter 1, states that the digestion products of soy protein, in the distal intestinal lumen, sequester bile acids to a greater extent than those of casein. As a consequence, there is increased fecal excretion of bile acids (Huff and Carroll, 1980b), and less bile acids are returned to the liver. This decrease in liver bile acids necessitates a greater conversion of liver cholesterol to bile acids, which would deplete liver cholesterol pools.

This would be compensated for by an increased uptake of cholesterol from LDL via receptor-mediated endocytosis, which will lower plasma cholesterol levels. Although studies in vitro have shown that casein and soy protein are both able to bind bile acids (Sklan, 1980; Woodward and West, 1984), there is no evidence from in vivo work in rabbits to support or refute the digestibility hypothesis. Studies by Kuyvenhoven et al., (1987) carried out in rats are not in support of this hypothesis, since these workers found no evidence for bile acid sequesteration in the rats fed the soy protein containing diet.

The experiments described in this chapter were designed to address this above hypothesis. Experiments were carried out to look at the role played by the stomach in the digestion of casein and soy protein and whether indeed the digestion products of soy protein were able to sequester bile acids in the distal intestinal lumen.

During the course of these experiments, some indirect results began to suggest the possible involvement of a gut hormone, and how this may be able to explain the differences in plasma cholesterol seen with casein and soy protein diets. Furthermore,the physiological action of this hormone provided a direct link between protein and cholesterol metabolism. The hormone suspected of having this potential role was Cholecystokinin (CCK). Therefore some preliminary

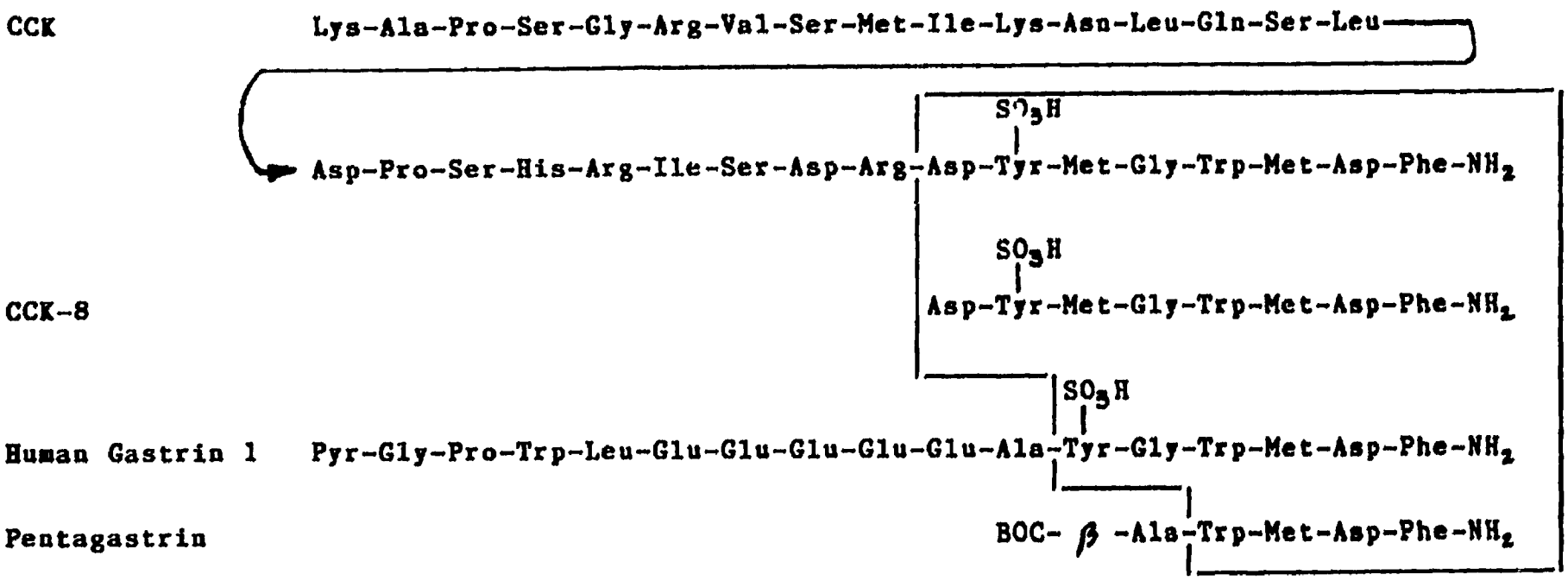


Fig. 5.1 Amino acid sequence for secretagogues of enzyme secretion.

experiments were carried out to see if indeed CCK might be the key to the mystery that has intrigued nutritionists since the early 1970's about the ability of dietary protein to influence plasma cholesterol levels.

Therefore before describing the experiments, there follows an introduction to CCK.

5.1.2 CCK

CCK is believed to be a major hormonal regulator of gallbladder contraction and pancreatic enzyme secretion (Ivy and Oldberg, 1928; Harper and Raper, 1943). The hormone is located in the I cells of the small intestine and is released after the ingestion of food (Mutt, 1980). Since its original isolation (as a 33 amino acid polypeptide, CCK-33, sharing an identical carboxy terminal pentapeptide with gastrin), several other molecular forms have been identified in the tissues and plasma of various species (Mutt, 1976; Dockray, 1976; Miller et al., 1984). The biological activity of this hormone is contained in the carboxy terminal octapeptide (CCK-8) portion of this molecule (Fig. 5.1) This smaller molecule is also present in the mucosa of the small intestine along with a larger variant, CCK-39 (Dockray, 1977; Rehfeld, 1978, Mutt, 1980).

In addition to its effects outlined above, CCK is now believed to be the physiological regulator of gastric emptying (Liddle et al., 1986), the

mediator of the feedback regulation of pancreatic enzyme secretion (Owyang et al., 1986) as well as a controller of satiety (Verbalis et al., 1986). In addition to its presence in the intestine, CCK also occurs in brain neurons (Saito et al., 1980). These multiple effects of CCK serve to coordinate the delivery of food into the intestine with the appearance of bile and pancreatic enzymes into that organ.

The effect of CCK on gastric emptying seems to constitute a feedback mechanism whereby CCK regulates its own release. When food leaves the stomach and enters the duodenum, CCK is released. The principal effectors of CCK release are dietary fats and proteins. The released CCK in turn inhibits gastric emptying. As a consequence, less food is delivered to the duodenum, thereby decreasing CCK secretion (Liddle et al., 1986).

Gallbladder emptying and small intestinal motility are both stimulated by CCK (Parker and Beneventane, 1970; Bertaccini and Agosti, 1971). Since artificially induced alterations in small intestinal transit time influence bile acid pool size by altering the synthesis rate of the primary bile acids (Duane, 1978), and both gallbladder emptying and small intestinal transit time are both correlated with bile acid pool size (Duane and Hanson, 1978), Jazrawi and Northfeld (1986) looked at the effects of a pharmacological dose of CCK on bile acid

kinetics in man. They found that CCK influenced bile acid kinetics by alterations in gallbladder emptying.

With the above ideas in mind, the following was formulated as a working hypothesis.

Increased digestion of soy protein in the stomach would produce peptides, which once in the duodenum, would stimulate the release of CCK. The latter would cause the gallbladder to contract, resulting in emptying of bile acids from the gallbladder. This would now result in the liver producing more bile acids from its cholesterol pool. The cholesterol would be replaced by cholesterol derived from LDL via receptor-mediated endocytosis, which would lower plasma cholesterol concentrations. For casein the converse would be true, i e., impaired CCK release, decreased gallbladder emptying, decreased conversion of liver cholesterol to bile acids, increased liver cholesterol, decreased uptake of LDL cholesterol resulting in increased concentrations of plasma cholesterol.

The above hypothesis still allows for possible effects at the intestinal level which may result from bile acid sequesteration by the digestion products of dietary protein. Additionally, CCK provides a link between protein and cholesterol metabolism, and suggests a method by which dietary proteins can exert their effects on the LDL receptor. One of the fudamental

tenets of this CCK hypothesis is that in rabbits fed soy protein, postprandial release of CCK should be higher than that seen in rabbits fed casein diets.

5.2 METHODS

5.2.1 Animals and Diets.

Rabbits were fed casein or soy protein diets following a one week adaptation period as outlined in Chapter 2. The diets were fed for the period of time indicated in the appropriate section.

5.2.2 SDS-PAGE of casein and soy protein.

500mg of casein or soy protein were suspended in 6ml of 0.1M potassium dihdrogen phosphate-potassium hydrogen phosphate buffer, pH 7.8. Solubility was arbitrarily defined as the protein concentration of the supernatant fraction following centrifugation at 10,000g for 30 min (Woodward and Carroll, 1985). 50-100µg aliquots were subjected to SDS-PAGE using 12.6% running gels (Laemmli and Favre, 1970). Following staining in Coomassie R-250, and destaining in acetic acid/methanol, the gels were scanned at 540nm using a LKB 2202 ultrascan laser densitometer equipped with a Hewlet Packard 2220 integrator. The percentage of protein in the stained bands was calculated by multiplying their percent distribution in the scanned gels by the total amount of protein loaded.

5.2.3 Enzymatic digestion of casein and soy protein in vitro

This was essentially done as described by Woodward and Carroll (1985) except that the accumulation of TCA-soluble peptides was used as the criteria for digestion. Casein and soy protein were solubilised as detailed in section 5.2.2. Aliquots were then incubated in 0.1M phosphate buffer with either trypsin, peptidase or pepsin. (The enzymes, obtained from ICN Pharmaceuticals Inc., Cleveland, OH., were isolates from pig pancreas, intestine and stomach mucosa respectively. The peptidase was a partially purified mixture of amino and carboxy peptidases). The pH of the incubation medium was 1.6 for the digestion using pepsin, whereas it was 7.8 for digestions with trypsin and peptidase.

Aliquots from the enzyme-protein mixture were then taken at timed intervals and added to ice-cold TCA (5% final concentration). Following centrifugation at 1500g for 15min the protein content of the supernatant, as well as the protein content of the precipitate was measured, following solubilisation of the precipitate in 2ml of the phosphate buffer. Results are expressed as the percent accumulation of TCA-soluble peptides.

5.2.4 Analysis of the stomach and intestinal contents of rabbits fed casein or soy protein.

5.2.4.1 Isolation of material

Rabbits were fed casein or soy

protein diets for 6-8 weeks. Blood was obtained from non-fasting anesthetised rabbits by cardiac puncture into EDTA-coated vacutainers and kept on ice. (In some studies rabbits were fasted for 24h. They were then allowed access to their diets, to which PEG 4000 had been added,(1g/100g diet) for 1 hour. Food was withdrawn and the rabbits sacrificed 6h later).

The abdominal cavity was now opened. The entire small intestine was now exposed (upto the ileo-cecal junction). Using silk threads, ligatures were placed around a) the point of entry of the oesophagus into the stomach, b) the proximal point of the duodenum, close to the stomach, c) the ileo-cecal junction and d) midway through the small intestine. The small intestine and stomach were now removed from the rabbit (In some studies the liver was also removed, blotted dry and weighed). The first half of the intestine was designated "proximal intestine" and the latter half as the "distal intestine".

The stomach, proximal and distal intestines were now emptied of their contents by flushing with fixed volumes of saline. The contents were homogenized at neytral pH and aliquots kept. The homogenized contents were centrifuged at 10,000g for 30 min. The supernatant was designated as the "soluble fraction" and the precipitate as the "insoluble fraction".

Aliquots of the soluble and

insoluble fraction were also kept and the remainder of the fractions were freeze-dried.

5.2.4.2 Gel-fractionation of stomach contents.

The soluble fraction from the stomach was subjected to gel filtration using a Sephadex-G25 column (90cm x 2 cm) equilibrated with 20mM Tris, 1mM EDTA and 0.02% sodium azide, pH 5.6 (Yashiro et al., 1985). Samples were eluted with the same buffer at 4°C and 2ml fractions were collected. The O.D. of the fractions were monitored at 280nm. The void volume of the column was determined using blue dextran.

5.2.4.3 Extraction of bile acids from freeze-dried intestinal contents.

Bile acids were extracted from freeze-dried samples of intestinal contents, according to the procedure of De Wael et al., (1977). (In one study, bile acids were also extracted from the feces of soy protein-fed rabbits collected over a 4 day period). 100mg of freeze dried intestinal contents (or feces) were mixed with 1ml KOH/ethylene glycol solution (3g KOH in 10ml ethylene glycol) and heated for 15min at 220°C. The solution was then cooled and 1ml of 3.4M NaCl added. The mixture was acidified with conc. HCl and diethylether added. The bile acids were extracted into diethylether by centrifugation. Following the evaporation of the ether at 40°C, the bile acid residue was dissolved in 1ml methanol. The recovery of ^{14}C-labeled deoxycholic acid

(added to the freezed dried aliquots) was 96%(±SD3%) (n=22).

5.2.4.4 Enzymatic determination of bile acids.

Bile acids were measured in the methanolic solutions using the enzyme 3α-hydroxysteroid dehydrogenase (Sheltawy and Losowsky, 1975) using deoxycholic acid as the standard. For this purpose the enzyme was dissolved in a solution of 0.1M hydrazine hydrate, 0.4mM NAD, 50mM sodium pyrophosphate, pH 9.5, to give a final enzyme activity of 0.07 IU/ml where 1 IU is the amount of enzyme that will convert 1umol of substrate per min at 25°C and pH 9.5.

The enzyme solution was mixed with the methanolic intestinal (or fecal) aliquot, and bile acids were quantitated by measuring the NADH produced following a 15 min incubation at 340nm.

This method is based on the ability of the enzyme to transfer the 3α-hydroxy group on steroids of the C_{19}, C_{21}, and C_{24} series, and the taurine and glycine conjugates of the C_{24} series, to their corresponding keto groups with the concomittant production of NADH from NAD^+. The reaction is reversible, but at alkaline pH and with the presence of the ketone trapping agent, hydrazine hydrate, the conversion of the 3α-hydroxysteroids to the 3-ketosteroids is quantitative. Hence the amount of NADH formed is directly proportional to the bile acids present in the

sample.

5.2.4.5 Measurement of protein and nitrogen.

Protein in the soluble fractions was measured by the method of Lowry et al., (1951). Nitrogen in the freeze-dried aliquots was measured using the micro Kjedahl method. In some cases aliquots of the soluble fraction were subjected to SDS-PAGE as detailed in section 5.2.2. To ascertain any qualitative differences in gel profiles as a result of feding the two diets, the percent distribution of proteins of a given molecular weight was determined following scanning of the gels (section 5.2.2).

5.2.4.6 Assay of Polyethylene glycol.

In studies where rabbits were fed PEG as part of their diets, the PEG in the soluble fraction isolated from the stomach and intestinal contents was measured according to the procedure of Boulter and McMichael (1970).

5.2.5 Determination of postprandial plasma CCK levels in rabbits fed casein or soy protein diets.

Rabbits were fasted for 24h. Blood was collected from the marginal ear vein of unanesthetized rabbits (for determination of fasting CCK levels).

They were then allowed access to their diets for 5 min. Food was withdrawn (all rabbits consumed 8-10g of their respective diet) and the rabbits were bled at various times thereafter. Plasma was isolated

and used for the measurement of CCK. For this purpose CCK was first isolated from plasma and then assayed. The protocol is essentially that of Liddle et al., (1984 & 1985) which was developed for quantitation of CCK in rat and human plasma, with the modifications of Owyang et al., (1986) and Louie et al., (1986).

5.2.5.1 Extraction of CCK from plasma.

Upto 6ml of plasma was adsorbed onto C_{18}-Sep Pak cartridges, previously washed sequentially with 10ml acetonitrile, 10ml methanol, 20ml deionised water. Cartridges were now placed in scintillation vials and kept at -20°C for a maximum of 48h. These cartridges, stored on wet ice, were now dispatched to the laboratory of Dr. Dexter Louie at the Gastrointestinal Peptide Research Center, University of Michigan.

The CCK was eluted from the cartridges with 1ml acetonitrile/water (1:1 v/v) into polyethylene scintillation vials and dried under nitrogen at 45°C. In order to assess the recovery of CCK from the cartridges, known quantities of CCK-8 (10 and 100fmol) dissolved in Tris-buffered Ringer solution (TR) were also eluted from Sep-Pak cartridges as detailed above for the plasma samples.

5.2.5.2 Quantitation of CCK.

In some of the early experiments CCK was quantitated by the bioassay described by Case and Clausen (1973). For this, baby rat uncinate pancreases

were incubated in TR solution and amylase release was stimulated by the addition of known amounts of CCK-8. The released amylase was quantitated by its ability to convert starch to maltose (Jung, 1980). This assay proved insensitive in its ability to detect CCK extracted from rabbit plasma samples and was accordingly not used further.

In all subsequent assays, CCK was quantitated by means of the bioassay which utilises rat pancreatic acinar cells, prepared from fasting male Sprague Dawley rats by collagenase digestion of the pancreases (Williams et al., 1979; Otsuki et al., 1982).

1ml aliquots of acini suspension were added to the vials containing the plasma extracts after they had been dried under nitrogen, or to vials containing known amounts of CCK-8. Following a 30min incubation at 37^{o}C, the amylase released into the medium and the total acinar amylase content was now measured using porcion yellow starch as substrate (Jung, 1980). The amylase released into the medium, expressed as the total acinar amylase content, by the known amounts of CCK-8 was used to generate a standard curve. Using this, and the percent amylase released by the plasma extracts, allowed for the quantitation of plasma CCK levels.

The time frame (from the initial collecting of blood from the rabbits upto quantitation of

CCK) was never more than 2 weeks.

5.2.6 Effects of injecting pharmacologicol doses of CCK-8 on plasma cholesterol levels in rabbits fed casein diets.

Rabbits were fed casein diets for the time period indicated. Following an overnight fast, their plasma cholesterol was determined. They were then divided into 2 groups of 4 rabbits each. Rabbits were allowed access to their diets from 9am to 5pm. Over a 4 day period, one group of 4 rabbits was injected i.p. with CCK-8 at 9am, 1pm and 5pm daily. Over the 4 day period, each rabbit was injected with 116µg of CCK-8. This corresponds to a dose of 3.9µg/kg of CCK-8 per injection. The other group of rabbits received i.p. injections of saline at the same times. On the morning of the 5th day, blood was withdrawn from all the rabbits for determination of plasma cholesterol levels.

5.3 RESULTS

5.3.1 SDS-PAGE of casein and soy protein.

Fig. 5.2 shows the gel profile of casein and soy protein. As can be seen casein was separated essentially into main components whose molecular weights were 37k and 29k. The former accounted for 66% and latter comprised 34% of the protein loaded.

Though soy protein was more heterogeneous in comparison to casein, it was essentially resolved into 4 components. The major one, molecular

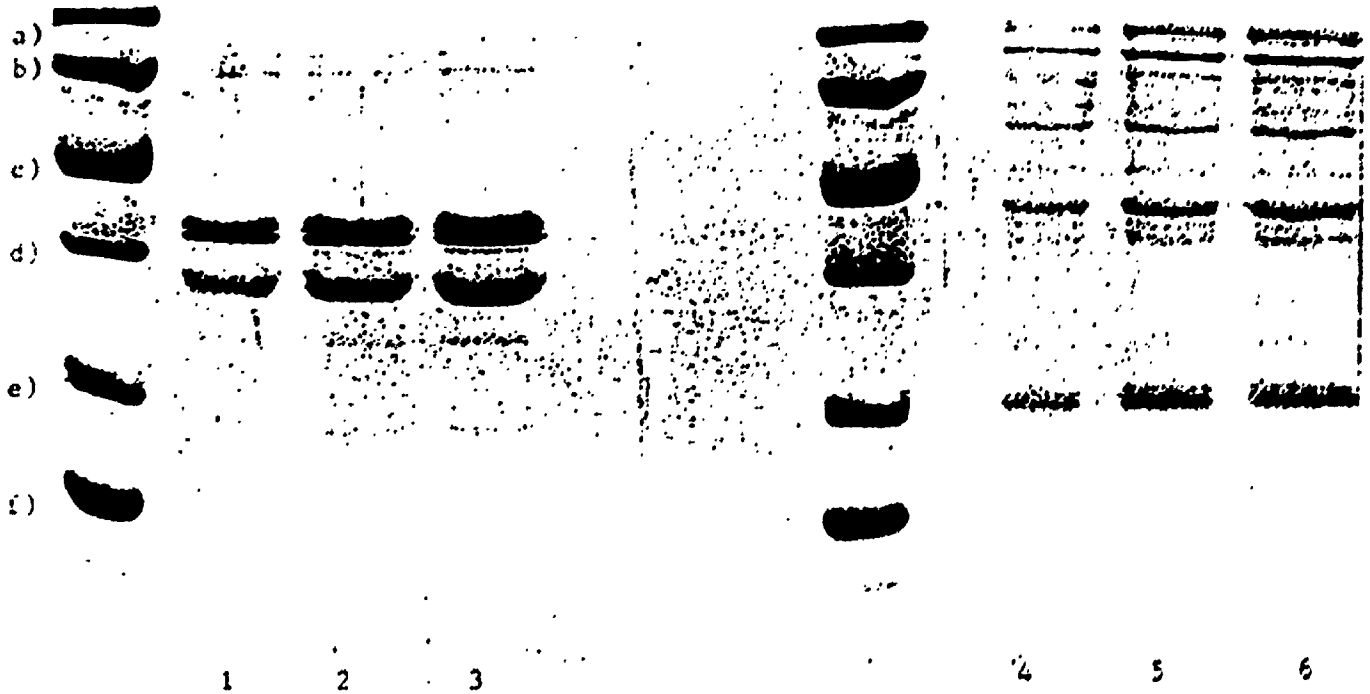

a)
b)
c)
d)
e)
f)
1
2
3
4
5
6

Fig. 5.2
SDS-PAGE of casein and soy protein

Casein and soy protein were solubilised and subjected to SDS-PAGE using 12.6% running gels as indicated in the methods.

Lanes 1-3 casein; lanes 4-6 soy protein

The low molecular weights standards are
a) Phosphorylase b (94k)
b) Bovine serum albumin (67k)
c) Ovalbumin (43k)
d) Carbonic anhydrase (30k)
e) Soybean trypsin inhibitor (20.1k)
f) α-lactalbumin (14.4k)

weight 39k, accounted for 58% of the protein. Two components, molecular weight 19k and >70k accounted for 20% and 19% of the protein respectively. In addition, a minor component of molecular weight 23k, and accounting for 3% of the protein was also resolved.

5.3.2 Enzymatic digestion of casein and soy protein in vitro.

Fig. 5.3 shows the time-course of the digestion of casein and soy protein, in vitro by a) pepsin, b) trypsin and c) peptidase, measured as the accumulation of TCA-soluble peptides (molecular weight <10k). Soy protein was essentially completely digested within the first 15min by pepsin, whereas digestion of casein proceeded more slowly, rising to a value of 80% after 90min incubation.

With trypsin, digestion of casein, was essentially complete within the first 30min, whereas at the same time, digestion of soy protein was only 45%. The digestion of soy protein, increased with prolonged incubation, and after 60 min was similar to that seen with casein.

With peptidase both proteins were digested more slowly in comparison with the other enzymes. The percentage of TCA-soluble peptides present after 90min was 3-7 times lower than that observed with pepsin or trypsin. Despite the lower digestibility, the extent of digestion of soy protein by peptidase was essentially

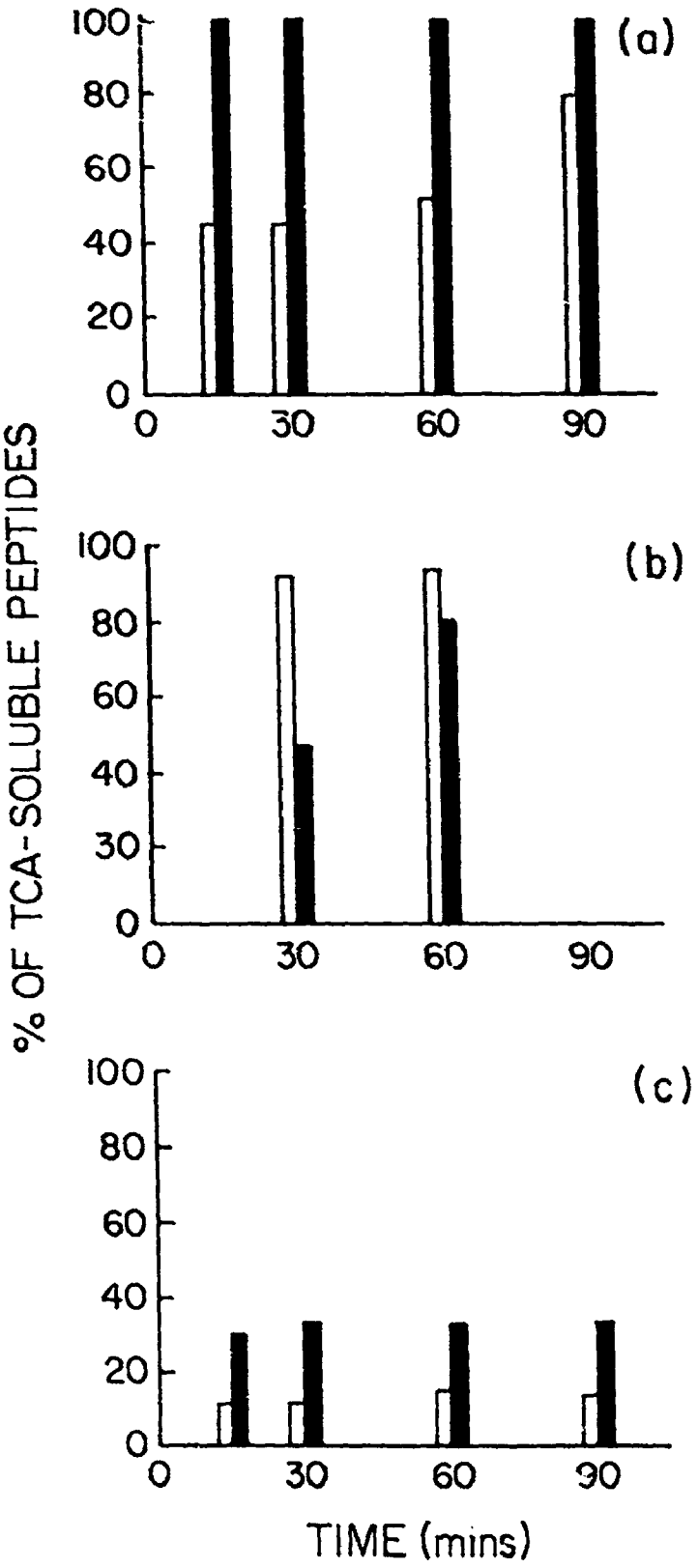

(a)
(b)
(c)
100
80
60
40
20
0
30
90
% OF TCA-SOLUBLE PEPTIDES
TIME (mins)

Fig. 5.3

Enzymatic digestion of casein (□) and soy protein (■) in vitro

Each value is the mean of 3 determinations

a) Pepsin, b) Trypsin and c) Peptidase

Table 5.1 Body weights, plasma cholesterol and liver weights of rabbits fed casein or soy protein diets

	BW	PC	LW
Casein	2.9±0.3	295±38	3.7±0.3
Soy protein	2.8±0.3	79±12	3.0±0.8
		$p<0.05$	

The values given are the means±SEM of 10 rabbits.

BW - Body weight (kg); PC - Plasma cholesterol (mg/dl)
LW - Liver weight as % body weight

3-fold higher than that observed with casein.

5.3.3 Stomach and intestinal contents of rabbits fed casein or soy protein.

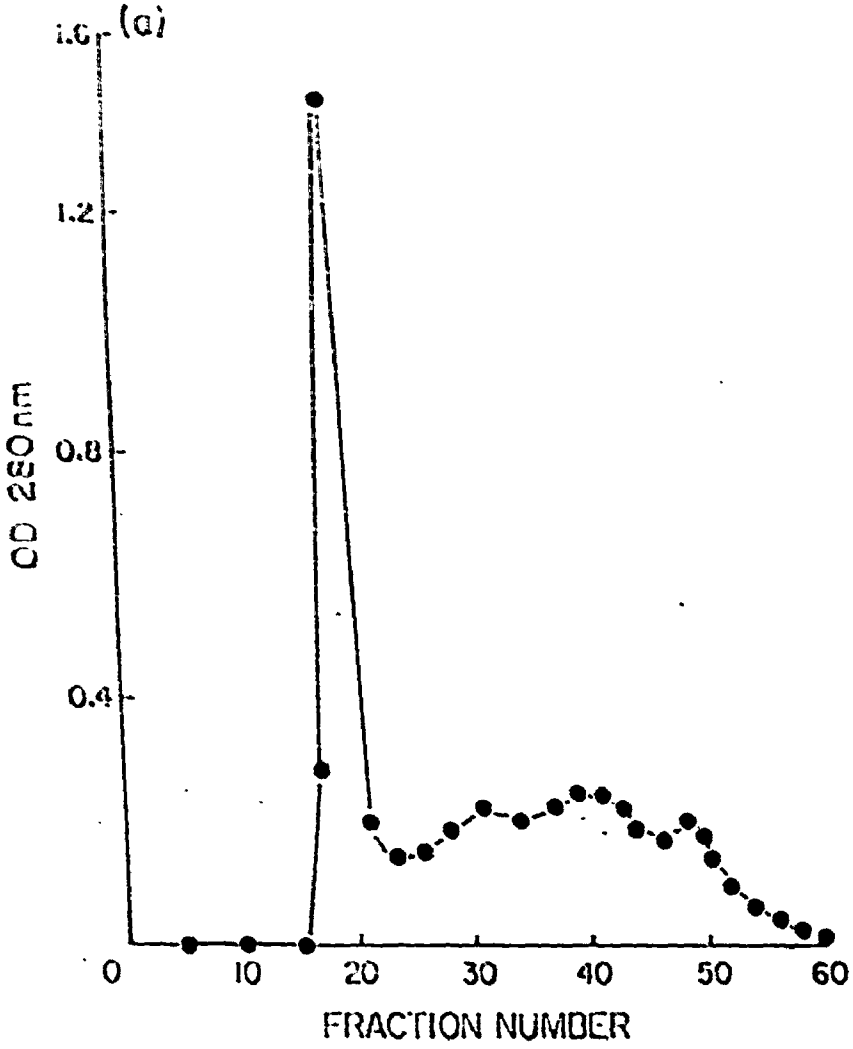
5.3.3.1 Body weights, liver weights and plasma cholesterol levels.

Table 5.1 shows that the body weights, weight gains and liver weights were comparable for the rabbits in the two dietary groups. Although the liver weights of the casein-fed rabbits were somewhat higher than those of rabbits fed soy protein, the difference was not statistically significant. As in all studies reported, the csein-fed rabbits were significantly hypercholesterolemic in comparison to the rabbits fed soy protein.

5.3.3.2 Gel fractionation of the stomach contents.

Fig. 5.4 shows the gel fractionation profile of the soluble fraction from the stomachs of rabbits fed a) casein and b) soy protein eluted on a Sephadex G-25 column. A representative profile from each rabbit is shown. (In all, the profiles from the stomach contents of 4 rabbits fed casein and 7 rabbits fed soy protein containing diets were examined). As seen, the material from the stomachs of the casein-fed rabbits eluted essentially with the void volume. This was seen in all the casein rabbits.

For the 7 rabbits fed soy protein, in addition to material eluting with the void volume, one


(a)
1.6
1.2
0.8
0.4
OD 280 nm
0
10
20
30
40
50
60
FRACTION NUMBER

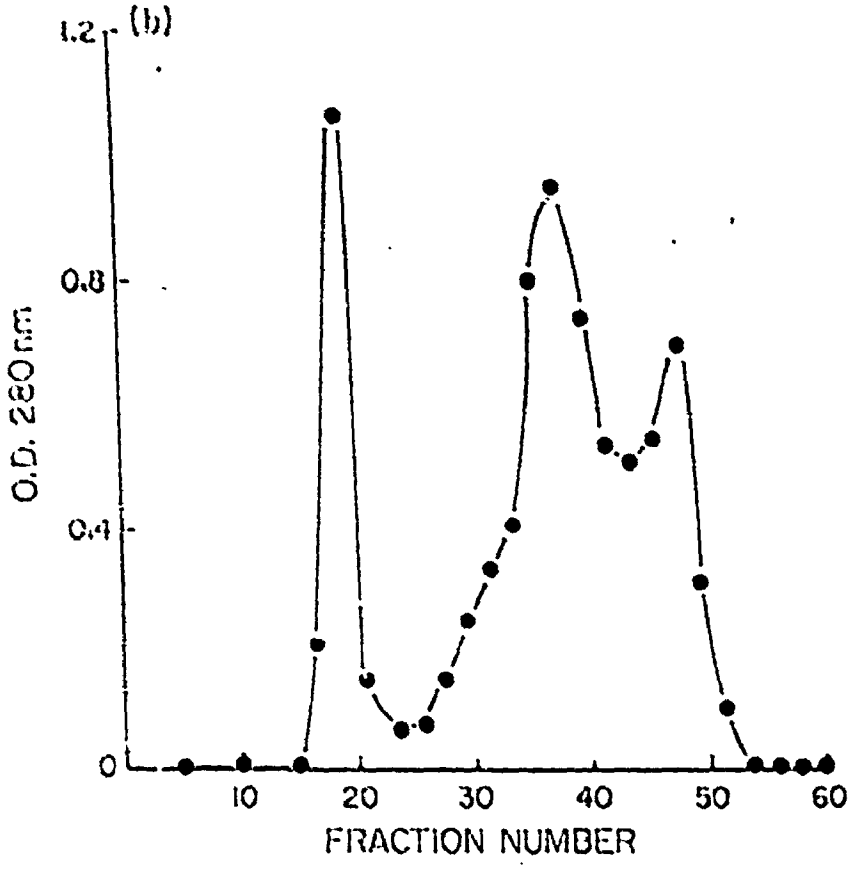

(b)
1.2
0.8
0.4
0
O.D. 280 nm
10
20
30
40
50
60
FRACTION NUMBER

Fig. 5.4

Elution profile on Sephadex G-25 of the soluble fraction from rabbits fed a) casein and b) soy protein

The profile from a representative rabbit in each group is shown.

Table 5.2 Intestinal contents of rabbits fed casein or soy protein diets

	Proximal		Distal	
	Casein	Soy Protein	Casein	Soy Protein
Freeze Dried Weights (g)				
Total	2.7(±0.3)	3.1(±0.25)	1.9(±0.3)	1.4(±0.12)
Soluble	1.8(±0.2)	2.1(±0.2)	0.9(±0.1)	0.7(±0.09)
Insoluble	0.9(±0.06)	1.0(±0.06)	1.0(±0.15)	0.7(±0.07)
Nitrogen (mg)				
Total	176(±11)	257(±19)	112(±7)	101(±9)
Soluble	129(±9)	197(±19)	84(±7)	73(±5)
Insoluble	47(±5)	60(±5)	28(±3)	28(±2)
Bile Acids (mg)				
Total	46(±4)	44(±4)	19(±1)	11(±1)
Soluble	17(±1.5)	17(±1.8)	2(±0.3)	1(±0.1)
Insoluble	28(±2)	27(±2)	17(±1.7)	10(±1.3)

Each value is the mean±SD of 8 rabbits.

The total and soluble nitrogen was significantly lower in the proximal intestinal contents of casein-fed rabbits, compared to the values for soy protein-fed rabbits ($p<0.05$).

or two additional low-molecular weight peaks were also resolved. The intact proteins elute in the void volume (Woodward and Carroll, 1985).

5.3.3.3 Dry weights, nitrogen and bile acid content.

Table 5.2 shows the freeze-dried weights, nitrogen and bile acid in the intestinal contents of the rabbits fed casein and soy protein diets. In the proximal intestinal contents, the dry mass appeared to be higher in the soy protein-fed rabbits, but this was not statistically significant. Similarly the apparently higher dry mass of the distal intestinal contents of the casein-fed rabbits was also not statistically significant. For both dietary groups the total dry mass decreased in going from the proximal to the distal intestine.

Despite the similarities in the dry mass, the nitrogen content in the proximal intestinal contents was significantly higher for the rabbits fed soy protein. This was attributable to a significantly higher nitrogen content in the soluble fraction. The soluble fraction accounted for 75% of the nitrogen in the proximal intestine for both dietary groups. Nitrogen content in the distal intestine was similar for both dietary groups. As with dry mass, the nitrogen content decreased significantly in going from the proximal to the distal intestine.

Table 5.3 Molecular weight distribution (%) of protein in the intestinal contents of rabbits fed casein or soy protein diets

	Proximal		Distal	
	Casein	Soy protein	Casein	Soy protein
Molecular weight				
> 40k	3.3(±0.4)	21.0(±2.7)	2.8(±0.2)	9.9(±1.2)
20-40k	43.0(±3.2)	22.7(±0.7)	53.0(±3.1)	8.9(±0.8)
< 20k	53.7(±2.5)	56.3(±3.5)	44.2(±2.8)	81.2(±1.7)

Aliquots of the soluble fraction were subjected to SDS-PAGE as detailed in the methods. Following destaining, gels were scanned using a densitometer.

Representative results (mean±SEM) from 4 rabbits per dietary group are shown.

The values for casein-fed rabbits were significantly different from the corresponding values for soy protein-fed rabbits ($p<0.05$) with the exception of the low molecular weight protein (<20k) in the proximal intestinal contents.

In the proximal intestine the content of bile acids was similar between groups. Though the bile acid levels in the distal intestinal contents were higher in the rabbits fed casein, compared to those fed soy protein, these differences were significant only at $p>0.10$. As with nitrogen and dry mass, bile acids decreased significantly in going from the proximal to the distal intestine.

In one study the enzymatic assay used to measure the fecal output of bile acids in 4 rabbits fed soy protein over a 4 day period was calculated to be 41 ± 6 mg/day. This compared with a value of 36 ± 3 mg/day obtained by GLC (Huff and Carroll, 1980).

5.3.3.4 SDS-PAGE of protein in the intestinal contents.

Table 5.3 shows the molecular weight distribution of protein in the soluble fraction from the proximal and distal intestinal contents of casein and soy protein-fed rabbits, determined from densitometric scans, following SDS-PAGE using 12.6% running gels. The proteins resolved were split into three groups, whose molecular weight ranges are also listed in Table 5.3.

21% of the proteins present in the proximal intestine of soy protein-fed rabbits were of high molecular weight (>40k) compared to a value of 3.3% for casein-fed rabbits. In the proximal intestine, intermediate molecular weight proteins (20-40k) were twice as abundant

in casein-fed rabbits as compared to soy protein-fed rabbits (43% v. 22.7%). Low molecular weight proteins (<20k) were similar between groups in the proximal intestine.

On going from the proximal intestine to the distal intestine, significant changes were seen only in the rabbits fed soy protein. In these rabbits high molecular weight proteins decreased significantly (21% to 8.9%) whereas low molecular weight proteins increased significantly (56.3% to 81.2%).

The distribution of these proteins in the distal intestine were significantly different between dietary groups. High molecular weight proteins and low molecular weight proteins were significantly higher for rabbits fed soy protein compared to rabbits fed casein (9.9% v. 2.8% and 81.2% v. 44.2% respectively). Intermediate molecular weight proteins were significantly higher for the casein group (53% v. 8.9%)

5.3.3.5. Migration of Polyethylene glycol.

To assess the mobility of the proteins, in vivo, some studies were conducted in which rabbits were fed their respective diets, containing the non-absorbable water-soluble marker PEG as detailed in the methods. PEG and protein were measured in the soluble fraction isolated from the stomach, proximal and distal intestinal contents. The results of these studies, for 6

Table 5.4 Protein/PEG ratios in the gut contents of rabbits fed casein or soy protein

	Stomach	Intestine Proximal	Intestine Distal
Casein	2.1±1.1	13±2	4.0±0.9
Soy protein	1.1±0.6	19±6	3.7±1.7

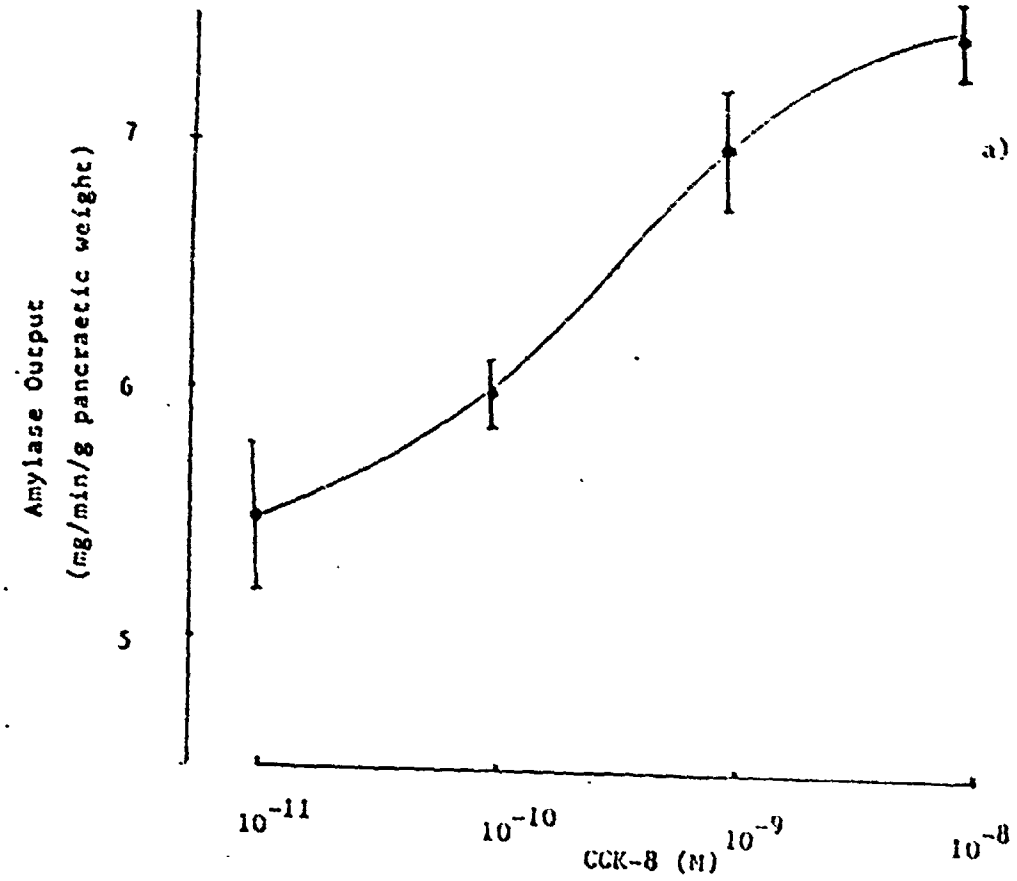
Protein and PEG were measured in the soluble fraction as indicated in the methods.

The values given are the means±SEM for 6 rabbits fed soy protein and 4 rabbits fed casein.

rabbits fed soy protein and 4 rabbits fed casein are shown in Table 5.4. Though the differences obtained were not statistically significant owing to overlap between groups, certain trends were apparent; a) soy protein migrated more rapidly than casein in the stomachs indicated by the lower ratio of protein/PEG, b) in the proximal intestine the migration of casein was faster than that of soy protein (lower protein/PEG ratio in the casein-fed rabbits) and c) in the distal intestine migration of casein was marginally slower than that of soy protein.

5.3.3.6. Fasting and post-prandial levels of plasma CCK in rabbits fed casein or soy protein.

In initial experiments to evaluate CCK levels the bioassay of Case and Clausen (1973) was used. In this assay, the amyalase released by the uncinate pancreases of baby rats (70-80g body weight) following stimulation by known concentrations of CCK, is used to quantitate CCK levels in unknown samples. Fig. 5.5a shows a typical dose response curve of amylase release v. CCK concentration obtained with this assay. The minimal concentration of CCK detected with any confidence is around 100pM. When rabbit plasma samples were used to assess CCK levels (both fasting and post-prandial), no CCK was detectable and it became apparent that this bioassay was too insensitive for the planned experiments. However, fortuitously a method was published in 1984 (Liddle et al, 1984) which allowed detection of CCK levels as low as 1pM.


Amylase Output
(mg/min/g pancreatic weight)
7
6
5
a)
10^{-11}
10^{-10}
10^{-9}
10^{-8}
CCK-8 (M)

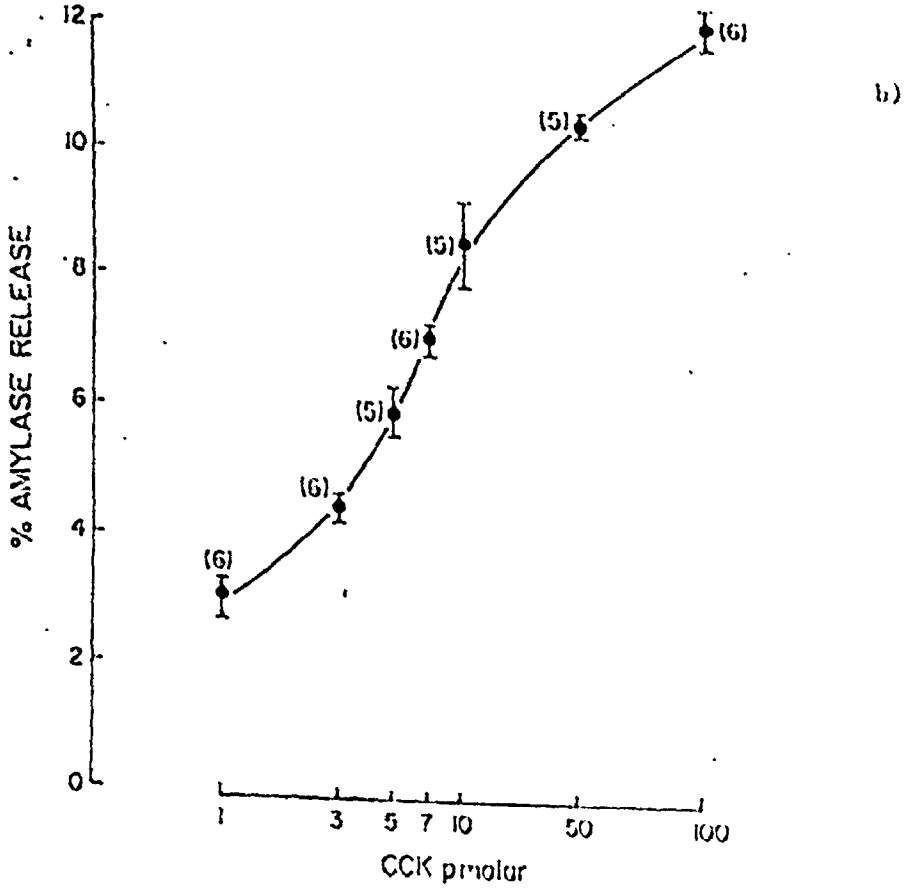

% AMYLASE RELEASE
12
10
8
6
4
2
0
b)
(6)
(5)
(5)
(6)
(5)
(6)
(6)
1
3
5
7
10
50
100
CCK pmolar

Fig. 5.5

Dose-response curve for CCK

The dose response curve was generated using either a) baby rat uncinate pancreases or b) isolated rat pancreatic acinar cells.

In a) each point is the mean±SD for 4 determinations and in b) the number of determinations (±SD) is indicated in the figure.

Furthermore, most CCK detection assays had been hampered by contamination due to cross-reactivity with gastrin but this assay did not suffer from this limitation. Therefore, accordingly, this assay was utilised, which works on the same principle as that of Case and Clausen (1973), but instead of using the whole uncinate pancreas, it utilises acinar cells prepared from collagenase digestion of the pancreas.

Fig. 5.5b shows a CCK-dose response curve obtained using this assay. In comparison to Fig. 5.5a, it can be seen that the detection levels are far more sensitive, and the assay can be used to measure fmol amounts of CCK.

Fig. 5.6 shows the time-course of post prandial release of CCK in rabbits fed casein and soy protein. The mean level of CCK in the plasma of 10 fasting rabbits determined individually was found to be 0.36 pM ($\pm$0.18 SD) regardless of the type of diet that the rabbits had been maintained on. The release of CCK following a casein or soy protein containing diet is shown.

In both dietary groups post-prandial levels of CCK rose above fasting levels and were maintained throughout the 1 h duration of the experiment. However as can be seen the response in the soy-protein-fed rabbits was markedly higher than in the rabbits fed casein. This was particularly prominent after 30 min. at which time the CCK levels were three-fold higher in the soy-fed rabbits.

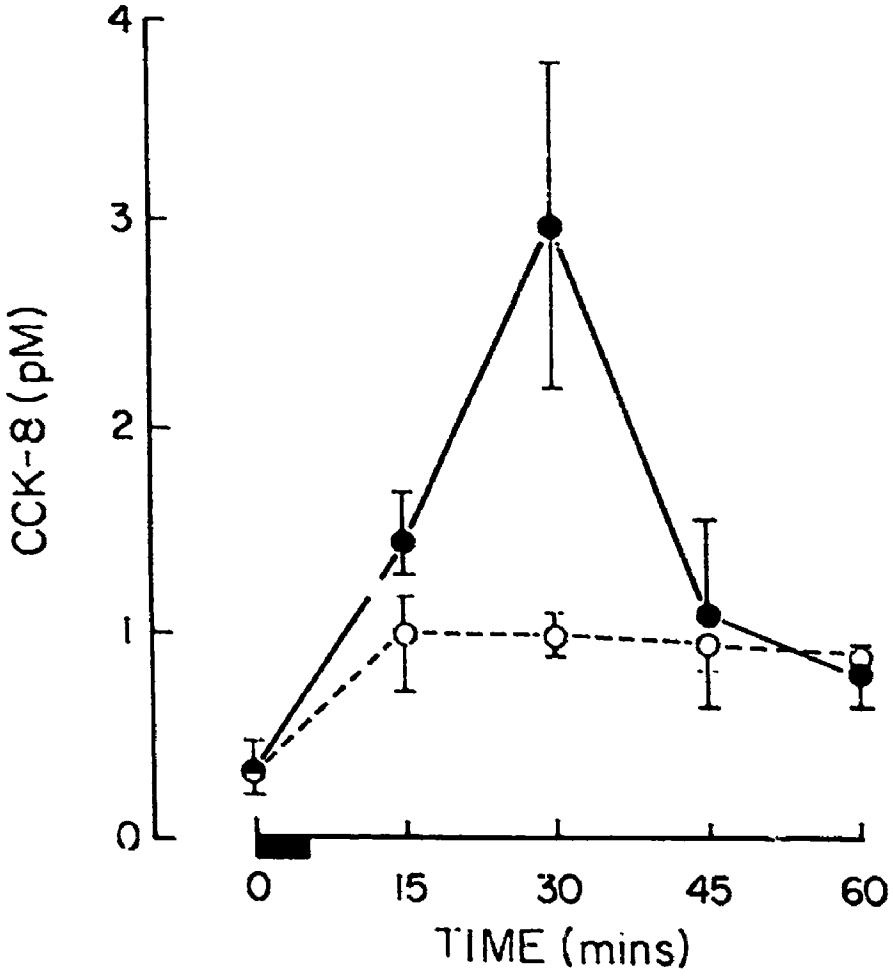

4
3
2
1
0
CCK-8 (pM)
0
15
30
45
60
TIME (mins)

Fig. 5.6

Post-prandial release of CCK in rabbits fed casein (O) or soy protein (●) diets

Each value is the Mean±SEM of 3 experiments. For each experiment 2 rabbits were used. Following a 24h fast, rabbits were fed for 5 min (shaded box) and then the food was withdrawn.

The 30 min CCK level were significantly higher in the soy protein-fed rabbits ($p<0.02$).

Table 5.5

Effect on plasma cholesterol (mg/dl) of injecting exogenous CCK-8 into rabbits fed casein diet

	Casein	Casein (CCK-8)
Day 0	226 (±7)	245 (±29)
Day 4	275 (±13)	209 (±6)
% change	+ 22%	- 15%

Casein-fed rabbits were injected either with saline or CCK-8 as indicated in the methods

Values given are the means(±SEM) of 4 rabbits per group. Over the 4 day period the treated group received a total of 116μg of CCK-8 each).

The CCK-8 was generously provided by Dr. Miguel Ondetti at the E.R. Squibb Research Institute, Princeton, NJ.

These levels then declined such that at 45 min. the CCK levels were similar between the two groups. The rapid rise in CCK levels in the soy protein-fed rabbits was not seen in the rabbits fed casein. In the latter group peak CCK levels were attained after 15 min. and did not change subsequently.

5.3.3.7 Effects of exogenous CCK on plasma cholesterol levels in rabbits fed casein.

Table 5.5 shows the result of one study in which 8 rabbits fed casein, were utilised. After determining plasma cholesterol levels, the rabbits were divided into two groups. Both groups continued on the same diets. One group received daily i.p. injections of CCK-8, whereas the other received i.p. injections of saline as outlined in the methods section.

As can be seen, the plasma cholesterol levels of the control rabbits, increased by a further 22% over the 4 day period (226 mg/dl to 275 mg/dl). Over the same time period, the plasma cholesterol level of the treated rabbits actually decreased by 15% (245 mg/dl to 209 mg/dl). The increased plasma cholesterol levels in the control group were apparent in each of the 4 rabbits. Similarly, the decrease in the experimental group, was also apparent in each of the 4 rabbits.

5.4. DISCUSSION

The enzymatic digestion studies of casein and soy protein in vitro confirmed the results obtained by previous workers in this laboratory (Woodward and Carroll, 1985). These differences in digestibility of casein and soy protein may be attributable to pH dependent changes in the solubility of the proteins since it was shown that casein and soy protein were most soluble at alkaline and acidic pH respectively (Woodward and Carroll, 1985). In vitro, both proteins were poorly digested by peptidase, since this enzyme acts specifically on amino and carboxy termini of peptides, and is consequently less effective against intact proteins. The fact that soy protein was more readily digested by pepsin, whereas casein was more readily digested by trypsin, suggested that in vivo, one would expect better digestion of soy protein by the stomach than casein, whereas the converse would be expected in the duodenum.

From gel fractionation of the proteins in the stomach contents this was indeed found to be true. For the rabbits fed soy protein, the elution profile on Sephadex G-25 showed extensive material of low molecular weight, whereas very little low molecular weight material was found in the rabbits fed casein. This is similar to the results obtained by Yashiro et al., (1986) using rats. These workers, additionally showed that the elution profiles of proteins from the stomachs of rats fed

soy protein were essentially identical to the elution profile of soy protein following its digestion by pepsin, in vitro. One of the low molecular weight peptides isolated from the stomachs of these soy protein-fed rats was found to have a hypocholesterolemic action when fed to mice. The hypocholesterolemic effect of soy protein in rabbits, may also reside in one of the peptides produced in the stomach but a feeding study in the rabbit with one of these isolated and purified peptides would prove quite impracticall. This set of studies, showed quite clearly that in vivo, soy protein is more readily digested in the stomach than casein.

From the nitrogen content of the proximal and distal intestine it appeared that soy protein was being digested more slowly than casein, owing to the fact that the total nitrogen content (sum of the proximal and distal contents) was higher for the soy protein-fed rabbits in comparison to the total mass in casein-fed rabbits (358 mg v. 288 mg).

The SDS gel profiles showed that there was an increase in the proportion of low molecular weight proteins of the soluble fraction, in going from the proximal to the distal intestine, in rabbits fed soy protein - 56% to 81%, whereas there was a decrease in the proporation of high and intermediate molecular weight proteins - 44% to 19% collectively. This is consistent

with a breakdown and digestion of the high and intermediate molecular weight proteins as they traverse the intestine. This suggests that in the case of soy protein, digestion occurs in the distal intestinal lumen. As an alkaline environment pervades in the proximal intestine, this does not favour solubilisation of soy protein, which will result in poorer digestion. For casein-fed rabbits, protein digeston occurred more proximally since the molecular weight distribution of the proteins was essentially similar in the proximal and distal intestinal contents. This may reflect the fact that the alkaline pH in the proximal intestinal lumen favours solubilisation of casein, facilitating better digestion by trypsin. These results are consistent with the results of enzymic digestion in vitro.

Although differences in protein digeston in vivo, may be dependent on the degree of solubilisation, they may also be due to differences in the pattern of enzymic activities in the proximal intestinal lumen. It is known that the enzyme content of the pancreas readily adapts to changes in dietary composition (Corring, 1977). Since the rabbits used for these studies had been on their respective diets for 6-8 weeks, these changes may already have taken place and consequently differing enzymic activities may have played a role in addition to differences in solubility. In mice, although decreased activities of pancreatic enzymes were observed in animals

fed soy protein compared to those fed casein, a significant reduction was only noted in chymotrypsin activity. Additionally, total enzymic activities in the small intestine were unchanged by the two diets (Roy and Schneeman, 1981).

One of the aims of these studies was to see if the digestion products of soy protein, in vivo, sequestered bile acids (digestibility hypothesis). Therefore, it was expected that if this hypothesis was true more bile acids would be present, especially in the distal inestinal lumen of soy protein-fed rabbits (due to the fact that they would have been sequestered by the digestion products of soy protein preventing their ability to be reabsorbed). However, the intestinal content of bile acids was found to be similar between the dietary groups. Higher levels of undigested protein have been correlated with higher levels of bile acids in the intestinal contents of mice fed a soy protein-cholesterol diet compared to mice fed a casein-cholesterol diet (Roy and Schneeman, 1981). A similar result was obtained with pigs consuming cholesterol containing diets although in this study plasma cholesterol levels in the two dietary groups were similar (Scholz et al, 1985). In studies with chicks fed atherogenic diets both casein and soy protein-fed chicks were able to sequester bile acids in their intestinal lumen to a similar degree (Sklan et al, 1979). In the most recent studies by

Kuyvenhoven et al., (1987), the digestibility of casein and soy protein was found to be similar and these authors concluded that the digestibility hypothesis cannot account for the effects of dietary protein on plasma cholesterol levels in rats.

The bile acids in the intestinal contents were measured enzymatically following their extraction from freeze dried intestinal contents. Though the extraction procedure was quantitatively able to extract free bile acids, as determined using radiolabeled deoxycholic acid, it may be that it was not able to extract bile acids that were sequestered with other materials in the freeze dried extracts. Even though the extraction procedure is quite harsh, this possibility cannot be totally excluded. One way to circumvent this problem, would have been to feed radiolabeled deoxycholic acid as part of the semipurified diet, allowing it to be sequestered by the digestion products, in vivo. Then isolate the intestinal contents, obtain the soluble fraction, and determine the radioactivity both before and after the extraction procedure. However, due to the amount and cost of the radiolabeled deoxycholic acid needed, this approach was not possible.

Another possibility to consider is that different dietary treatments lead to differences in the bile acid profile in the animal per se. If the bile acids themselves are qualitatively different, then one may

not get a true quantitative value since the enzymatic method is based on reference to a deoxycholic standard. This is also unlikely since precvious work has shown that in the rabbit, the major bile acid is deoxycholic, and though there may be changes in its pool size following dietary manipulations, no qualitative differences result (Hellstrom and Sorjvall, 1962). Futhermore the fecal bile acid output in soy protein-fed rabbits, measured enzymatically, was found to be similar to values measured by GLC (Huff and Carroll, 1980).

Therefore although the results cannot rule out the possibility of sequestering of bile acids by the digestion products of soy protein, they suggest that this may be the same in the two groups (if indeed it takes place). With similar levels of intestinal bile acids, how can one explain the increased fecal output of bile acids in rabbits fed soy protein (Huff and Carroll, 1980; Kuyvenhoven et al., 1986). This can be reconciled if there is increased mobility in the gut of soy protein-fed rabbits. If the digesta is traversing the distal intestine faster, this can lead to increased fecal output of bile acids. Faster migration of digesta in soy protein-fed rats was found by Kuyvenhoven et al., (1987).

Attempts to measure gut transit using PEG were inconclusive. These results suggested that soy protein was migrating faster in the stomach than casein. In

the proximal intestine migration was slower for soy protein. In the distal intestine migration was faster for soy protein. Hence even though the distal intestinal contents of bile acids were similar for the two groups of rabbits, an increased mobility in the distal intestire may be an explanation for increased fecal output of bile acids in rabbits fed soy protein. Further experiments are needed to completely resolve this point. Roskowski et al., (1986) could not find any differences in overall digestion of casein and soy protein in vivo of rabbits fed these diets. However these workers made no measurements of intestinal bile acids. A faster migration through the gut in soy protein-fed rabbits may also explain the presence of more nitrogen and could account for the decreased apparent digestion, since a faster transit time would mean that the protein is exposed to the digestive enzymes for a shorter time period.

Postprardial release of CCK was clearly different between the two dietary groups. The results presented are from 3 experiments per dietary group. In each experiment pairs of rabbits were bled at the times indicated, so that in any experiment, the time-course profile of CCK release is from the same two animals. In another earlier series of pilot experiments, a total of 28 rabbits were used and the time course profile generated by using 1 rabbit to provide a CCK value at a given time (results not shown). This profile was essentially identical

to the one shown in Fig. 5.6 with peak CCK levels in soy protein-fed rabbits ocurring after 30 min.

This is the first assessment of CCK levels in the rabbit with the use of this sensitive bioassay. The fasting levels obtained in rabbits is essentially similar to those observed in rats, 0.18pM (Liddle et al., 1984) and man, 0.2pM, (Liddle et al., 1985). The postprandial levels in rats and man are 6-8pM which is in the same order of magnitude for the soy protein-fed rabbits. However although this result of the effect of casein and soy protein on CCK release is consistent with the hypothesis outlined, several points have to be considered whilst interpreting the data.

Firstly it is not known what are the circulating forms of CCK in rabbits. It may be CCK-8, CCK-33, CCk-39 or even a longer variation of the parent molecule CCK-58. As the assay is based on a CCK-8 staadard, the results should really be expressed as CCK-8 "equivalents". The measuremeats reported are based on the ability of rabbit CCK to stimulate amylase release from rat acinar cells. The CCK receptors on the rat acinar cells may not be fully able to recognize the rabbit CCK. This may be a factor responsible for the lower l vels of CCK found in the rabbit.

Secondly the normal effectors of CCK release in the rabbit are not known. Based on work with

rats and humans (Liddle et al., 1984 & 1985; Owyang et al., 1986; Louie et al., 1986) the breakdown products of protein digestion are likely to stimulate CCK release, but it is not known whether these will be specific amino acids or peptides. In the rat it has been shown that proteins, but not anino acids stimulate CCK release (Liddle et al., 1986).

Thirdly the roles of CCK which have been established in man, namely regulation of gastric emptying, regulation of gallbladder contraction etc., have to be shown to be operating in the rabbit.

Nevertheless, despite this, the increased release of CCK in soy protein-fed rabbits was consistent with the basic tenets of the proposed hypothesis. However it should be mentioned that for these experiments, 2.5kg rabbits were being used. As 4 blood samples were obtained from each animal over the 1h of the experiment's duration, this corresponds to 20% of their blood volume. A decrease in blood volume of this magnitude could result in the release of catecholamines, which might have contributed to the values of CCK observed.

The results from the exogenous injections of CCK were essentially in agreement with the proposed hypothesis. Although the results obtained were from a few animals, and the changes in plasma cholesterol were not that dramatic, the trend was quite clear. [Again though, it should be stressed that as the

food consumption of the rabbits was not monitored, the observed results may have been due to a satiety effect of CCK. The dose injected (3.9 μg/kg body weight) is certainly within the range (1-10 μg/kg) at which satiety effects are seen in other species (Smith and Gibbs, 1984), although there are no published data for the rabbit.] A more ideal experiment might have been to use a larger number of rabbits fed casein for a longer time period, injecting CCK into half of them and saline into the rest, then switch treatments and see what this effect this had on plasma cholesterol levels. However this would have necessitated the use of large amounts of CCK, and besides the cost involved, the long term effects of injecting CCK is not known.

In summary, the results from the series of studies reported in this chapter showed,

1) Increased digestion of soy protein and casein by pepsin and trypsin, respectively, in vitro.

2) Gel-fractionation of the stomach contents of soy protein-fed rabbits showed soy protein to be better digested than casein.

3) Analysis of intestinal contents from casein and soy protein-fed rabbits suggested that, in vivo, the slower digestion of soy protein may be due to an increased mobility, which would decrease the exposure time to the intestinal enzymes.

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4) Intestinal contents of bile acids were similar between the two dietary groups, suggesting that both proteins may sequester bile acids in vivo, to similar degrees (if indeed they sequester bile acids).

5) Fasting levels of CCK were measured in rabbits and were found to be 0.36 pM, regardless of the diet that the animals had been maintained on.

6) Post-prandial release of CCK was 3-fold higher after 30 min in soy protein-fed rabbits compared to the CCK release in casein-fed rabbits.

7) Over a 4 day period, exogenous injections of CCK into casein-fed rabbits resulted in a 15% decrease in plasma cholesterol levels. In contrast the plasma cholesterol in casein-fed rabbits, not receiving any CCK, rose by 22%.

CHAPTER 6

GENERAL DISCUSSION AND SUMMARY

Although dietary proteins have the ability to modulate plasma cholesterol levels, especially in the rabbit, their role in human nutrition is less clear cut. With the exception of Sirtori's group in Italy, and a few scattered reports in the literature, their beneficial effects in man remains unresolved. One of the reasons for this may be that the level of protein fed to a rabbit, to produce an effect, is generally much higher than the level of protein consumed in a typical North American diet, and this may be one reason why their is insufficient evidence for the lack of an effect in man. Nevertheless, even from an academic viewpoint, it would be mentally rewarding to sort out the mechanism. From the introductory chapter it will be noted that there differences within species. It may therefore be that there is no all encompassing hypothesis or mechanism which will explain the effect. There may be several possibilities in any one species, let alone several different species.

The rabbit has been much utilised in studying the effects of dietary protein on cholesterol metabolism. Ironically, the rabbit is a vegetarian, and feeding it dietary animal protein is definitely not "physiological". This fact will still have to be remembered if and when the mechanism is elucidated fully.

Given this limitation, the physiological scheme for cholesterol metabolism was outlined in Chapter 1. The response to dietary protein may be due to differences at more than one stage, such that the response seen after feeding casein or soy protein, may itself be a multi-step process. Deciphering which is the first step, may be fraught with difficulties. Even if the action of casein and soy protein can be explained, one then has to explain the effects of a variety of dietary animal and plant proteins which have been shown to be hyper and hypocholesterolemic, respectively (Hamilton and Carroll, 1976).

The kinetic experiments collectively have shown that the LDL pool rises because of impaired receptor-dependent catabolism. These studies have shown for the first time that it is the protein component of the diet that is sponsible for the effect in rabbits, and more generally these are also the first studies using cholesterol-free diets unlike the studies in rats which utilised cholesterol-containing diets (Sirtori et al., 1984; Cohn et al., 1984; Lovati et al., 1985; Cohn et al., 1985). The fact that LDL receptors were down-regulated within 5 days of feeding casein diet (Chapter 4) suggests that this is an early event.

The rapidity of down-regulation of LDL receptors seems to favour an initial, rapid trigger

switched on by the protein content of the diet. The postprandial release of CCK supports this argument, since dietary protein is one of the effectors of CCK release. CCK also provides for a quick route to regulate liver cholesterol levels, by means of feedback inhibition of liver cholesterol synthesis as a consequence of gallbladder bile acids. The alternative route (implicated by the digestibility hypoythesis) is one where bile acids first have to reach the distal intestine, and then find their way back to the liver. This route is longer. Furthermore it is dependent on the ability of the ingested material to traverse the stomach, the proximal intestine and eventually find its way to the distal intestine, the site of bile acid reabsorption. If CCK has a role in mediating the effect, then the ingested material has simply to traverse the stomach and it will stimulate CCK release.

Additionally CCK provides a theoretical explanation for how other plant and animal proteins could mediate their effects on plasma cholesterol. This could be reconciled by the differing solubilities of plant and animal proteins in the stomach, and their subsequent ability to release peptides into the duodenum which will stimulate CCK release. Since the rabbit is a vegetarian, its normal physiology will be well suited to digesting plant proteins, more effectively than animal proteins.

It could be that CCK is simply the initial trigger and subsequent events are mediated by events in the

intestine. This is also consistent if one considers the situation in the rabbit when hypercholesterolemia is developing (non steady-state) as opposed to the rabbit with a steady-state level of cholesterol. If impaired CCK release is the cause of events which lead to increases in plasma cholesterol than the latter would continue to increase indefinitely, unless the body adapted. This adaptataion to the steady-state is by increasing bile acid and cholesterol absorption from the intestine.

Of course the above speculations stem from the observation that postprandial levels of CCK are lower in casein-fed rabbits in comparison to soy protein-fed rabbits. These effects of CCK have not been correlated with effects on gallbladder emptying. Recently, Bauer (1988) found that gallbladders of casein-fed rabbits were larger than rabbits fed soy protein. He postul ted that the differing respose of dietary protein on plasma cholesterol may be mediated by differences in gallbladder contraction. If this effect can be established and some of the experiments outlined at the end of the previous chapter (with regards to characterization of rabbit CCK) are carried out, one will be in a better position to appraise the role of CCK. If the hypothesis holds true, then it should be possible to prevent impaired receptor-dependent catabolism seen in casein-fed rabbits (over the first five days of feeding the casein diet) by injecting the rabbits

i.v. with CCK to match postprandial CCK levels seen in soy protein-fed rabbits.

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Given this limitation, the physiological scheme for cholesterol metabolism was outlined in Chapter 1. The response to dietary protein may be due to differences at more than one stage, such that the response seen after feeding casein or soy protein, may itself be a multi-step process. Deciphering which is the first step, may be fraught with difficulties. Even if the action of casein and soy protein can be explained, one then has to explain the effects of a variety of dietary animal and plant proteins which have been shown to be hyper and hypocholesterolemic, respectively (Hamilton and Carroll, 1976).

The kinetic experiments collectively have shown that the LDL pool rises because of impaired receptor-dependent catabolism. These studies have shown for the first time that it is the protein component of the diet that is responsible for the effect in rabbits, and more generally these are also the first studies using cholesterol-free diets unlike the studies in rats which utilised cholesterol-containing diets (Sirtori et al., 1984; Cohn et al., 1984; Lovati et al., 1985; Cohn et al., 1985). The fact that LDL receptors were down-regulated within 5 days of feeding casein diet (Chapter 4) suggests that this is an early event.

The rapidity of down-regulation of LDL receptors seems to favour an initial, rapid trigger