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APOLIPOPROTEIN B METABOLISM IN HAMSTER LIVERS, STUDIED IN VITRO.

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ABSTRACT

This study aimed to investigate lipoprotein metabolism in male hamsters fed diets considered to be atherogenic in humans. Livers from adult male hamsters were selected to study aspects of apolipoprotein B metabolism. Isolated hepatocytes in suspension were compared with those maintained under tissue culture conditions. Liver slices were also prepared and compared with isolated suspended heptaocytes.

Freshly prepared hepatocytes from the animals were incubated with radiolabelled precursors in suspension, or they were maintained under tissue culture conditions; liver slices were also investigated. The rates of total protein synthesis were of the same order in each of these systems, but protein secretion was impaired in liver slices, probably as a result of diffusion problems associated with the altered architecture of the sliced tissue. Albumin constituted 40 - 50% of the secreted proteins in each system. The rates of VLDL synthesis were increased in cells and slices prepared from animals previously fed sucrose- or fat-rich diets, but the secretion of VLDL was inhibited when diets contained unsaturated fat. The overall synthesis of apolipoprotein B was enhanced by fat-feeding; in the case of suspended hepatocytes, secretion of this protein was decreased when the preceding diet contained fats that were unsaturated; while in the case of liver slices, secretion was paradoxically enhanced. Apolipoprotein B was not degraded at significant rates in hepatocytes prepared from either control or fat-fed hamsters.

DEDICATION

For Neil,from whom (even in this endeavour) I learn so much.

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ABBREVIATIONS

The following is a list of the abbreviations and symbols used in the text:

A_{340}	= Absorbance at 340 nanometer wavelength
Apo	= Apolipoprotein
ATP	= adenosine 5'-triphoshate
BSA	= bovine serum albumin
β (prefix)	= beta
cDNA	= copy deoxyribonucleic acid
Ci	= curie (3.7×10^{10} disintegrations per minute)
DMSO	= dimethylsulfoxide
DNA	= deoxyribonucleic acid
dpm	= disintegrations per minute
EDTA	= ethylenediamine tetraacaetic acid
EGTA	= ethyleneglycol-bis-(B-aminoethyl ether) tetraacaetic acid
ER	= endoplasmic reticulum
FCS	= foetal calf serum
Fig.	= figure
g	= gram
HDL	= high density lipoprotein
HEPES	= 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
IDL	= intermediate density lipoprotein
k (prefix)	= kilo (10^{3} x)
KRB	= Krebs-Ringer bicarbonate buffer
l	= litre
LDH	= lactate dehydrogenase
LDL	= low density lipoprotein
Lys	= lysine
m (prefix)	= milli (10^{-3} x)
m	= meter
M	= molar

MEM = minimal essential medium

mRNA = messenger ribonucleic acid

n (prefix) = nano (10^{-9} x)

NADH = nicotinamide adenine dinucleotide (reduced form)

PBS = phosphate buffered saline

PMSF = phenylmethylsulphonyl fluoride

rpm = revolutions per minute

S = svedberg units (rate of particle sedimentation in a centrifugal force)

SDS = sodium dodecyl sulphate

SDS-PAGE = SDS-polyacrylamide gel electrophoresus

T3 = 3,3´,5-triiodothyronine

TCA = trichloracaetic acid

TLC = thin-layer chromatography

μ (prefix) = micro (10^{-6} x)

U = units of activity

Vo = void volume

x g = acceleration due to gravity (9.8 m/s^2)

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Chapter 1: INTRODUCTION AND GENERAL REVIEW

1.1 Introduction

The liver plays a central role in the ordered function of the body. It is therefore not surprising that this organ has been extensively investigated in the past. Its roles in the metabolism and storage of carbohydrates, lipids and proteins, the "detoxification" of foreign and endogenous molecules and many other processes have been studied in a number of model systems, ranging from *in vivo* studies of the whole organ to the investigation of cultured hepatoma cell lines. A common theme in all these efforts has been a concern with the physiological validity of the chosen model system; that is, the question as to whether the system was capable of providing an accurate reflection of events in the *in vivo* organ and body.

Studies of whole livers *in vivo* are possible in certain experimental animals but are almost impossible in humans because of the ethical issues involved. Unfortunately, small-animal models often differ substantially from humans in their responses to various conditions and treatments. This species-difference problem has accordingly been superimposed on that relating to physiological relevance. An understanding of species differences in metabolism together with the special features of liver structure and of the cells that make up the organ, are therefore prerequisites in choosing the most appropriate model system for the study of a particular problem.

1.2 The Structure Of The Liver

The liver is a large abdominal organ that develops from an outgrowth of the primitive entoderm into the mesoderm (1). It

exists in close proximity to the gut and is well supplied with blood vessels.The arterial blood supply maintains cellular oxygenation and the portal vessels supply nutrients absorbed from the gut (2).Bile produced by the liver is removed via bile ducts (3).

Unlike many other organs,the liver has proved difficult to describe in terms of functional and/or structural units;the most recent (and most widely cited) concept is that of the liver acinus,which is defined as a collection of hepatocytes that is arranged around the afferent blood vessels (portal and arterial) (4,5).The efferent vessels (hepatic venules) lie on the outer limits of the acinus and thus drain more than one acinus.This has led to the description of three zones of cells in each acinus:Zone 1 consists of periportal cells which are richly supplied with nutrients and oxygen and have high gluconeogenic activity (and low rates of bile acid synthesis),Zone 2 has intermediate cells and Zone 3 is made up of perivenous cells which have low gluconeogenic activity and high rates of bile acid synthesis (6 - 9).Thus opposing gradients exist across the acinus:one of high to low gluconeogenic activity (and supply of plasma nutrients and oxygen) and the other of low to high bile acid synthesis (and production of plasma carbon dioxide).

1.3 The Cells Of The Liver

1.3.1 Parenchymal Hepatocytes

The parenchymal cells are called hepatocytes because they are the most abundant cell type in the liver,making up 60% of the total cell number and 80% of the liver volume (4).Hepatocytes are 17 - 35 μm in diameter and are polygonal in shape (3).Their cell membranes consist of three kinds of surfaces:vascular,biliary and intercellular (5).The vascular surfaces are characterised by

numerous microvilli which project into the spaces of Disse,biliary surfaces are specialized membrane regions tightly bound together by junctional complexes which prevent leakage of bile while intercellular surfaces consist of the membranes of two adjoining cells that are separated by about 15 nm with gap junctions at intervals.

1.3.2 Sinusoidal cells

The sinusoidal cells constitute 8% of the liver volume (4).Four cell types have been classified as being sinusoidal:Endothelial cells,Kupffer cells,fat-storage cells and pit cells.Each of these cell types is found in the architecture of the liver sinusoids.The sinusoids are "spongework" areas filled with plasma from which the nutrients of the blood reach the cells (3).The endothelial cells have fenestrations and thus when they are grouped they form "sieve plates" which allow movement of all non-cellular plasma components into the sinusoids (5).The Kupffer cells are phagocytic cells which often bulge into the sinusoids (4).The fat-storage cells are found between the hepatocytes and the cells lining the sinusoids.There are a number of filaments and microtubules found in cytoplasmic extensions in these cells which suggests that they may have some role in anchorage or support and they also play a role in vitamin A metabolism (5).The pit cells are characterised by cytoplasmic granules and may have some endocrine function (4).

1.3.3 The intercellular spaces

The extracellular spaces account for the remaining 12 - 15% of the volume of the liver.They consist of the sinusoidal lumens (discussed above),the spaces of Disse,bile canaliculi and collagen-rich matrixes (10).Between the sinusoidal cells and the

hepatocytes lies the Space of Disse into which the microvilli of the hepatocytes extend. It is from these spaces that the hepatocytes absorb molecules carried to the liver by the blood, and it is also into this area that they secrete most of the products of hepatic metabolism. The bile canaliculi are small spaces between hepatocytes that form a duct system which eventually becomes the bile duct and transports the bile to the gall bladder and the intestine (3). Glycoproteins and proteoglycans make up the connective tissue, the extracellular matrix also contains collagen. These molecules are complex in structure and are not simply a cement holding the liver cells together - they provide certain signals to the cells and play a role in maintaining specific hepatic functions (10).

1.4 Experimental Systems Used To Study The Liver

The ideal system in which to investigate the functioning of an organ is one in which the setting up of the preparation does not adversely affect the activities of the system or cause other significant disturbances, the preparation is viable for the minimum period of time required for a particular metabolic process, adequate controls and multiple samples can be furnished and it is possible to prepare subcellular fractions from the tissue (11). A survey of the systems used to investigate liver functions shows that no single preparation complies with all of the above criteria.

1.4.1 Intact animals

Experiments which make use of intact animals are rarely undertaken because they are expensive and provide few or no satisfactory controls or multiplicates. Because the sacrifice of many animals is required, the ethical issues involved in such

studies have also become prominent. This is a pity from the experimental point of view because such studies come closest to the ideal: the activity of the system is very high and influences such as those of nervous stimuli and hormones are present and can be controlled. Use is frequently made of constant-rate infusions or of pulse-labelling with a particular marker (11). As an example, in studies of the control of hepatic cholesterol metabolism, the whole-animal approach has been undertaken by Dietschy and co-workers with considerable success (12 - 15). Other aspects of liver function have not been accessible in this way, however.

1.4.2 Isolated perfused livers

A recirculating or non-recirculating perfusion system in which oxygenated fluids are pumped through the vessels of the liver (either *in situ* or in the isolated state) characterizes this system. Perfused livers have been extensively used because the system is less expensive and simpler than the use of intact animals. While the activities of various metabolic processes are somewhat lower than normal, and nervous and hormonal controls are absent or altered, perfused livers are active for many hours. Unfortunately, few controls or multiplicates are again possible. A factor that is both an advantage and a disadvantage is that the combined and organized function of all of the cells of livers is observed. In addition it is possible to alter the flow rates of the perfusate, as well as altering the concentrations of various substances (16).

1.4.3 Liver slices

Many thin slices can easily be prepared from a single freshly-removed liver, making possible the provision of

appropriate controls,multiplicates and time points (11).Liver slices also have the feature of containing all the cell types of the organ in an ordered arrangement even though the vascular system is disrupted and nerve and hormonal influences are removed.Liver slices are active for up to five hours but disturbances caused by their preparation renders them generally less active than either of the two systems described above.

1.4.4 Freshly-isolated hepatocytes

Isolated hepatocyte have been the most widely used of the systems currently available for studying liver function *in vitro* .The most common donor animals have been rats,but biopsies from human livers have also been successfully used to prepare hepatocytes (17).In a widely used procedure described by Seglen,a two-step process is applied in which the livers are first perfused with a calcium-free buffer to loosen intracellular junctions,followed by perfusion with partially purified bacterial collagenase,which digests the collagen and other proteins in the extracellular matrix leading to the release of individual cells in large numbers (18,19,20).Many modifications of this method have been made (for a review see 21) and some authors prefer to prepare cells using the calcium-chelator EDTA without collagenase (22).The type and batch of collagenase used markedly affects the cell yield and viability (23).Overall,the procedure remains the most popular method of isolating hepatocytes.

Whilst a large number of disturbances clearly do occur in such cells,their activities remain high for a number of hours,and the yield of cells permits adequate experimentation in the sense of providing duplication,controls and time points.Also,the technique allows the experimenter to prepare hepatocytes as a pure population so that their functions can be studied in isolation

(24 - 30). It has been shown that isolated hepatocytes synthesize and secrete albumin, fibrinogen and transferrin as well as lipoprotein particles containing the apoproteins known to be secreted by the liver (31,32,33). Isolated hepatocytes have proved easy to maintain under tissue culture conditions, where they remain active for many days and attain a measure of organization (34,35). They have also been successfully used as freshly isolated, incubated single-cell suspensions (36).

1.4.5 Transformed hepatoma cell lines

In recent years, a number of fairly well-differentiated transformed hepatoma cell lines, from various species have become available. These include Hep G2, Fao, BW1 and Hep 3B (37 - 40). The most common cells used in the study of lipoproteins have been human Hep G2 cells. These immortal cell lines are convenient because they can be stored, they continue to divide in culture and no time-consuming preparative work is required. They are cancerous (transformed) cells, however, and as such often differ from their untransformed parent cells. Hep G2 cell have been shown to secrete most of the major apoproteins synthesized by the liver, but the particles with which the apoproteins were associated are different from those found *in vivo* (40 - 43). Similarly, CaCo2 a transformed intestinal cell line, whilst making the appropriate apoproteins, does so in an abberent fashion (44). The interpretation of data from such cells can therefore be difficult.

1.4.6 The choice of model system

As already stated, while the ideal conditions under which to study various aspects of the functioning of an organ such as the liver are *in vivo*, *in vitro* experimentation is often necessary or

preferable for a number of reasons. Clayton and Darnell have investigated an aspect which affects the choice of model systems, namely the pattern of transcription as a measure of the differentiated activity of the liver-cell systems (39,45 - 48). They investigated isolated hepatocytes (from mice), liver slices and a number of transformed (rodent) hepatoma cell lines. In the case of liver-specific proteins, the various mRNA concentrations were decreased in hepatocytes after twenty four hours in culture (45). While similar results were obtained in the case of liver slices incubated in culture for the same period of time, the decline in liver-specific mRNA transcription was significantly less. In slices prepared from livers that had been perfused with EDTA to disengage the cells, and then re-perfused with calcium to reengage them, transcription of mRNA was at a higher level than in the cells that had not been re-engaged (45). These results suggest that the cellular architecture of the liver is important in determining the rate of transcription of liver-specific mRNA. Alterations of some nutrients and hormones made little difference to the rates of mRNA transcription, and co-culture of hepatocytes with other hepatic cells was also not important in this respect (45). Transformed cell lines also showed variations in mRNA concentration and transcription rates in the case of liver-specific mRNA species (39). The above-mentioned considerations suggest that liver slices may be a system of choice for studies in which the rates of biosynthesis of liver proteins are to be studied over a period of several hours in an experimentally manipulable system. In Peters' general description of the liver slice technique, he makes the point that protein synthesis in incubated liver slices is linear for up to six hours after preparation (11). It is interesting that protein synthesis in the liver slices studied by Clayton and Darnell differed from that observed in hepatocytes when both were maintained under tissue culture conditions: in the liver slices, both mRNA

transcription and the rate of total protein synthesis dropped during long incubation periods while in hepatocytes the rate of total protein synthesis increased and the transcription of liver-specific genes decreased (45,46). Unfortunately the actual rates of synthesis of the liver-specific proteins was not determined and other measures of viability (trypan Blue staining, cytosol release, respiration rates or stimulation of respiration by succinate (49)) were not carried out.

A study that compared isolated hepatocytes (incubated in suspension) with perfused livers, used a number of criteria to assess the competence of each system (50). The metabolic rate of each system was measured using glucose synthesis, fatty acid and urea synthesis, ketone body production and the uptake of oxygen. The adenine nucleotide content was also measured as an indicator of the energy status of the cells. The results suggested that the cells required some preincubation to allow them to reach a steady state after their preparation; after such treatment the systems were quite comparable (50).

Numerous studies have been conducted to define the ideal conditions under which to culture hepatocytes. It has been shown that the cells can be maintained for up to forty days in tissue culture, and while the synthesis of liver-specific proteins drops over this period, the cells remain metabolically viable (47). Incubation with DMSO and nicotinamide has been shown to prolong the survival of cultured hepatocytes without loss of liver-specific function (47,51). Co-culture of hepatocytes with a line of biliary epithelial cells was found to be associated with prolonged survival of the cells, with albumin secretion occurring throughout (52). Variations in culture medium have been extensively tested, with special reference to the rates of protein catabolism. Thus nett protein degradation has been reported to occur at 2 - 6% per hour in cultured hepatocytes (53 -

59). Addition of pyruvate stimulated protein synthesis, whilst glucocorticoids and insulin helped prolong survival (53). Hepatocytes attached well to tissue culture plates lined with collagen which prolonged survival and enhanced the performance of the cells (60,61,62).

Freshly-isolated hepatocytes have been used as single-cell suspensions (for review see 36), in which the rates of total protein synthesis were linear for up to six hours and the cells secreted albumin, fibrinogen, VLDL and other liver-specific proteins throughout this time (33,36,63). The advantage of single-cell suspensions is that the stringent requirement for sterility of the tissue culture environment, is lessened and the preparation and subsequent treatment of the cells is quicker and easier. Uptake kinetics for various fluids and molecules have been measured in isolated hepatocytes (cultured and suspended) and it appears that the uptake of some substrates drops with time in culture while that of others is unaffected. Suspended cells incubated for six hours are similar to six-hour cultures in respect of the uptake of most substrates (64).

It is likely (although it may not hold for all cases) that cultured cells begin to adapt to tissue culture conditions after about 6 hours, losing the "memory" of some of their liver-specific functions. Liver slices differ in that the architecture of the acinus is retained throughout if the cells are well oxygenated. It may be assumed that acina- or liver-like structure is essential for the well-differentiated behaviour of hepatocytes. Liver slices contain all the sinusoidal cell types as well, but after a number of hours of incubation, the risk of anoxia in cells furtherest from the medium becomes very real (11). These factors are all important in the process of selecting a model system and interpreting the data obtained from it.

1.5 Lipoproteins

Lipoproteins are complex associations of lipids and protein many of which are glycoproteins. The liver plays a vital role in the orchestration of the metabolism of the various lipoproteins in the body. It takes up and metabolizes the lipoproteins that are delivered to it, and secretes new lipoprotein molecules essential for other organs. There are four classes of lipoprotein that have been distinguished according to their size (or density in the plasma). The interactions of these molecules with one another have been closely studied especially because of the roles that they may play in the development of coronary artery disease.

1.5.1 The lipoprotein classes and their metabolism

Each of the lipoprotein classes constitutes a lipid-transport mechanism which is soluble in the plasma: the solubility is conferred on the lipids by the unique structure of the individual lipoprotein particles. At the core of a spherical lipoprotein lie the hydrophobic lipids - mainly triglycerides and cholesterol esters. The shell contains phospholipids (with their polar head groups on the outer surface), cholesterol, and protein molecules (apoproteins) woven into the lipid shell or attached loosely to the surface (65) as depicted in Fig. 1.1.

Chylomicrons are the largest of the lipoprotein molecules with a density range of 0.92 - 0.96 g/ml (66). They are assembled in the small intestine reflecting the packaging of incoming dietary lipids. They consist almost entirely of triglycerides with some phospholipids and cholesterol esters, the apoproteins found on the surface are apo B48, apo AIV and apo CII. Chylomicrons are transported to the plasma via the lymph. The triglycerides undergo hydrolysis catalysed by lipoprotein lipase bound to the endothelial wall of the capillaries of muscle and adipose

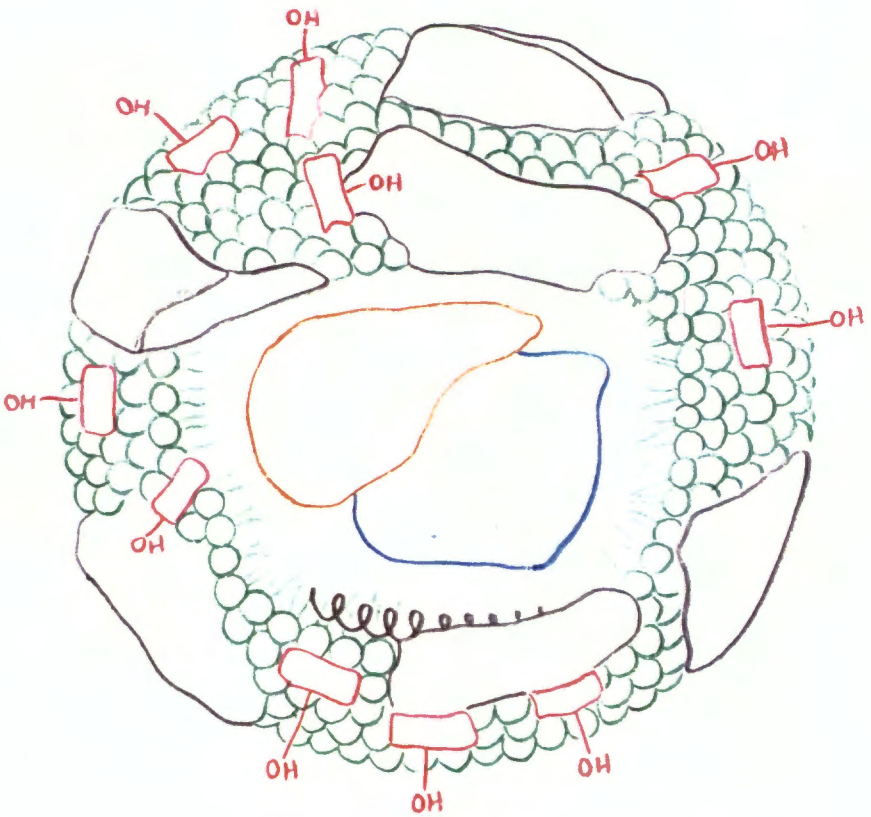

Figure 1.1 Plasma lipoprotein structure
The "iceberg-sea model" of the structure of plasma lipoproteins (from Brewer B. (1981) Klin. Wochenschrift 59, 1023 - 1035). Triglycerides (brown) and cholesterol esters (blue) are found in the core of the particle while cholesterol (red), phospholipids (green) and the apoproteins (black) make up the shell. The phopholipids are oriented so that their polar head groups face the aqueous environment and folding of the apoproteins ensures that hydrophobic regions are embedded in the lipid. There may be interactions between the individual apoproteins.

tissue;the particles become smaller in a heterogeneous fashion until finally a population of particles known as chylomicron remnants remains.These each still contain one molecule of apo B48 and acquire many molecules of apo E from HDL circulating in the plasma.The remnants are taken up by the hepatocytes by means of "remnant receptors" on the hepatic surface,after sequestration in the space of Disse (67).

Very low density lipoproteins (VLDL) are made by the liver and their density ranges from 0.96 - 1.006 g/ml.Triglycerides are packaged with cholesterol,cholesterol esters and phospholipids all synthesized in the liver cells.Apo B is an essential component of VLDL (one molecule per particle) and the other apoproteins associated with it (in varied numbers) are apo E,apo AI and all three of the apo C´s (68).Once in the plasma,the VLDL particles are also acted upon by lipoprotein lipase in the capillary beds of the muscle and adipose tissue,yielding smaller molecules depleted of almost half of their triglycerides and called intermediate density lipoproteins (IDL) (69).Some of the IDL particles undergo a series of exchanges and hydrolyses that result in the loss of all the apoproteins (except apo B100) together with much of the cholesterol and phospholipids - while cholesterol esters are added (67);the resulting particles are low density lipoproteins (LDL).Some of the smaller VLDL and IDL particles are removed by receptor-dependent processes directly by the liver cells,and do not form LDL.

LDL are the lipoprotein particles most directly associated with the formation of atherogenic plaques in the coronary arteries (70).They have a density range of 1.019 - 1.063 g/ml and are cholesterol ester-rich particles with some triglycerides and phospholipids;apo B100 is the sole apoprotein (67).LDL are cleared from the plasma via LDL receptors,mainly in the liver (12).If the LDL receptors cannot cope with the amount of LDL

present in the plasma then the risk of arterial plaque formation is greatly enhanced by the resulting hyper-LDLaemia. Reasons for elevated LDL levels are either a poorly functioning LDL receptor pathway, or continued excessive production of LDL which may be associated with consumption of the so-called "Western Diet". Other factors like stress, smoking, lack of exercise and vitamin imbalances may place an extra strain on the body and thus also play a role in the formation of atheroma (71).

High density lipoproteins (HDL) range in density from 1.063 - 1.21 g/ml. HDL form from the lipolysis of chylomicrons and VLDL, and may also arise via the production of precursor disks of lipid and protein by the liver and other organs (69). HDL contain apo AI, apo AIV, apo E and all of the apo C's. The role that these particles play in cholesterol homeostasis in the body is vital, and the negative correlation between the concentration of plasma HDL and the onset of coronary artery disease is an indication of its importance.

1.5.2 Assembly of very low density lipoproteins

VLDL production by the liver may have important consequences relating to the formation of heart disease, and has been well studied specifically for this reason. Two aspects of VLDL metabolism that are of current interest are the intrahepatic assembly pathway and the factors regulating it, and the overall regulation of VLDL synthesis and secretion by the dietary habits of an individual. While many aspects of these processes have already been elucidated, "grey areas" still exist.

Without an apo B molecule, VLDL cannot be assembled (68). Apo B is unique as perhaps the largest secretory protein made in the body and is only water-soluble while tightly associated with lipid. The apo B gene (47 kbases) is found, in humans, on the short arm of

chromosome 2;it has 28 introns and 29 exons,with exons 26 and 29 coding for almost 66% of the protein (72).The 4563 amino acid sequence of the protein has been determined from cDNA clones (73,74) and its mRNA also codes for a 27 amino acid signal sequence (75).Post-transcriptional insertion of a stop codon in the mRNA or its precursor gives rise to apo B48,a truncated form of apo B100 containing the N-terminal 2152 amino acids of the protein (76).In humans,apo B48 is made exclusively by intestinal cells whilst apo B100 is made by the liver;in the rat,the liver makes both apo B100 and apo B48 (76).Studies in humans show that the foetal intestine makes both forms of the apo B;as gestation continues the relative proportion of apo B48 increases until the adult gut makes only apo B48 (77).Recent studies have shown that thyroid hormone induces the production of apo B48 (78).The livers of hyperthyroid rats synthesize almost no apo B100 and thus the hormone is directly related to the induction of the stop codon.

Apo B100 has a molecular mass of 512 kdal.It runs anomalously on SDS-polyacrylamide gels probably because it is a glycoprotein.At the N terminal is a cysteine-rich region that is highly disulphide-bonded,probably giving it a globular structure (73,74).Alternating hydrophobic/hydrophilic regions (the hydrophilic being beta-sheet structures) along the length of the protein are probably essential to the interaction of the protein with the lipids in the VLDL (79).The hydrophobic regions are very similar in structure to membrane-spanning regions in other proteins (75).Further regions of amphipathic alpha-helixes and beta-sheets are probably also involved in the binding of the protein to the lipoprotein (75).There is a region at the C terminus that shows homology to the receptor-binding domain of apo E and thus is probably the area involved in binding of LDL-bound apo B to LDL receptors (73,74,79).Apo B is cotranslationally N-glycosylated with a high proportion of the

carbohydrates in the high-mannose form. There is a clustering of glycosylation sites between amino acids 2900 and 3500, and the oligosaccharide chains are processed while moving through the Golgi apparatus, so that the molecular mass of apo B changes as it moves through the secretory pathway (80,81). There are two lysine residues at position 1297 and 3249 which are susceptible to cleavage by thrombin and kallikrein (82). Thrombin cleaves preferentially at lys 3249 to give the large N terminal region called T1 and the smaller C terminal T2 fragment. T1 is cleaved upon further exposure to thrombin at lys 1297, to give the N terminal fragment T4 and internal fragment T3. Kallikrein cleaves first at lys 1297, and then at lys 3249. Apo B has also been reported to be fatty acylated (83), while phosphorylation of the protein has been shown by some investigators (84,85).

About ten minutes is required for the translation of an apo B100 molecule, but a further 30 to 35 minutes elapse before the protein is secreted by liver cells, associated with mature VLDL particles (75). In the case of most secretory proteins, the translation of the signal sequence (usually hydrophobic in nature) facilitates a mechanism by which the protein is translocated across the membrane of the ER and into the lumen (86). This process has been considered to be continuous: that is to say that the translocation of the protein across the membrane is coupled to translation and is an ongoing process (86). Unlike soluble secretory proteins, apo B is unable to be extruded into the lumen of the rough ER. Therefore following its translation, the protein probably becomes woven into or through the rough ER membrane in a way different from that in which it eventually associates with the VLDL particles (75). Recent studies have suggested that apo B translocation across the ER membrane happens in a "stop-start" manner (87): a short section of newly synthesized chain becomes translocated into the lumen of the ER, translocation is halted while further chain elongation takes place followed by

translocation of further regions of the nascent chain at the same or at different places on the ER membrane (88).Sections of the protein become integrated into the ER membrane and either span the entire membrane (88) or become woven into the luminal aspect of it (75) in such a way that the protein is protected from proteolysis,as shown in Fig. 1.2.This explains why careful investigation of the first ten minutes of apo B synthesis have shown numerous low molecular weight proteins,immunoprecipitable with anti-apo B antibodies,to be synthesized in a "stop-start" manner until finally after ten minutes the completed apo B appears (89).It is unclear whether bulk membrane flow moves the protein within the RER or whether it is specifically packaged into vesicles.At some point,probably at the junction between smooth and rough ER,the apo B buds off from the membrane in association with sufficient lipid for this to be possible,forming nascent VLDL particles.This has been proposed to occur in a pre-Golgi assembly compartment which contains such VLDL particles (90).

In the Golgi apparatus,complex carbohydrate modification of the apo B takes place.A substantial fraction of the phospholipid is added to the particles at this stage (91,92).It has been shown by Vance and co-workers that VLDL secretion requires the active synthesis of phospatidylcholine in the Golgi apparatus and that it is the choline head group that is specifically required (93,94).It is difficult to visualize the addition of so much new surface material to the VLDL particles so late in their transit through the secretory pathway.There is the possibility that phosphatidylethanolamine (which is abundant in ER membranes) makes up the surface phospholipid in the nascent particles,and that in the Golgi apparatus phosphatidylethanolamine and phosphatidylcholine exchange takes place.Apo E and other apoproteins may also be added in the Golgi,where further cleavage

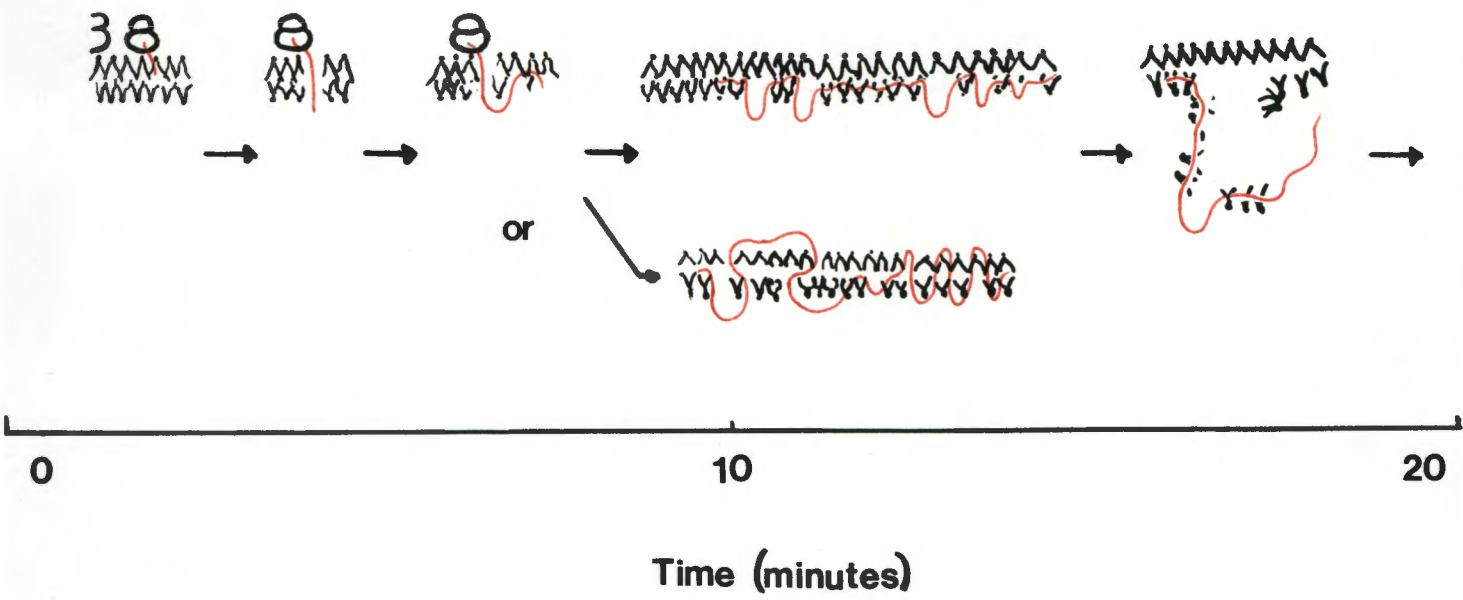

Figure 1.2 Apo B translocation across the ER membrane
Apo B transcription takes about ten minutes during which time regions of the protein are thought to be translocated across the ER membrane in a "stop-start" manner. The protein (red) either spans the membrane or becomes woven into the luminal aspect of the ER membrane (black) and is moved towards the smooth and rough ER junction where it becomes associated with sufficient lipid to be freed from the membrane, as a nascent lipoprotein particle.

and tidying-up of the VLDL takes place,before the mature particle becomes packaged into a secretory vesicle and is secreted (68).While a study by Morré using electron microscopy has suggested that an alternative pathway of VLDL secretion that by-passes the Golgi may exist, there has been no further evidence to support these findings (95).

There is presently great controversy concerning the rate-limiting step in the assembly of VLDL particles.Since the assembly of the particles is a multi-step process (see Fig. 1.3),one of the steps in the assembly should be rate-limiting.In rat hepatocytes,movement from the ER appears to be rate-limiting (96) while in cultured liver cells prepared from oestrogen-induced chicks,the transit through the Golgi may be the rate-limiting step (97).It has been suggested by others investigating Hep G2 cells,that a large pool of apo B in a membrane-bound "preassembly" pool exists and that the transfer of apo B from the membrane to the lumen is likely to be the rate-limiting step in its secretion;thus the initial assembly compartment in human Hep G2 cells constitutes the limiting process (90,98).Unfortunately,each of these studies made use of different species and different fractionation systems for preparing intracellular organelles.

Apo B remains membrane-bound until it is associated with sufficient lipid to become luminal.There have been some studies in which rough and smooth ER and Golgi fractions have been prepared and the proportion of membrane-bound and luminal apo B in each fraction has been measured (sodium carbonate-mediated release of the contents of the microsomes made these studies possible (99,100,101)).Even in the rough and smooth ER,apo B was present in a form that was no longer membrane-associated while in the Golgi, membrane-bound apo B was still present (97,102).Endosomal contributions to the microsomal apo B

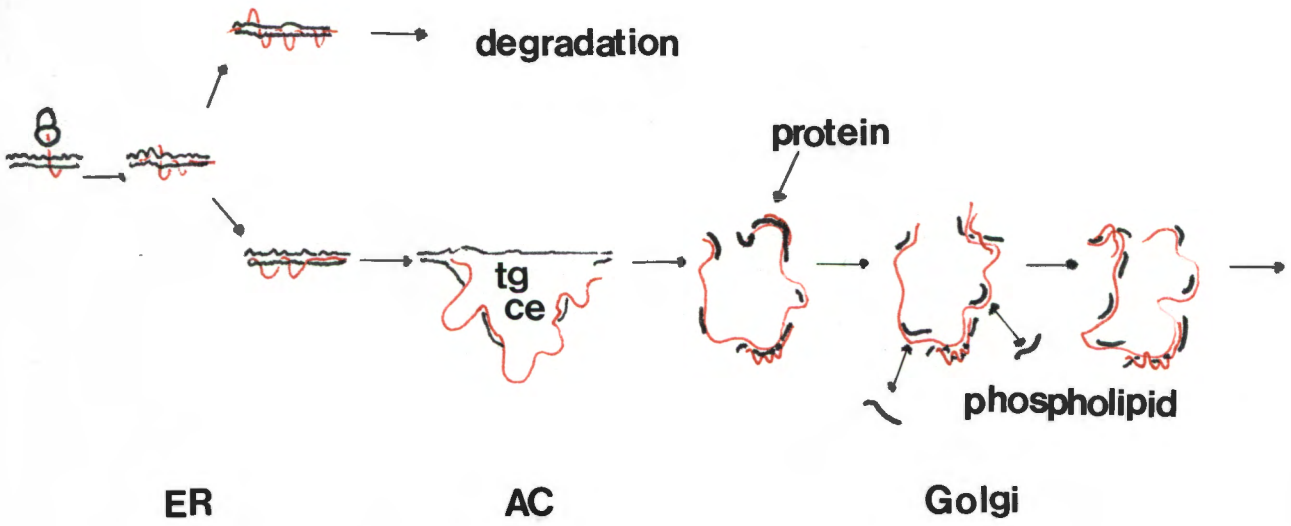

Figure1.3 The assembly of VLDL particles

The assembly of VLDL particles is a multi-step process. Apo B synthesis is associated with its translocation across the ER membrane where it is separated into two pools: one is degraded and the other becomes associated with lipid at the junction of the smooth and rough ER in a putative assembly compartment (AC). The nascent particles move into the Golgi apparatus where addition and modification of the apoproteins takes place and phospholipid addition or exchange occurs.

fraction were shown to be minimal; thus little re-endocytosis of secreted apo B occurs in such systems (103). Unfortunately, in these studies as in others, the methods of subcellular fractionation were probably not sufficiently efficient to completely separate the different microsomal subfractions, and contamination of the fractions means that the results were difficult to interpret in the light of the other data already collected. Gibbons, in a recent review of VLDL secretion has interpreted these data in a different manner, however (104). If apo B conformation becomes changed as the various modifications and lipid additions occur (in the ER and Golgi complex), then it might be forced to bind to the microsomal membrane in a transient fashion while triglyceride or phospholipid additions were happening. This means that whether apo B was membrane-bound or luminal would be no indication of where in the secretory pathway it was; it also suggests that triglyceride synthesis and addition might take place in the Golgi as well as in the ER - postulates which are very different from the general model presented above.

The question as to whether some molecules of apo B are degraded during their intracellular processing and if so, where this occurs, hold a possible key to solving the problem of the regulation of apo B synthesis. Controversy reigns in this area. Studies using Hep G2 cells have shown that under various conditions the rate of apo B secretion can undergo an increase or decrease of up to four-fold in either direction (105, 106, 107). Under these conditions, the cellular content of apo B mRNA remained constant; the mRNA was shown to be very stable and to have a half life of 16 hours (107). It was accordingly concluded that in Hep G2 cells, while the apo B gene was constitutively expressed, variable degradation of the protein was responsible for changes in the amounts of apo B secreted under different conditions (107). It has subsequently been shown that apo B in Hep G2 cells undergoes posttranslational degradation at a site not

yet identified (98). In cultured rat hepatocytes subjected to pulse-chase protocols, up to 40% of the synthesized apo B100 was degraded and the figure was 50% for apo B48 while albumin was not degraded at all (96). In another study on rat hepatocytes in culture, apo B degradation was stimulated by the presence of physiological concentrations of insulin (108). The degraded apo B was apparently separated from the secreted proteins in the ER (87). In perfused rat livers not all of the apo B that was synthesized was secreted on a nett basis; it seemed to be recycled in the liver and not to be degraded (109). In cultured oestrogen-induced chick hepatocytes, no degradation of apo B could be determined (97).

The observations concerning apo B integration and translocation across the ER membrane gave rise to further investigation which showed that there were two distinct pools of apo B in rat hepatocytes: one pool was integrated with, and translocated across, the ER membrane and finally secreted with mature VLDL particles, while proteins in the other pool were retained in the ER membrane thus targeting them for degradation - the specific degradation site remains to be identified (87). Apo B molecules that were targeted for degradation were accessible to trypsin when ER vesicles were treated with the enzyme, the molecules remained accessible to trypsin during a pulse-chase experiment. Apo B has been shown to be phosphorylated and it could be speculated that phosphorylation might target a particular apo B molecule for degradation thus preventing its translocation across the membrane. There is a possibility that apo B48 plays a role in determining the fate of both apo B100 and apo B48. Apo B48 appears to be phosphorylated more readily than apo B100 (84) and hepatocytes that were prepared from fasted rats displayed increased "editing" of apo B: apo B48 was increased as a proportion of the total apo B species, there was more degradation

of apo B100 in these hepatocytes than in cells from control animals (discussion with those who attended the Gordon conference 1990). If apo B48 does play a role in apo B100 degradation then it is possible that apo B degradation in humans and rats differ since human livers do not normally synthesize apo B48.

1.5.3 Factors affecting VLDL synthesis

The number of VLDL particles synthesized and secreted, and the size and composition of the particle population are influenced by diet, hormonal and other environmental factors (for review see 68). The genetic make-up of the individual also plays an important role. Thus while generalizations can be made concerning the effects of a particular diet or hormone when compared with the "basal situation" in a population, specific individuals may fail to conform. It is the question of factors such as these that have made the integration of information from numerous studies using animals models or human subjects so difficult. Investigations using small animal models usually make use of one of two approaches: a set of animals may be pretreated with a diet or hormone for a period of time after which the animals are sacrificed and the liver investigated; alternatively an untreated set of animals may be used and the modifications made, in vitro, by the addition of various agents to cell systems.

The links between diet and hypertriglyeridaemia have been recognized for many years; numerous studies have shown that an increased intake of carbohydrate or lipid foods results in raised serum triglyceride levels (68, 110). Such increases could reflect elevated plasma concentrations of chylomicrons and of chylomicron remnants, or they could be caused by increased VLDL concentrations. In normally functioning individuals, rapid chylomicron clearance prevents the build-up of chylomicron-associated triglycerides in the plasma under

subsequent fasting conditions.Under most circumstances,it is therefore an increased production of VLDL,or a poor clearance of these particles which is associated with diet-related hypertriglyceridaemia.There are two mechanisms by which triglyceride output by the liver can be increased:by increasing the number of VLDL particles secreted from the cells (and thus increasing the number apo B molecules secreted) or by increasing the size of the particles so that the cores contain more triglyceride (68).

Fasting lowers the hepatic synthesis and secretion of VLDL (111).Hepatocytes from fasted rats secreted only 20% of the triglyceride that was produced by cells prepared from chow-fed animals;the secretion of apo B100 was unchanged but apo B48 secretion was reduced by 50% (112).The activities of the lipogenic enzymes acetyl-CoA carboxylase,fatty acid synthase,glucose-6- phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were decreased by fasting (113);while the refeeding of carbohydrate to fasted animals resulted in the intracellular accumulation of triglycerides with an apparent block in secretion,probably maintained until nutritional equilibrium was reattained (112,114).

Prolonged carbohydrate feeding was associated with raised serum triglyceride levels and an increase in VLDL secretion by hepatocytes prepared from treated animals (68,115 - 120).The type of carbohydrate used - glucose or fructose - affected the response of the hepatocytes (115,118):fructose feeding gave a more marked hypertriglyceridaemia than did glucose feeding,but the composition of the VLDL particles was similar in the case of both diets.The rate of removal of plasma VLDL particles,in animals previously fed glucose or fructose,was faster than in normal chow-fed individuals.Carbohydrate feeding caused an increase in both the number of VLDL particles secreted as well as

the enrichment of these particles with triglycerides (121). The in vivo alterations in VLDL composition and number brought about by carbohydrate feeding have not only structural but also functional implications: that is that the changed compositions were associated with altered rates of removal of particles from the plasma, which was probably caused by altered rates of lipoprotein lipase-catalysed hydrolysis of the VLDL particles and possibly changes in the receptor-dependent uptake of particles from the plasma.

Addition of free fatty acids to cultured hepatocytes caused an increase in the triglyceride content of the VLDL particles but did not change the number of particles secreted (122, 123, 124). In perfused livers, the infusion of oleate resulted in increased apo B and triglyceride secretion (125). In the case of cultured Hep G2 cells, the addition of albumin-bound free fatty acids to the incubation medium caused an increase, in one set of studies, in the secretion of apo B and triglyceride (38, 126, 127) but in another study it was associated with an increase in triglycerides secreted (128). Fatty acid chain length modulated the increase in triglyceride secretion (68), while the extent of fatty acid saturation was directly proportional to the induction of VLDL synthesis (129). This effect has been seen most strikingly in the suppression that fish oils (very unsaturated) exert on VLDL production (130, 131). Sunflower oil, when compared with lard (a saturated animal fat), caused much reduced VLDL secretion (129). The composition of VLDL particles also changed depending upon the extent of saturation of the dietary fat (124), and since this factor affects the function of VLDL in carbohydrate-fed animals, this may explain why dietary unsaturated fats generally have a "lipid lowering" action. Kalopissis found that hepatocytes prepared from rats fed a high saturated fat diet (71% calories lard) showed marked reduction in VLDL synthesis, but it was

suggested that this was due to inhibition of hepatic lipogenesis by the unphysiologically high dietary intake of total fat (132,133).

A high intake of dietary cholesterol alone had no effect on rat hepatic apo B or apo E synthesis and secretion,but it increased serum apo B concentrations and decreased that of apo E (134).Rabbit hepatocytes loaded with cholesterol derived from the uptake of β-VLDL (a plasma lipoprotein species produced in animals overfed cholesterol) showed increased apo B and apo E secretion,but apo B mRNA was unchanged (135).Bile acid synthesis, a pathway for cholesterol excretion,was increased in hepatocytes from rats fed a diet containing 1% cholesterol (136,137,138).

The effects of dietary fat saturation and cholesterol on hepatic LDL receptors has been extensively studied by Dietschy and co-workers (12-15,139,140).Not only do these factors influence the secretion of VLDL by the liver,but they also alter the number and possibly the behaviour of the LDL receptors on the hepatocyte membranes.For example,receptor-dependent uptake of LDL by the liver was reduced by the feeding of cholesterol-containing diets.The effect of the cholesterol was enhanced by the feeding of saturated fat with the cholesterol,but diminished by unsaturated fat (14).Feeding of saturated fat alone was found to affect only the LDL concentration in the plasma but not receptor-dependent LDL transport (15).The long-chain saturated fats had a more pronounced effect on receptor-dependent LDL uptake in the presence of cholesterol,than did short chain saturated fat (15).Fat saturation appeared to affect the number of LDL receptors but not the affinity of the receptors for LDL (141).In monocytes,cis-unsaturated fatty acids changed the pysical properties of the plasma membranes which was proposed to be associated with increased clearance of LDL (141).This was also suggested by results obtained from monkeys fed saturated fatty

acid-rich diets (142).

Amongst the hormones that regulate VLDL synthesis and secretion are the oestrogens, thyroid hormones, glucagon and insulin. VLDL secretion in women fluctuates with their menstrual cycle. In the case of female rats, perfused livers secreted more VLDL per unit mass than did those from male rats (68). The VLDL particles secreted by livers from female rats were larger and more enriched with triglycerides than were those secreted by livers from male animals. Oestrogen-treated chickens have been used by Lane to investigate VLDL metabolism (80,81,97,143) and in this species the hormone causes increased VLDL and apo B synthesis and secretion. Apo AI is unaffected by oestrogen treatment but the avian apoprotein apo-II is also induced.

Thyroid hormone induced a change in the proportion of apo B48 synthesized by rat livers, apparently by the induction of the "stop codon" mechanism for mRNA editing (77); reduced VLDL triglyceride secretion also resulted (68). Hepatocytes from rats rendered hypothyroid exhibited increased rates of VLDL triglyceride, apo B and apo E secretion (144). Glucagon treatment in perfused livers, like thyroid hormone *in vivo*, exercised an inhibitory effect on the secretion of VLDL by hepatocytes (145,146). In the light of the role that glucagon plays in carbohydrate metabolism this result is not unexpected.

The role that insulin plays in the regulation of VLDL synthesis and secretion is an area of controversy. The hormone has been shown both to stimulate VLDL production (147,148) and to inhibit it (108,146,149). It is difficult to be sure what physiological concentrations of insulin might be in such experiments, especially in the light of the regular fluctuations in insulin levels that occur *in vivo*. Two experimental model systems - streptozotcin-diabetic rats, and genetically obese Zucker rats - both of which have impaired insulin responses, have been used to

investigate insulin effects.

Genetically obese Zucker rats are members of a mutant strain that is obese and hyperinsulinaemic (150). In these animals, the rate of hepatic VLDL triglyceride secretion is twice that of normal animals (151). The livers show no elevation of the rate of apo B synthesis and thus the triglyceride content, and not the number, of the plasma particles, is increased (150). Diabetes can be induced in rats and hamsters by injection with streptozotocin (152); in such animals, VLDL triglyceride, cholesterol and apoprotein secretion was reduced by 60%, 70% and 66%, respectively (153, 154). The results from both of these systems thus support the notion that hepatic VLDL synthesis and secretion requires the presence of insulin, but insulin is also implicated in the degradation of apo B (108) - the dose-respone curves for these events should thus be elucidated.

A further use of streptozotocin-induced rats has been in the study of the phosphorylation of apo B. Davis has reported that apo B48 and possibly apo B100 from cultured rat hepatocytes undergo phosphorylation (84). The apo B48 molecules were secreted in the phosphorylated form and contained phosphorylated serine residues (84). Sparks and Sparks showed later that in hepatocytes prepared from streptozotocin-induced diabetic rats, while there was a reduction in overall secretion of apo B, the secreted apo B was more heavily phosphorylated when compared to normal animals (85). Furthermore, both forms of the apoprotein were phosphorylated and there were a significant number of phosphotyrosine residues; control animals showed only phosphoserine residues (85). Since apo B48 was more readily phosphorylated than apo B100, it is interesting to speculate on the role that thyroid hormones might play in this phenomenon. Furthermore, there is the possibility that extensive apo B phosphorylation may be particularly characteristic of rodents.

1.6 Small Animal Models

1.6.1 Rats

Rats have been used extensively for the preparation of isolated hepatocytes, most commonly from animals of the Sprague-Dawley strain. Unfortunately, rats are not suitable models for the development of atherosclerosis to which they are extraordinarily resistant. This is not to say, however, that rats do not respond at all to metabolic challenges of a cholesterol and lipid-rich "western" diet. Rats have been described by Dietschy as "HDL" animals (155). Normally, their rate of cholesterol synthesis is high and they respond by suppression of this process to an increased cholesterol intake, never exhibiting hyper-LDLaemia or atherosclerosis. Rat livers also differ from those of humans in their synthesis and secretion of both apo B48 and apo B100, which may or may not be a factor in the development of coronary artery disease.

1.6.2 Golden Syrian Hamsters

The Golden Syrian Hamster (Mesocricetus auratus Waterhouse) was first described in 1797 by a physician working in Aleppo in Syria who took it to be the same as the European hamster (156). It was not until 1839 that George Waterhouse presented it as a new species to the London Zoological Society. It was described as "....this species is less than the common Hamster (Cricetus vulgarus) and is remarkable for its deep golden yellow colouring. The fur is moderately long and very soft, and has a silk-like gloss; the deep gold yellow colouring extends over the upper parts and sides of the head and body, and also over the outer side of the limbs: on the back the hairs are brownish at the tip, hence in this part the fur assumes a deeper hue than on the

sides of the body: the sides of the muzzle, throat, and upper parts of the body are white but faintly tinted with yellow: on the back, and sides of the body, all hairs are of a deep grey or lead colour at the base. The feet and tail are white. The ears are of moderate size, furnished extensively with deep golden-coloured hairs, and internally with whitish hairs. The moustaches consist of black and white hairs intermixed..."

In 1930, Saul Adler, a parasitologist in Jerusalem, decided to try to capture and breed the animals because he could not breed Chinese hamsters. After much trouble - animals escaping or hurting each other in ruthless antisocial behaviour - three males and one female reached his laboratory: it was from these four animals that much of the world's population - both laboratory and domestic - stems (156).

Hamsters show some interesting feeding patterns that are important when studying their response to different diets. Hamsters eat every two hours, and if fasted do not compensate for the lost food when they are refed (157). This means that they rapidly become emaciated under conditions of food deprivation. Their natural measure of calorie intake is very accurate and they control this precisely (158-161). The ingestion of a high fat diet results in unusual weight gain even though the total calorie intake is unchanged or lowered (157). Lipid metabolism follows the patterns observed for hibernating animals - as the length of day decreases so the synthesis of stored lipids increases (157).

Hamsters have been suggested as a good small-animal model for the study of lipoprotein metabolism (155, 162, 163). Dietschy and co-workers showed that the response of hamster receptor-dependent LDL transport was very similar to that hypothesized for the human (155). In addition, atherosclerotic plaques have been induced in

hamsters by a diet containing 3% cholesterol and 15% saturated fat (162). Cholesterol does not accumulate in the liver under these conditions but there is substantial hypercholesterolaemia (162,163). Apo AI and apo B from hamsters have been characterized: while the molecular weight of apo AI is the same as in the human, it contains a higher threonine- to-serine ratio and has a higher content of methionine and leucine than does human apo AI (164). Apo B in turn contains more methionine and valine and has a higher ratio of aspartic acid to glutamic acid than does the human form. It also appears to consist of three major forms of differing molecular masses, the largest being 635 kdal, the second 305 kdal (which could correspond to apo B48, or to a product of proteolysis) and a third with molecular mass of 460 kdal. The anomalous behaviour of apo B on SDS gels could also be an explanation for the vastly differing molecular masses. Hamsters fed a diet containing 2% cholesterol and 5% lard developed raised serum cholesterol, triglyceride, LDL and HDL concentrations and their plasma apo B content was increased 5 times (164).

Hamster hepatocytes have been compared with those from rats, mice and rabbits (165); they are more difficult to culture than the others, having the lowest 24 hour survival rate. Conditions under which their survival can be improved, have been described (165).

1.6.3 Other models

Mice, Guinea pigs, gerbils and hedgehogs have all been used to study lipoprotein metabolism and the effects of various drugs. Mice and gerbils respond poorly to drugs used in the regulation of lipoprotein synthesis while hedgehogs show great seasonal variations in cholesterol metabolism (166). Rabbits, hares, dogs and non-human primates have also been widely used, but these animals are larger, more difficult to handle and much more

expensive than the small rodents.

1.7 Summary

In an attempt to mimic diet-induced changes in human lipoprotein metabolism, hamsters appear to be a very good small-animal model. Provided that the subtle ways in which they differ from humans are bourne in mind, experimentation using hamsters should be helpful in elucidating mechanisms by which diets regulate lipoprotein metabolism. The tools with which to study the role that the liver plays in these processes have been discussed. Since *in vivo* studies of intact animals, and isolated perfused livers are expensive to undertake and provide few controls or duplicates, liver slices and freshly prepared hepatocytes maintained under tissue culture conditions or as single cell suspensions were selected for the present study.

The study set out to characterize protein synthesis in liver slices and hepatocytes prepared from male hamsters and then to study lipoprotein metabolism in male hamsters fed diets considered to be atherogenic in humans. The data was to be used to compare isolated hamster hepatocytes in fresh suspensions with those maintained under tissue culture conditions. Isolated hamster hepatocytes were also to be compared to liver slices prepared from similar animals.

Chapter 2: CHARACTERIZATION OF ISOLATED HEPATOCYTES

2.1 Materials and Methods

2.1.1 Materials

Acrylamide and Bis-acrylamide used in the preparation of SDS polyacrylamide gels were purchased from ICN Biomedicals (Cleveland,USA) while Amplify used for enhancing gels for fluorography was obtained from Amersham (Buckinghamshire,England). Aprotinin (trasylol) was from Novo Industries (pharmaceuticals) (Pty) Ltd. (Johannesburg,South Africa). Anti-human serum albumin antibodies were bought from Calbiochem (La Jolla,USA). Azide was from BDH chemicals Ltd. (Poole,England). Benzamidine was purchased from Sigma Chemical Co. (St. Louis,USA). Betadine was from Biocide (Cape Town,South Africa) and B-mecaptoethanol was purchased from E. Merck .A.G. (Darmstadt,West Germany). BSA,fatty acid free,Fraction V and Cab-O-Sil (fumed silica gel) were from Sigma. Chloramphenicol was obtained from Warner Lambert S.A. (Retreat,South Africa). Colchicine and Collagenase,type IV from *Clostridium histolyticum* were from Sigma. Coomassie brilliant blue R25 stain was obtained from E. Merck and cycloheximide was from Sigma. FCS (a number of batches) was obtained from the State Vaccine Institute (Cape Town,South Africa) and was heat-inactivated at 56°C for 30 minutes before being used. Fungizone was from Squibb Laboratories (Pty) Ltd. (Isando,South Africa) and Gentamycin was purchased from Scherag (Pty) Ltd. (Isando,South Africa). 2-H^3-glycerol and 9,10(n)-H^3-oleic acid were obtained from Amersham and H^3-l-leucine was from New England Nuclear (Boston,USA). HEPES was purchased from Sigma. Insulin (actrapid) was from Novo Industries and Kodak X-Omat film was purchased from Sigma. Leucine-free MEM was from Gibco Ltd. (Paisley,Scotland) while MEM was from Flow Laboratories (Ayrshire,Scotland). Methionine-free MEM was

purchased from Highveld Biologicals (Pty) Ltd. (Kelvin,South Africa).NADH was from Sigma.OSAN (anti-human LDL apo B antibodies) was purchased from Behring (Marburg,France) and was heat inactivated at 56°C for 30 minutes before being used. Penicillin G was from Novo industries and Percoll was purchased from Pharmacia fine chemicals (Uppsala,Sweden).PMSF and Protein-A-Sepharose CL4B were from Sigma.Primaria tissue culture flasks and dishes were purchased from Falcon Labware (Oxnard,USA).Rainbow molecular weight markers were obtained from Amersham.The sonicator was from Heat systems Ultrasonics inc.(model W385) and sterile surgical cannulae were from Sabax (Johannesburg,South Africa).Streptomycin sulphate was from Novo Industries while T3 (Tertroxin) was purchased from Glaxo (Pty) Ltd. (Wadeville,South Africa).TCA was from E.Merck and TLC polyester-silica gel plates were obtained from Sigma.Trans S^{35}-label was used as the source of S^{35}-l-methionine and contained 70% S^{35}-methionine, 20% S^{35}-cysteine, 7% S^{35}-methionine sulphoxide and 3% other labelled S^{35} compounds,it was obtained from ICN Radiochemicals (Irvine,USA).Triton-X 100 was from BDH and Trypan blue stain was from E.Merck.Tunicamycin was purchased from Calbiochem and Whatman GF/C glass filter disks were from Whatman Int.Ltd. (Maidstone,England).

All other reagents were analar grade and were of the highest chemical quality available.

2.1.2 Animals

Male Golden Syrian Hamsters (weighing 120-200 g) were maintained on a twelve hour dark,twelve hour light cycle,with *ad libitum* access to water, standard rat chow and sunflower seeds.Half an hour prior to sacrifice the food was removed - sacrifice was undertaken in the middle of the dark cycle.

2.1.3 Preparation of hepatocytes

The hepatocytes were prepared using a modification of the method described by Seglen (18,28).The solutions and instruments used for the preparation of cultured cells were all sterilized by autoclaving or immersion in 70% (v/v) ethanol.Each animal was anesthetized with ether and then weighed and washed in warm water and betadine.It was secured,ventral side upwards,on a dissection board,and the exposed area of the abdomen was saturated with 70% ethanol.The liver was exposed and the inferior vena cava was cannulated just below the liver,and ligated above but as close to the diaphragm as possible.The portal vein was cut to allow efflux of the buffers.Pre-perfusion buffer (150 mM NaCl, 6.7 mM KCl,1,5 mM EDTA, 10 mM HEPES, pH 7.4 at 37°C)was pumped through the liver at low speed (0 - 5 ml/min),gradually increasing the rate over the following 8 minutes.The liver began to blanch almost immediately and became quite swollen.

While the pre-perfusion was taking place the liver was carefully cut free of all the connective tissues,and once it was fully blanched the pump was switched off to change to the perfusion buffer (66 mM NaCl, 6.7 mM KCl, 5 mM $CaCl_2$, 100 mM HEPES,0.05% (w/v) collagenase, pH 7.6).The liver was then transferred to a gauze bed in a recirculating system while the perfusion buffer was pumped through it.An infrared lamp was placed some distance from the liver to warm the isolated organ.After 5 - 8 minutes, when the liver had lost its swollen appearance and looked as though it was filled with air bubbles,the perfusion was halted.The gall bladder was then carefully removed with the cannula and any other debris,and the liver was placed in a beaker of ice-cold washing buffer (150 mM NaCl, 6.7 mM KCl, 12mM $CaCl_2$, 10 mM HEPES, 5% (v/v)FCS, pH 7.4).It was gently cut with scissors and the cells were dispersed by careful shaking using forceps.The cells were filtered through a nylon gauze and then harvested by

means of a low-speed spin in a Beckman centrifuge (250 X g for 45 sec.).The cells were washed thrice,and then finally resuspended in the medium in which the incubation or culture was to take place.Viability was tested by microscope using trypan blue (0.01%(w/v)for 1 minute).

2.1.4. Cultured Hepatocytes

Hepatocytes to be cultured were finally resuspended in commercially available MEM containing 10% (v/v)FCS and Penicillin (100 U/ml),Streptomycin (20 µg/ml) and Fungizone (0.5 µg/ml).They were counted using a coulter counter and the cell density was adjusted to give 10^6 cells/ml.They were plated on Primaria Tissue culture dishes and placed in the incubator.After 4 hours,the medium was replaced, after 24 hours the medium was altered to methionine-free MEM and experiments were begun.

2.1.5 Suspended Hepatocytes

Cells were retained without shaking at room temperature for 30 minutes to recover,prior to their final wash.They were then resuspended in Krebs-Ringer bicarbonate buffer (118.5 mM NaCl, 4.47 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.88 mM $NaHCO_3$, 20 mM HEPES, 2.5 mM $CaCl_2$, 10 mM glucose, 2.5 mM sodium lactate, 100 U/ml Penicillin, 100 µg/ml Streptomycin, pH 7.4) or methionine-free or leucine-free MEM.The incubations were carried out in clean 10ml Erlenmeyer flasks,stoppered with a rubber bung and sealed with parafilm (following thorough gassing with 5% CO_2 and 95% O_2).The cells were incubated on a rotating shaker (75 per min) at 37°C,for the required period.Unless otherwise stated, incubation volumes were 1 ml with 1 µCi S^{35}-L-methionine added per incubation.

2.1.6 Protein Synthesis

Protein synthesis was measured by the incorporation of S^{35}-L-methionine or H^{3}-L-leucine into cellular and secreted proteins. Cells were incubated in the presence of varying amounts of the radiolabelled precursor, for various times. At the end of the incubations, the cells and medium were separated by a 1 minute high-speed spin in a microfuge. The cells were resuspended in the same volume of homogenization buffer (150 mM NaCl, 1.4 mM Na_2HPO_4, 2mM methionine, 1 mM PMSF, 1 mM benzamidine, 1 mM EDTA and 1% (v/v) Triton X-100, pH 7.0)) and sonicated on ice with 2 x 5 second bursts with the microtip set on 3. The incorporation of radiolabelled amino acids into the total intracellular or medium protein was determined by TCA precipitation (167) followed by measurement in a Packard liquid scintillation counter. One-dimensional SDS-PAGE analysis was performed (as described by Laemmli (168)) to detect the proteins synthesized and secreted by the cells. Coomassie Blue staining and enhancement with Amplify was followed by fluorography at -70°C of the dried gel using Kodak X-Omat film. The Coomassie stain identified all proteins (whether radiolabelled or not) while fluorography detected the radioactivity contained in individual proteins. Quantification of the bands on the fluorograms was done using sodium hydroxide elution according to the method of Suissa (169). Some fluorograms were scanned using a Helena scanner equipped with an integrater. Total cellular protein was measured as described by Lowry et al (170).

2.1.7 Pulse-chase protocol

In some experiments, pulse-chase protocols were set up in order to investigate turnover and secretion rates of certain proteins. Cells were incubated or cultured with radiolabelled

methionine for up to 90 minutes. The medium was then removed and the cells were washed twice, new unlabelled medium containing 10mM L-methionine (96) was added and the cells were incubated for a further period of time. Azide (10 mM, freshly prepared in KRB or MEM), colchicine (100 μM), tunicamycin (10 μg/ml) and cycloheximide (10 μM) were added to the chase medium in some experiments.

2.1.8 Quantitation of Fluorograms

Fluorograms were quantified by extraction of selected bands with sodium hydroxide (169) or by scanning the lanes with a Helena Scanner. For the extraction procedure, labelled standards were prepared by running C^{14}-casein (prepared by Dr J. Arnold in our laboratory) on a one-dimensional SDS-PAGE. The lanes were run in duplicate and there was a single band per lane. One band in each duplicate was oxidized and the radioactivity was measured in a Packard liquid scintillation counter, while the other was retained as a standard in which the exact radioactivity was recorded. Because C^{14}-casein had been used, there was no significant effect of radioactive decay on the standard, and its activity was comparable with that of S^{35} used as a radioactive tracer in the above experiments. Each dried gel was placed in an X-Ray cassette with the standard sample which was visible on the fluorogram (Fig. 2.1). Bands were cut out and weighed (to allow for a standard background subtraction) and the radioactivity in the samples was accurately determined from the known figure in the case of each standard. The standards provided the added advantage of allowing one to be sure that one was within the linear range of response of the X-ray film for all the sample bands.

In the case of media samples, the TCA-precipitable results were often spuriously high because of labelled amino acid

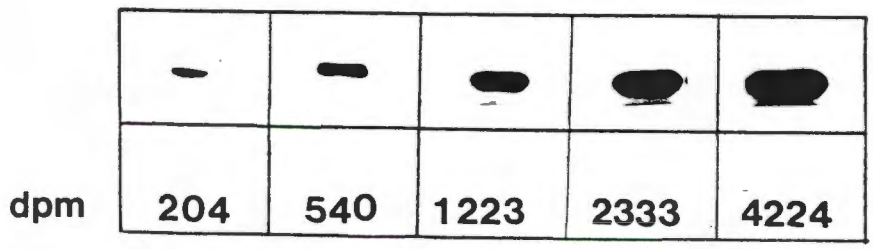

Figure 2.1 Radiolabelled standards for fluorography

C^{14}-casein was prepared by Dr J. Arnold and aliquots were run on a one dimensional 5 - 20% SDS-polyacrylamide gel, in duplicate. One band in each duplicate was oxidised and the radioactivity was measured (values indicated) in a Packard liquid scintillation counter, the other (of known radioactivity) was retained to be used as standard for fluorography (see 2.1.8). C^{14} and S^{35} have comparable energy, and the slow radioactive decay of C^{14} ensured that the standards remained valid.

contamination. Media samples were thus run on SDS-PAGE and the fluorograms were scanned to ascertain the exact percentage of the total pattern that the albumin band represented. Knowing this, the albumin band was extracted, and the total medium radioactivity could be calculated.

2.1.9 VLDL isolation

VLDL was isolated both from the cells and from the medium: cells were suspended in fractionation buffer (0.25 M sucrose in 10 mM HEPES, 1.5 mM EDTA, 1mM PMSF, 1mM benzamidine, pH 7.4) and homogenized with ten passes through an Embo-Lab cell cracker (internal diameter 8.020 mm with ball-bearing diameter 8.008 mm). The nuclei and mitochondria were sedimented by centrifugation at 10 000 x g for 5 minutes. The supernatant was then layered onto 1 ml of the fractionation buffer containing 2 M sucrose, and pelleted microsomes were prepared at 2°C by means of a high-speed spin in a Beckman Ultracentrifuge (100 000 x g for 60 minutes in an SW 40 rotor). The microsomal pellet (at the interface between the 2 M sucrose and the 0.25 M sucrose) was collected and suspended in 1M sodium carbonate to give a final concentration of 100 mM Na_2CO_3 in 2 ml with vigorous mixing. After standing for 30 minutes on ice, with further vigorous mixing thrice, the solution was neutralized with drops of 0.1 M HCl and retained for the preparation of VLDL (see below).

Human VLDL, prepared as a carrier, was added either to the medium or to the microsomal extracts (1 ml per sample) and the density was adjusted to 1.31 g/ml with KBr. The samples were placed in tubes for the SW 40 rotor, and overlayered with saline/EDTA (0.9 % NaCl (w/v), 1 mM EDTA, pH 7.4). The tubes were were centrifuged at 100 000 x g for 22 hours in a Beckman ultracentrifuge at 15°C. At the end of the spin, the milky VLDL layer at the top of the tubes

was aspirated and retained; the infranatant was also stored. Precipitation of proteins in samples of the particles was done by addition of TCA for a final concentration of 10% (w/v), on Whatman glass fibre filters by the method of Mans and Novelli (167) and SDS-PAGE analysis, followed by fluorography was performed on another aliquot of each sample.

2.1.10 Immunoprecipitation

Synthesis and secretion of Apo B and albumin was quantified by immunoprecipitation of medium and cell samples using the method described by Kessler (171) and modified by Davidson and Glickman (172). Commercially available antibodies prepared to human antigens were used (OSAN, an antibody prepared to the apo B in human LDL and an anti-human serum albumin antibody) unless otherwise stated. Cells were sonicated in homogenization buffer and the debris was removed by a 3-minute spin in the microfuge. Triton X-100 (1% v/v) was added to the medium, and the medium and cellular extracts were incubated with either 25 ul of anti-apoB or 50 ul of anti-albumin antibodies overnight at 4°C on a rotating mixer. Protein A-Sepharose was prepared in immunoprecipitation buffer (150 mM NaCl, 5 mM EDTA, 65 mM Tris-HCl, 2 mM methionine, 1% (v/v) Triton X-100, 0.1% BSA, 0.02% NaN3, pH 7.4), washed and added to the samples (10 mg per sample). After further rotation in the cold for 2 hours, the samples were centrifuged in a Beckman bench-top centrifuge at 2000 rpm. The protein A-Sepharose pellet was washed twice in immunoprecipitation buffer and a further three times in immunoprecipitation buffer prepared without BSA. In the immunoprecipitation of albumin, the BSA was substituted with casein. The final pellet was suspended in solubilizing mix (65 mM Tris-HCl, 2.5% SDS, 10% glycerol, 5% B-mercaptoethanol, pH 8.0). TCA precipitation and SDS-PAGE followed by fluorography were

carried out on the samples as described above.

2.1.11 Lactate dehydrogenase assay

Aliqots of cells and media were used for the LDH assay which was used to assess the viability of cells. To a cuvette 1 ml LDH reaction mix (100 mM Tris, 5 mM Na pyruvate, 0.2 mM NADH, pH 7.4) was added and the absorbance was read at A340. A sample of cell extract or medium (10 - 50 ul) was added to the cuvette and mixed in quickly. The absorbance was read at 30 sec intervals over the next 3 minutes at A340 and the nMoles of pyruvate reduced per minute at room temperature was calculated for each sample.

2.1.12 Lipid Synthesis

The synthesis of triglycerides, phospholipids, cholesterol and cholesterol esters was measured using the incorporation of H^3-glycerol or H^3-oleic acid. The latter was added to the cells bound to BSA. (The complex was prepared by adding 250 μCi to 11.3 mg of unlabled oleic acid in a test tube. 120 umoles of NaOH in 3.8 ml of 0.9% (w/v) NaCl was added and the solution was carefully brought to the boil and then cooled to 40°C . BSA (680 mg) was added to 6 ml 0.9% NaCl and heated to 40°C. The fatty acid solution was transferred with a heated pasteur pipette in drop-wise fashion to the BSA solution. The solution was made up to 10 ml with 0.9% saline, aliquoted and stored). Cells were suspended in MEM and the radiolabelled glycerol or oleic acid complex was added. The cells were incubated for the required time.

2.1.13 Lipid extraction

Cells and medium were separated and the lipids were extracted

using methanol/chloroform. Cells were suspended in 1.75 ml 0.9% saline and 4.4 ml 100% methanol (1.75 ml medium was added to 4.4 ml methanol). Chloroform (2.2 ml) was added and the solution was vortexed; a few drops of methanol were added if the solution was milky. The samples were then spun for 20 minutes at 2000 rpm in a Beckman bench-top centrifuge. To the supernatant fluids were added 2.5 ml chloroform and 1.5 ml $CaCl_2$ (0.04%); the solution was vortexed and then spun for 10 minutes at 2000 rpm in a Beckman bench-top ultracentrifuge. The top water layer was discarded and the lower chloroform layer was dried under nitrogen.

2.3.14 Lipid separation

The lipids were separated by TLC. Silica plates were marked and prepared. The dried lipids were spotted in chloroform and dried between applications. A standard solution was also applied which contained each of the lipid classes. The plates were developed with freshly prepared solution (150 ml hexane, 50 ml ether, 2 ml glacial acetic acid). Once it had run 15 cm the plate was allowed to air-dry and then placed, for 5 minutes, in a desiccator containing iodine crystals. The yellow bands of the standards identified the bands in the sample lanes. These were cut out, placed in scintillation fluid and counted in a Packard liquid scintillation counter.

2.2 Hepatocyte Preparation

The effects of various steps in the preparation of the hepatocytes were investigated to optimize conditions and to give the highest viability and cell yield.

2.2.1 Method of dispersion

Collagenase and/or calcium chelators have both been used for the isolation of metabolically active hepatocytes (18,21,22,23,28,45,).Preparations (using various batches of collagenase) were attempted with collagenase,EDTA or both (Table 2.1).There was considerable variation between the batches of collagenase as has been reported elsewhere (28).EDTA alone gave very low cell yields and the mechanical forces required to free the cells were such that the viability of the cells was also very low. Pre-perfusion of the liver with pre-perfusion buffer containing EDTA was helpful in releasing more cells,however,and did not adversely affect their viability.Viability was measured by trypan blue exclusion and cells were counted using a Coulter counter.

The appearance of the collagenase was a good general indicator of its performance;batch 125F-6822 was mustard in colour and fine and powder-like and was difficult to dissolve.The other batches were chocolate-brown,crystalline and dissolved readily in perfusion buffer.Batches 106F 6827/8 were used for all future experiments.

2.2.2 Percoll gradients

It has been reported that Percoll (a colloidal silica,low in viscosity and having good osmotic properties) can be used to separate viable from non-viable hepatocytes (173,174).Percoll has also been used to separate hepatocytes from other hepatic cells because of the size differences between the cells (174). Self-generating gradient centrifugation was attempted but did not give results as reported by others:the cell viability was not significantly improved and many cells were lost in the procedure.The cells were sticky and difficult to resuspend in

Table 2.1 Enzymes and chelators used to prepare hepatocytes from male hamsters.

Hepatocytes were prepared from male hamsters (as described in 2.1.3) using a number of different batches of collagenase (1 - 6). In some experiments (7,8,9) the livers were perfused with pre-perfusion buffer containing EDTA (1.5 mM) and the collagenase-containing perfusion step was omitted. In other preparations (10,11) the livers were pre-perfused with pre-perfusion buffer containing EDTA (1.5 mM), followed by perfusion with collagenase to liberate the hepatocytes. Cell number was counted using a coulter counter, and the viability of the cells was assessed with trypan blue as described in 2.1.3.

PREPARATION	YIELD [i]	VIABILITY [ii]
1 Collagenase batch 81F 6828	0.7	75
2 85F 6845	10	88
3 106F 6827	10	87
4 106F 6828	20	92
5 116F 6808	5	90
6 125F 6822	1.3	37
7 EDTA	0.64	5
8 EDTA	0.37	14
9 EDTA	1.2	8
10 Collagenase (EDTA pre-perfusion)	50	93
11 Collagenase (EDTA pre-perfusion)	42	89

i Yield is expressed as cells x 10^6

ii Viability is expressed as trypen blue-negative cells as a proportion of total cell number.

medium following this treatment.

2.3 Isolated Hepatocytes as Suspensions

2.3.1 Necessity of pre-incubation of cells

It has been reported that hepatocytes to be used in suspension require a pre-incubation period to overcome the trauma of their preparation (18). Conditions of preincubation were accordingly investigated, cell viability and protein synthesis being used to assess the effects of the treatments. Pre-incubations were carried out prior to the final washing step. Cells were kept on ice (unshaken) for up to 4 hours in KRB; the viability was assessed with trypan blue. Fig. 2.2 shows that after 2 hours on ice the viability of the cells declined significantly. Fig. 2.3 shows how preincubation under various conditions affected the incorporation of H^3-leucine into total proteins synthesized: cells that were incubated immediately incorporated less radiolabel into protein than did any of the preincubated cells. The data suggested that preincubation for half an hour at 37°C would enable cells to recover maximally, while longer than this would cause a fall in recovery. Half an hour at room temperature was almost as effective as the same period at 37°C, while one hour was the optimum time on ice. In all further experiments, the cells were retained for 30 minutes at room temperature before the final wash.

2.3.2 Problem of bacterial contamination

The possibility that a specific portion of the protein synthesized was due to contaminating bacteria was investigated by the addition of cycloheximide and chloramphenicol at various concentrations to the incubations. Cycloheximide inhibits eukaryotic protein synthesis by inhibition of the binding of the

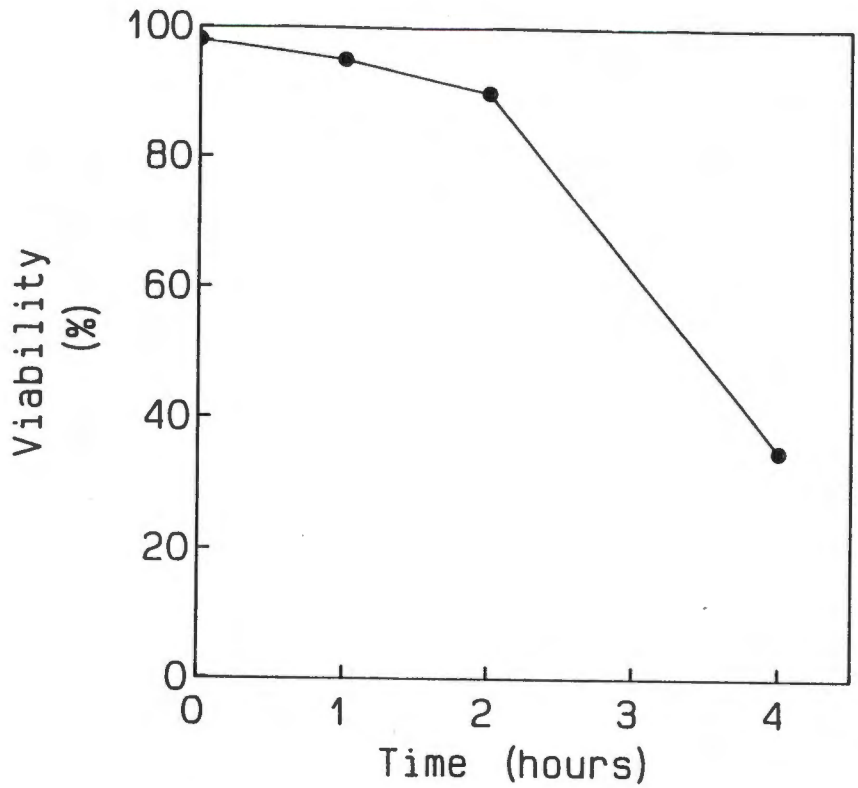

Figure 2.2 The effect of incubation at 0°C on the viability of hamster hepatocytes

Hamster hepatocytes were prepared as described in 2.1.3 and then incubated at 0°C for up to four hours in well-oxygenated MEM in stoppered 10 ml Erlenmeyer flasks. Each flask contained 2 ml MEM and 2 x 10^6 cells. The viability of the cells was assessed using trypan blue (as described in 2.1.3) at the indicated times. The viability is expressed as the trypan blue negative cells as a proportion of the total cell number. The resuls were obtained from a single representative experiment.

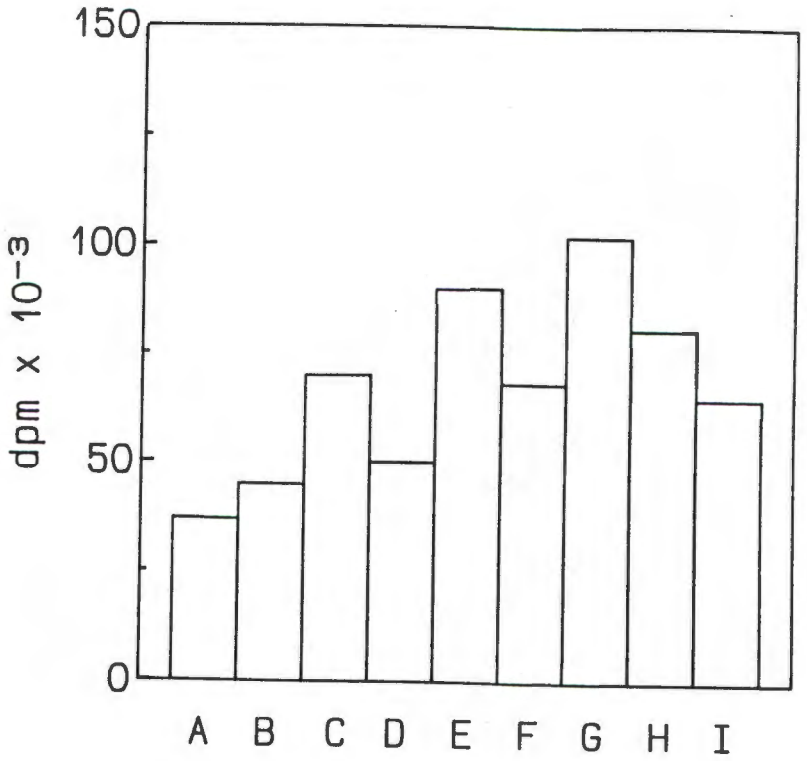

Figure 2.3 The effects of storage on protein synthesis in incubated hamster hepatocytes.

Hamster hepatocytes were prepared as described in 2.3.1. The cells (2×10^6) were placed in 2 ml of well-oxygenated MEM in stoppered 10 ml Erlenmeyer flasks and stored under various conditions: immediate incubation (A), 30 minutes at 4°C (B), 1 hour at 4°C (C), 2 hours at 4°C (D), 30 minutes at room temperature (E), 1 hour at room temperature (F), 30 minutes at 37°C (G), 1 hour at 37°C (H) and 2 hours at 37°C (I). After the appropriate storage time the cells were separated from the medium by centifugation (45 seconds at 250 x g) and resuspended in 2 ml leucine-free MEM. After gassing 2 μCi H^3-leucine was added to each flask and the stoppered flasks were incubated at 37°C for 2 hours in a shaking water bath. At the end of the period the cells and medium were sonicated as described in 2.1.6 and samples were taken for TCA-precipitation (see 2.1.6) to assess the incorporation of radiolabel into total proteins over the 2 hours. Results are expressed as dpm of incorporated radiolabel per system and are the means of three such experiments.

80S ribosomal subunit (66) and has no effect on prokaryotic protein synthesis. Chloramphenicol, on the other hand, inhibits prokaryotic and mitochondrial protein synthesis. All radiolabelled protein synthesis was of eukaryotic origin and thus carried out by the hepatocytes (Fig. 2.4).

2.3.3 Optimizing the incubation medium

The medium in which the cells were incubated was varied with respect to various components, using the incorporation of S^{35}-L-methionine into proteins to assess the effects. Seglen has extensively investigated media and additions such as antibiotics, amino acid, hormones and nutrients in the case of cultured rat hepatocytes and has shown that penicillin, streptomycin and gentamycin have no effect on protein synthesis in such cells (175). Various amino acids affect the degradation of proteins in hepatocytes (176-182) and hormones have been added in varying concentrations. The best fuel source for cultured hepatocytes is pyruvate (rather than lactate or glucose) (181, 182).

Methionine-free MEM supported the incorporation, by hamster hepatocytes, of S^{35}-methionine into total protein better than did KRB. Addition of pyruvate to the MEM gave no significant improvement, but there was a noticeable effect when cells were incubated in KRB. Cells incubated in KRB with 100 uM pyruvate incorporated less radiolabel into total protein over the 2 hour incubation period than did cells incubated in methionine-free MEM. Addition of glucose and lactate had no significant effect in any system. Gentamycin (20 µg/ml) had a significant inhibitory effect on the incorporation of radiolabelled amino acid into protein, while penicillin, fungizone and streptomycin were without any effect at the concentrations used. Table 2.2 shows the effect

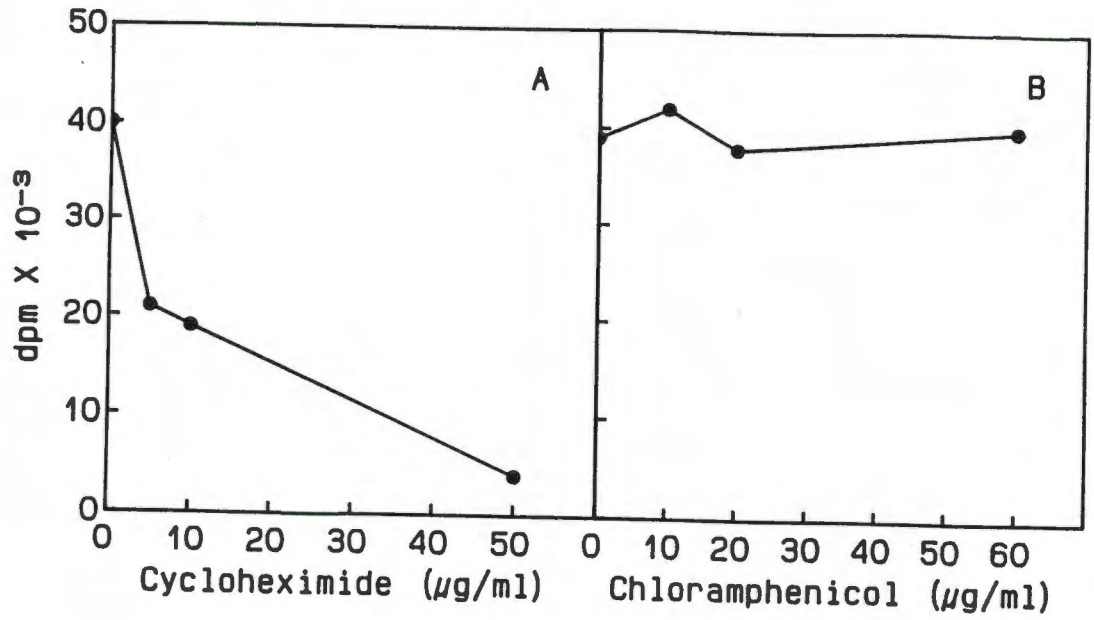

Figure 2.4 The effects of either cycloheximide or choramphenicol on total protein synthesis in incubated hamster hepatocytes.

Hamster hepatocytes were prepared as described in 2.1.3. The cells (2×10^{6}) were placed in 2 ml well-oxygenated leucine-free MEM in 10 ml Erlenmeyer flasks, 2 µCi H^{3}-leucine and the indicated concentration of either cycloheximide [panel A] or chloramphenicol [panel B] was added to each flask. Flasks were stoppered and the cells were incubated at 37°C for 2 hours in a shaking water bath. After 2 hours the cells and medium were sonicated as described in 2.1.6 and samples were taken for TCA-precipitation (section 2.1.6) to assess the synthesis of total proteins by the hepatocytes. Results are expressed as dpm of incorporated radioactivity per 2 ml system and are results obtained in a single representative experiment.

Table 2.2 The effects of different media, fuels and antibiotics on total protein synthesis in incubated hamster hepatocytes.

Hepatocytes were prepared as described in 2.1.3 and placed in either KRB buffer (1,2) or leucine-free MEM (3 - 7). Each 10 ml Erlenmeyer flask was well oxygenated and contained 2 x 10^6 cells in 2 ml medium with the indicated additional fuel or antibiotics. 2 uCi H^3-leucine was added to each flask prior to it being stoppered and incubated at 37°C in a shaking water bath for 2 hours. After 2 hours the cells and medium were sonicated as described in 2.1.6 and samples were taken for TCA-precipitation to assess the effect of each of the media upon the incorporation of radiolabel into total protein. Results are expressed as dpm of incorporated radioactivity in each system and are the means of two experiments.

	MEDIUM	ADDITION	INCORPORATION[i]
1	KRB	-	68 022
2	KRB	pyruvate	114290
3	MEM	-	127383
4	MEM	penicillin, streptomycin	126454
5	MEM	penicillin, streptomycin, fungizone	127477
6	MEM	gentamycin	36 043
7	MEM	gentamycin, fungizone	34 214

i Expressed as dpm per system.

of medium and antibiotic addition on protein synthesis by suspended hamster hepatocytes.

Insulin, glucagon and dexamethazone, or combinations of these hormones had no significant effects on total protein synthesis in suspended cells. A dose-response curve for insulin showed that at concentrations greater than 20 U/ml insulin did produce an inhibitory effect (Fig. 2.5).

The catabolic state of the cells was investigated by measuring protein degradation during a pulse-chase experiment. The rate of total protein synthesis was unaffected by the addition of amino acids (asparagine, glutamine, histidine and leucine) known to inhibit protein degradation (176-180). The rate of protein degradation was found to be between 2 and 4 %/hour, depending on the cell preparation: the cells were pulse-labelled for one hour after which the medium was removed and the cells were washed thrice. The appearance in the medium of TCA-soluble radioactivity and the disappearance of cellular TCA-precipitable radioactivity over the next 3 hours was measured in the whole system (medium + cells). In three experiments this was respectively 1.66%, 1.54%, 4.2% per hour, well within the range of published values for isolated hepatocytes (data not shown).

The effects of exogenous proteins were investigated by the addition of increasing concentrations of BSA to the medium; no differences were found (data not shown). Calcium was removed by the addition of the calcium- chelator EGTA (Fig. 2.6); this showed a requirement for calcium reflected by lowered synthesis of total protein in the presence of the chelator. pH affected the incorporation of radiolabelled amino acids into protein: the pH optimum lay between 7.2 and 7.7; on either side of these values, protein synthesis was significantly inhibited. The temperature optimum was 37°C: at 33°C and 40°C, the incorporation

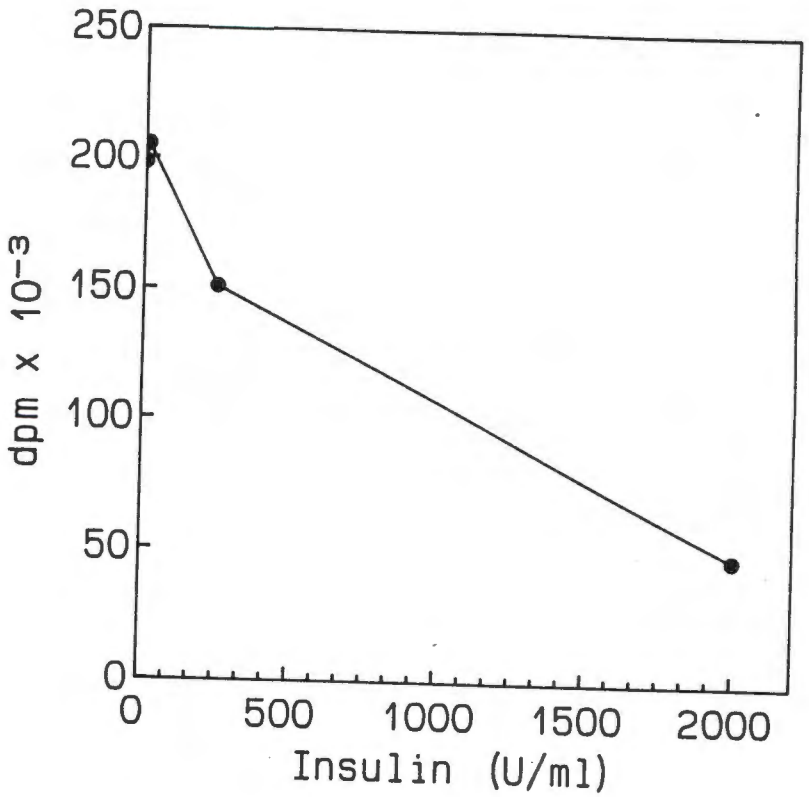

Figure 2.5 The effect of insulin on protein synthesis in incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3 and suspended in well oxygenated methionine-free MEM. Each 10 ml Erlenmeyer flask contained 2 x 10^6 cells in 2 ml medium, 2 µCi S^{35}-methionine and the indicated concentration of insulin was added prior to it being stoppered. The cells were incubated for 3 hours at 37°C in a shaking water bath. At the end of each incubation the cells and medium were sonicated as described in 2.1.6 and samples taken for TCA-precipitation to assess the effect of insulin on incorporation of radiolabelled amino acid into total proteins. Results are expressed as dpm of incorporated radioactivity per system and from are a single representative experiment.

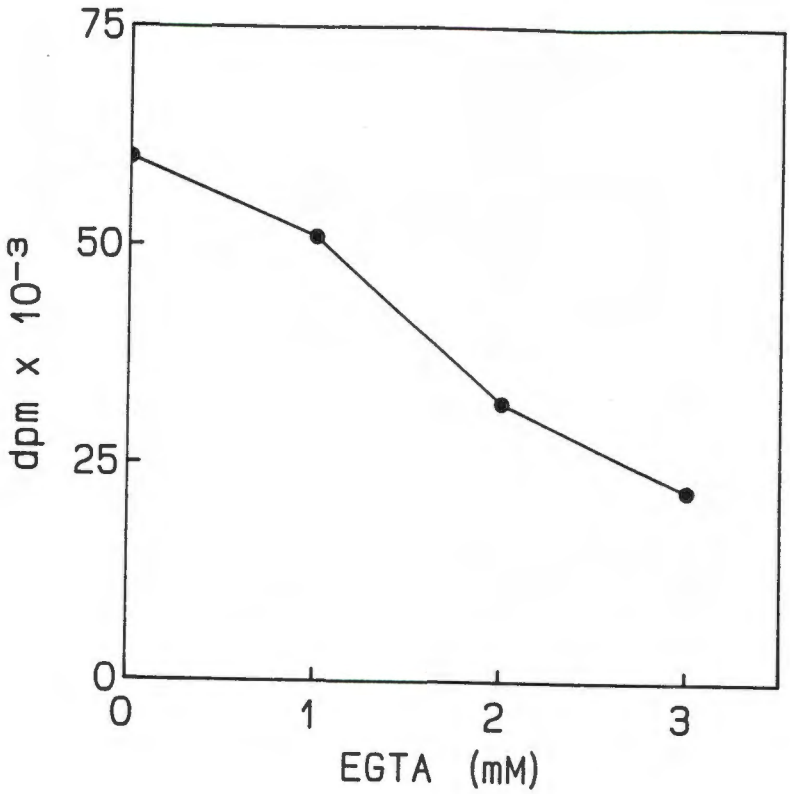

Figure 2.6 The effect of removing calcium on total protein synthesis in incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells were (2×10^6) were placed in 2 ml well-oxygenated methionine-free MEM in 10 ml Erlenmeyer flasks with the indicated concentration of the calcium chelator EGTA as well as 2 µCi S^{35}-methionine. The flasks were stoppered and the cells were incubated for 2 hours at 37°C in a shaking water bath. After 2 hours the cells and medium were sonicated as described in 2.1.6 and samples were taken for TCA-precipitation to assess the effect of calcium removal on the incorporation of radiolabelled amino acids into total proteins. The results are from a single representative experiment and are expressed as dpm of incorporated radioactivity per flask.

of radiolabelled precursors was half of the value obtained at 37°C. Shaking improved the performance of the cells, probably because it enhanced gas exchange in the case of suspended cells.

2.3.4 Protein synthesis

The linearity of protein synthesis and factors affecting this were investigated using S^{35}-methionine incorporation. Hepatocytes were incubated with increasing amounts of labelled methionine (Fig. 2.7). A point was reached where the addition of more S^{35}-methionine had no further effect on the incorporation of amino acid into protein. Increasing cell number per incubation was associated with a linear increase in the incorporation of amino acid into protein: under the conditions measured, no plateau was reached (Fig. 2.8). Increasing both the cell number, the medium volume and the added radiolabelled amino acid, gave an increased incorporation of amino acid into protein. Thus in experiments that required maximal labelling, incubations were carried out in 5 ml (with 5 x 10^6 cell) and 50 uCi S^{35}-methionine was added. The dilution effects of added cold amino acid on the incorporation of the labelled amino acid were also tested (Fig. 2.9).

Separation of the cells from the medium showed that over a five-hour time course the incorporation of S^{35}-methionine into both cellular and medium proteins was linear, but after six hours the cellular incorporation began plateau (Fig. 2.10). In all experiments, the TCA-precipitable counts in the medium were about 15 - 20% of the total TCA-precipitable counts, provided that the measurements were carried out in the linear time zone of cellular incorporation.

A number of proteins were synthesized and secreted by isolated hepatocytes (Fig. 2.11). Albumin represented 40 - 50% of total

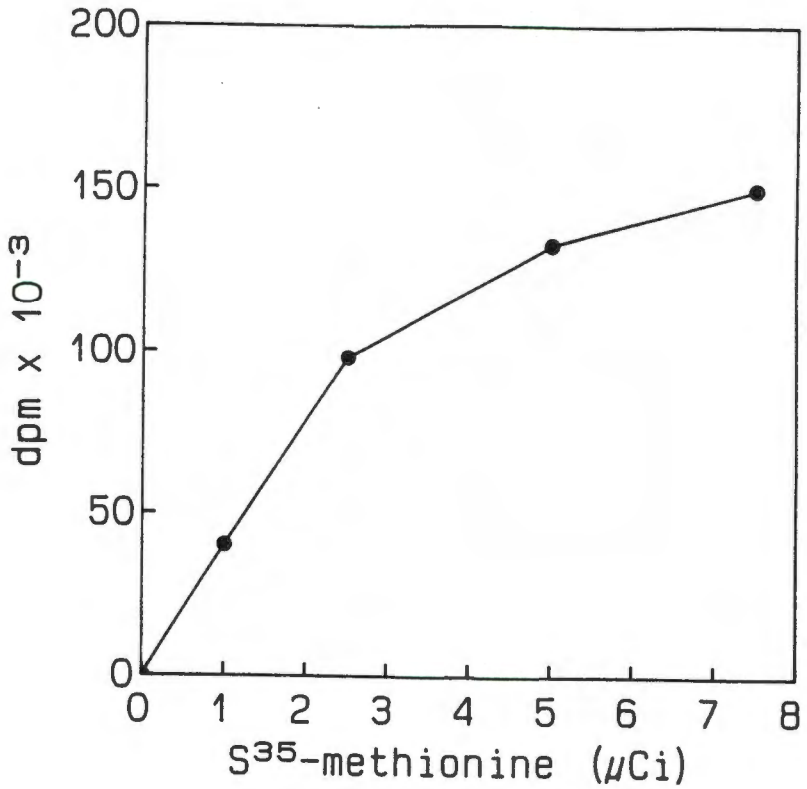

Figure 2.7 The effect of increasing radiolabelled amino acid on the incorporation of radioactivity into total proteins in incubated hamster hepatocyes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells (2×10^6) were placed in 2 ml well-oxygenated methionine-free MEM in 10 ml Erlenmeyer flasks with the indicated amount of S^{35}-methionine. The stoppered flasks were incubated for 2 hours at 37°C in a shaking water bath. After 2 hours the cells and medium were sonicated as described in 2.1.6 and samples were taken for TCA-precipitation and the effect of increased radiolabel on incorporation into total proteins was assessed. Results are the means of duplicates from a single representative experiment and are expressed as dpm of incorporated radioactivity per flask.

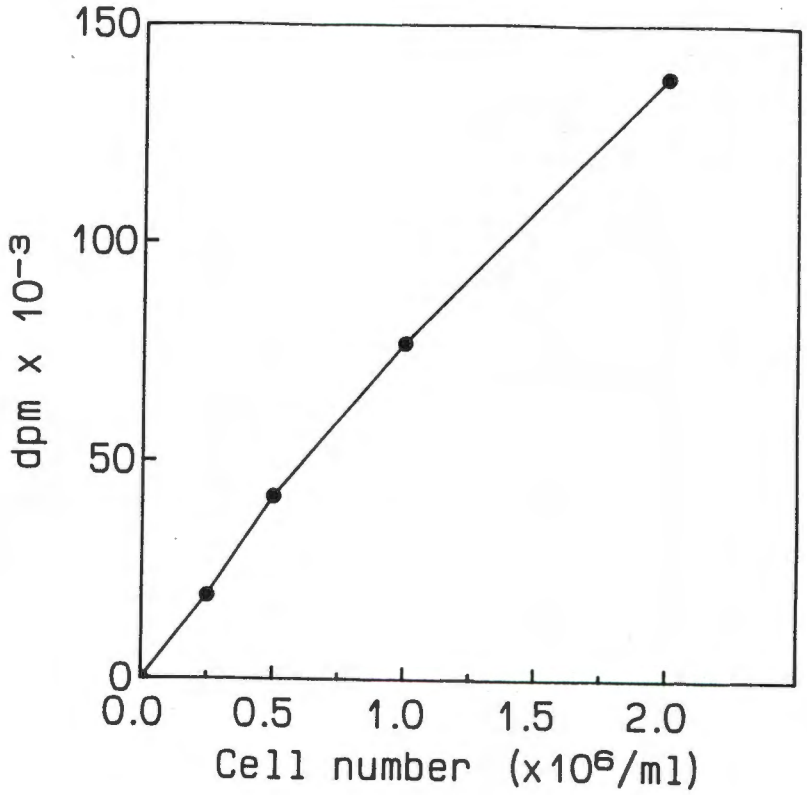

Figure 2.8 The effect of increasing cell number on the incorporation of radiolabelled amino acid into total proteins by incubated hamster hepatocytes. Hamster hepatocytes were prepared as described in 2.1.3. The indicated number of cells was placed in 2 ml well-oxygenated methionine-free MEM in 10 ml Erlenmeyer flasks with 2 μCi S^{35}-methionine. The stoppered flasks were incubated for 2 hours at 37°C in a shaking water bath. After 2 hours the cells and medium were sonicated as described in 2.1.6 and samples were taken for TCA-precipitation to assess the effect of increasing cell number on total protein synthesis. The results are representative data from a single experiment and are expressed as dpm of incorporated radioactivity per flask.

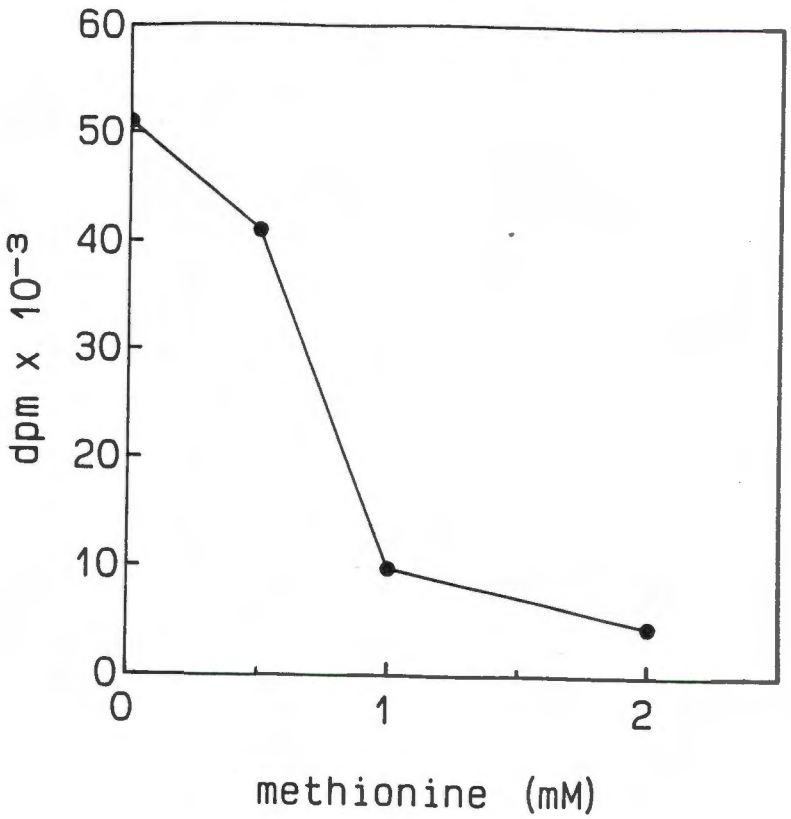

Figure 2.9 The effect of added "cold" methionine on the incorporation of S^{35}-methionine into total proteins by incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells (2 x 10^6) were placed in 2 ml well-oxygenated methionine-free MEM in 10 ml Erlenmeyer flasks with the indicated concentration of l-methionine and 2 µCi S^{35}-mthionine. The stoppered flasks were incubated in a shaking water bath at 37°C and after 2 hours the medium and cells were sonicated as described in 2.1.6. Samples were taken for TCA-precipitation to assess the effect of added methionine on the incorporation of radiolabelled methionine into total proteins. The results are representaive data from a single experiment and are expressed as dpm of incorporated radioactivity per flask.

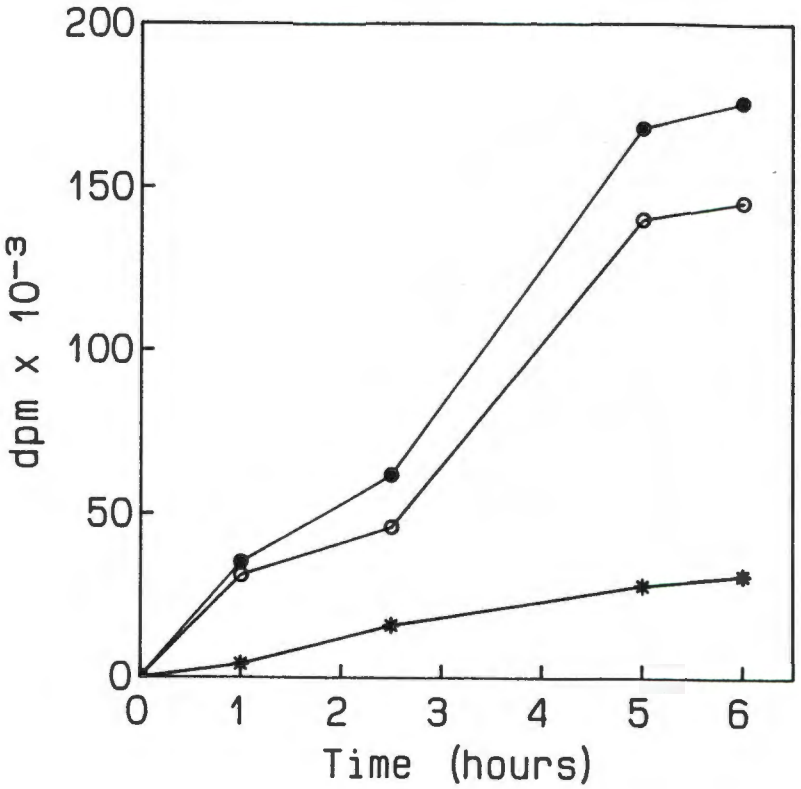

Figure 2.10 A six-hour time-course of the incorporation of radiolabelled methionine into cellular and medium proteins by incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells (2×10^6) were placed in 2 ml well-oxygenated methionine-free MEM in 10 ml Erlenmeyer flasks with 2 μCi S^{35}-methionine. The stoppered flasks were incubated for the indicated times at 37°C in a shaking water bath, after which the cells and medium were separated as described in 2.1.6. The cells were sonicated as described in 2.1.6 and samples were taken for protein determination and TCA-precipitation; medium samples were also taken for TCA-precipitation and the time course for the incorporation of radiolabelled methionine into total (●), cellular (o) and medium (*) proteins was determined. Results are expressed as dpm of incorporated amino acid per milligram of hepatocyte protein and are the means of duplicate flasks in a single representative experiment.

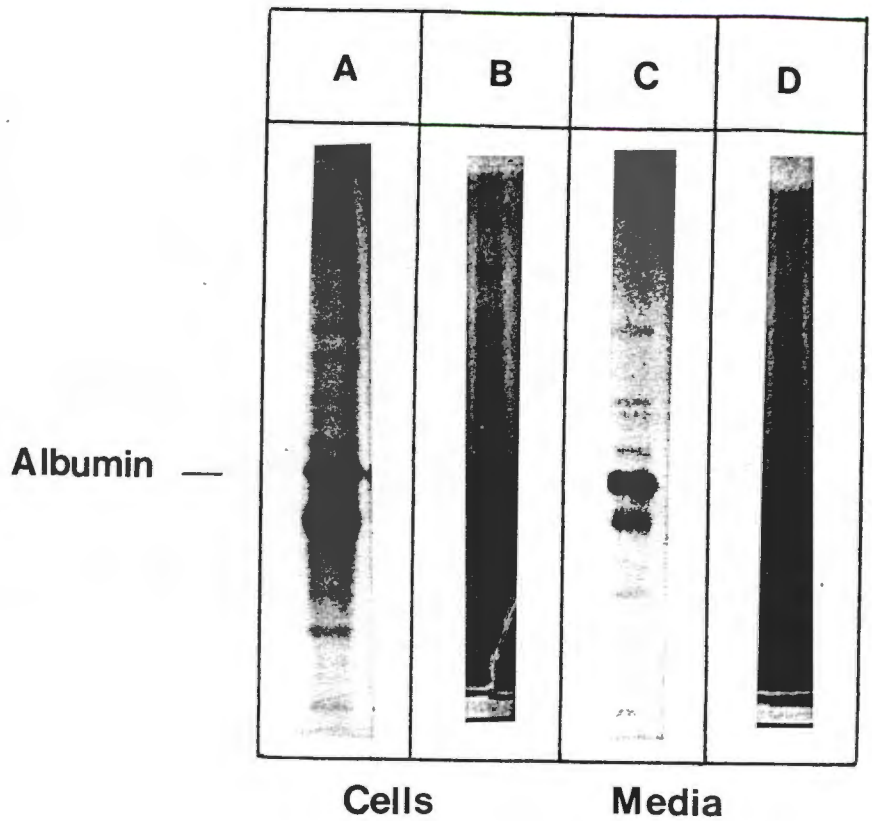

Figure 2.11 Coomassie blue-stained SDS-polyacrylamide gels and fluorograms of the proteins synthesized and secreted by incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells (2×10^6) were placed in 2 ml well-oxygenated methionine-free MEM in 10 ml erlenmeyer flasks. 2 μCi S^{35}-methionine was added to each flask and, once stoppered, the flasks were incubated for 3 hours at 37°C in a shaking water bath. After three hours the medium and cells were separated as described in 2.1.6 and the cells were sonicated. Samples of the cellular extracts and media were subjected to SDS-PAGE (see ref 168) to separate the proteins. The gels were stained with Coomassie blue and once dried, were exposed to Kodak X-Omat film (see 2.1.6) for a number of days. The fluorograms were developed and the radioactive patterns (lanes A and C) were compared with those seen with Coomassie blue stain (lanes B and D).

scanning the fluorogram and extracting the albumin band with sodium hydroxide - see methods). The protein pattern on the stained gels in the case of the cellular extracts and medium samples were similar, but on the fluorograms (radiolabelled pattern) they differed. This suggested that the medium represented the products of true secretion and not the lysis of damaged cells. The release of LDH into the medium during an incubation of five hours was only 5% of the total LDH activity in the system (data not shown).

The effects of azide on the secretion of proteins by the hepatocytes was next investigated. It has been reported that azide inhibits the secretion of proteins by reducing the ATP available to drive the secretion process (183). Cells were pulse-labelled for 1 hour with S^{35}-methionine and then chased for 2 hours in the presence of azide and cold methionine (Fig. 2.12). The much reduced medium albumin level was very clear, while the cellular albumin appeared, as expected, to be more plentiful than in its absence.

2.3.5 VLDL Synthesis

The synthesis and secretion of VLDL was investigated during five-hour incubations of suspended cells (5 ml adding 50 uCi S^{35}-methionine). Both the intracellular and the secreted VLDL could be isolated by the methods described above and secretion was shown to be linear over five hours (Fig. 2.13). Cab-O-Sil was also used used as a lipoprotein-binding device as described by Vance (184) but there was serious contamination of the lipoproteins by other proteins and the fluorograms were very difficult to interpret. Fluorograms of the particles isolated by ultracentrifugation showed little contamination with albumin or other proteins (the albumin observed in some preparations may in

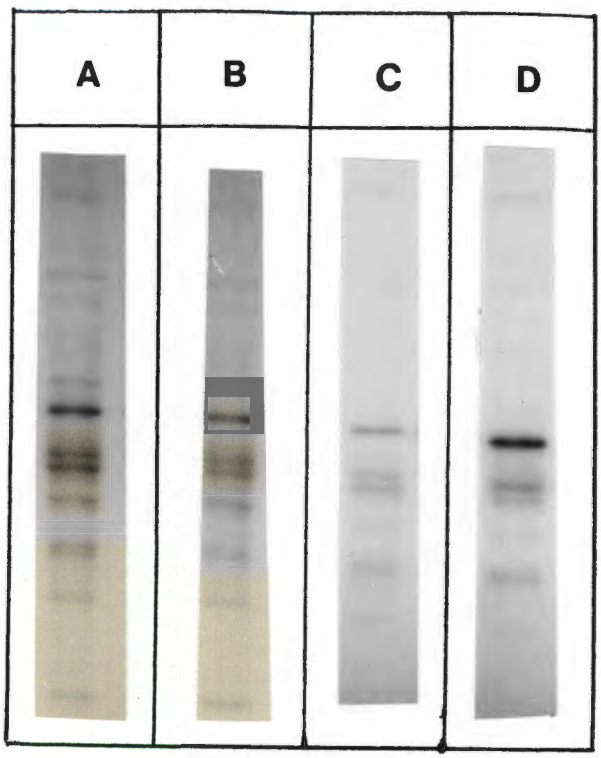

Figure 2.12 The effect of freshly prepared azide upon the secretion of proteins by incubated hamster hepatocytes.

Hamster hepatocytes were prepared as described in 2.1.3. The cells (5×10^6) were placed in 5 ml methionine-free MEM with 10 µCi S^{35}-methionine in stoppered 10 ml Erlenmeyer flasks. The cells were incubated for 1 hour in a shaking water bath at 37°C, after which the medium was removed and the cells were washed as described in 2.1.7 and placed in 5 ml MEM with or without 10 mM azide (freshly prepared in MEM). The cells were incubated for a further 90 minutes and the medium and cells were separated as described in 2.1.6. Samples were subjected to SDS-PAGE and the dried gels were used to obtain the fluorographic patterns of each sample (cellular proteins with (A) and without (B) azide and secreted proteins in the presence (C) or absence (D) of azide) to observe the effect of azide on the secretion of proteins by hamster hepatocytes.

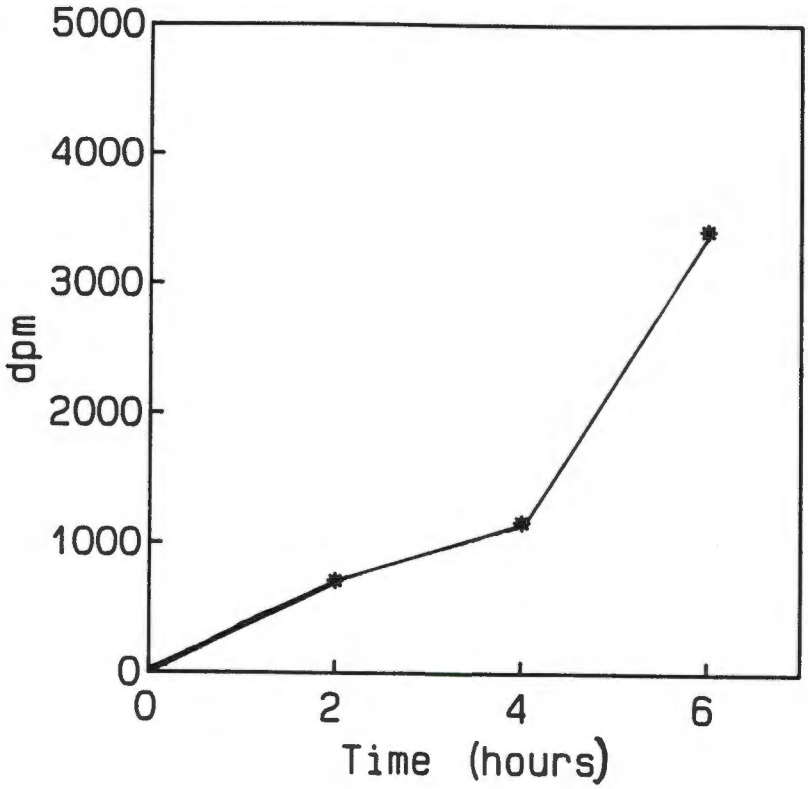

Figure 2.13 A time course of the secretion of VLDL by incubated hamster hepatocytes.
Hamster hepatocytes (5 X 10^6), prepared as described in 2.1.3, were placed in 5 ml methionine-free MEM with 50 µCi S^{35}-methionine in stoppered 10 ml Erlenmeyer flasks. The cells were incubated for the indicated times in a shaking water bath at 37°C, and then the cells and medium were separated as described in 2.1.6. VLDL was prepared from the media samples as described in 2.1.9. The floated samples were TCA precipitated in order to observe the time course for the synthesis and secretion of VLDL by incubated hamster hepatocytes. Results are expressed as dpm of radioactivity associated with VLDL-protein in each flask and are duplicate samples from a single representative experiment.

any case have been associated with the lipids on the particle and not been a contamination of the preparation). In Fig. 2.14, the fluorogram of the cellular and medium VLDL shows apo B100 and apo E to have been the predominant apoproteins in both the cells and the medium, but some proteolysis of the intracellular apo B appeared to have taken place. The cellular samples were prone to reflect this, even though a number of antiproteases were used in the homogenization of the cells. A faint apo A I band was also visible in the medium VLDL preparations but not in those from the cells.

An experiment was next carried out to confirm that the VLDL "apo B" was indeed true apo B. VLDL was prepared from a medium sample without using carrier VLDL in the preparation. An immunoprecipitation was carried out on the floated particles with an antibody specific for rat apo B (gift from Dr G. Getz, Chicago). Fig. 2.15 shows the single apo B100 band derived from the immunoprecipitation of the medium flotation with no band in the supernatant fluid of the immunoprecipitation.

Column analysis of the lipoproteins prepared from the cells and medium was performed: the medium particles less dense than 1.21 g/ml were isolated in an ultracentrifugation spin of 30 hours. They were placed on a Sephadex CL4B column and eluted at 0.2 ml/min in the cold. The only particles eluted at the Vo and were thus VLDL-sized particles (Fig. 2.16). The eluted fractions were too dilute for lipid analysis or SDS-PAGE to be carried out.

After three-hour incubations, the TCA-precipitable radioactivity in medium VLDL was compared with VLDL present in the cells, in order to investigate the secretory activity of the cells. The ratio was 2.23 while that for albumin was about 1; this suggested that VLDL was being actively secreted.

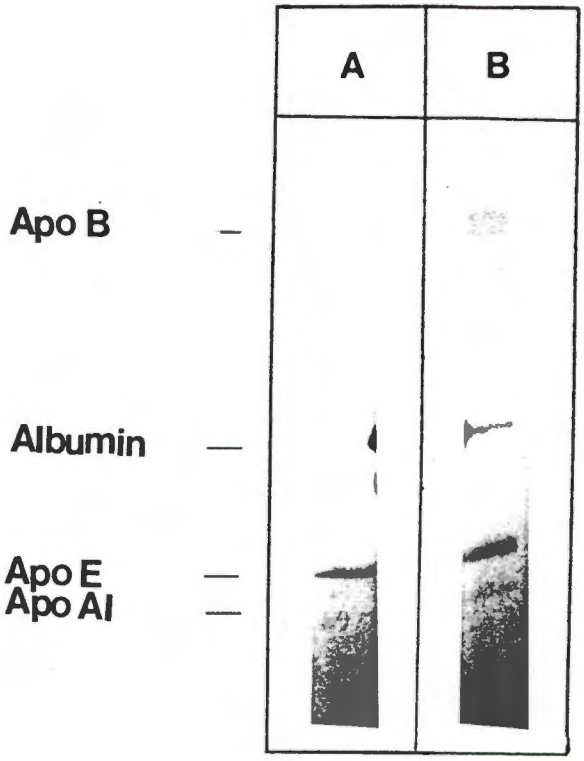

Figure 2.14 The fluorographic appearance of VLDL synthesized and secreted by incubated hamster hepatocytes.
Hepatocytes were prepared for incubation as described in figure 2.13. The cells were incubated for 5 hours and then VLDL was prepared from the separated cells and media (as described in 2.1.9). Aliquots from the floated samples were subjected to SDS-PAGE followed by fluorography (2.1.6) to observe the proteins associated with the floated particles in both the cellular (A) and medium (B) samples.

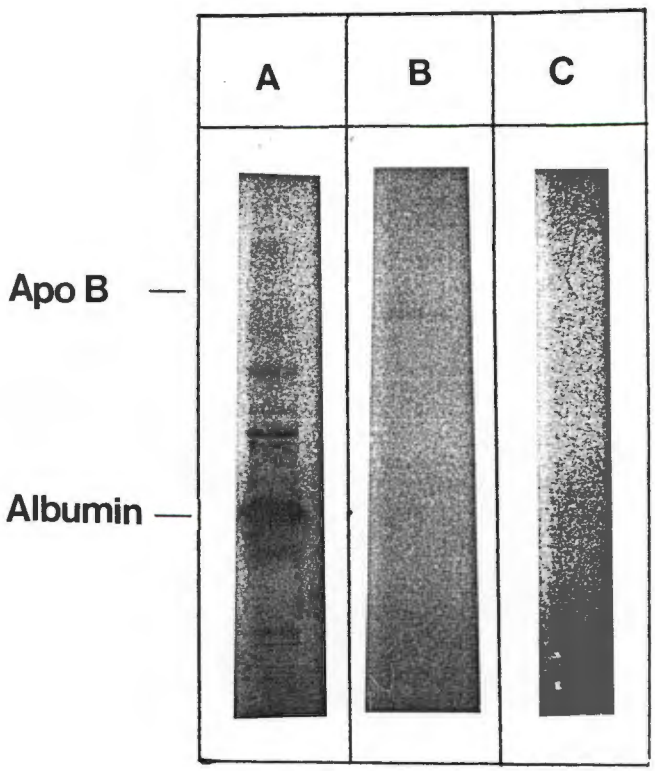

Figure 2.15 The immunoprecipitation of apo B from VLDL particles secreted by incubated hamster hepatocytes.
Hamster hepatocytes were prapared for incubation as described in fig. 2.13. The cells were incubated as described in fig. 2.14 and VLDL was prepared from the medium only. VLDL preparation was without human carrier VLDL so that immunoprecipitation of the apo B could be carried out on the floated particles. Immunoprecipitation, as described in 2.1.10, was carried out. The total medium (lane A), immunoprecipitated VLDL (lane B) and VLDL immunoprecipitated with pre-immune serum (lane C) were subjected to SDS-PAGE followed by fluorography (2.1.6) to show that the flotable apo B was immunoprecipitable.

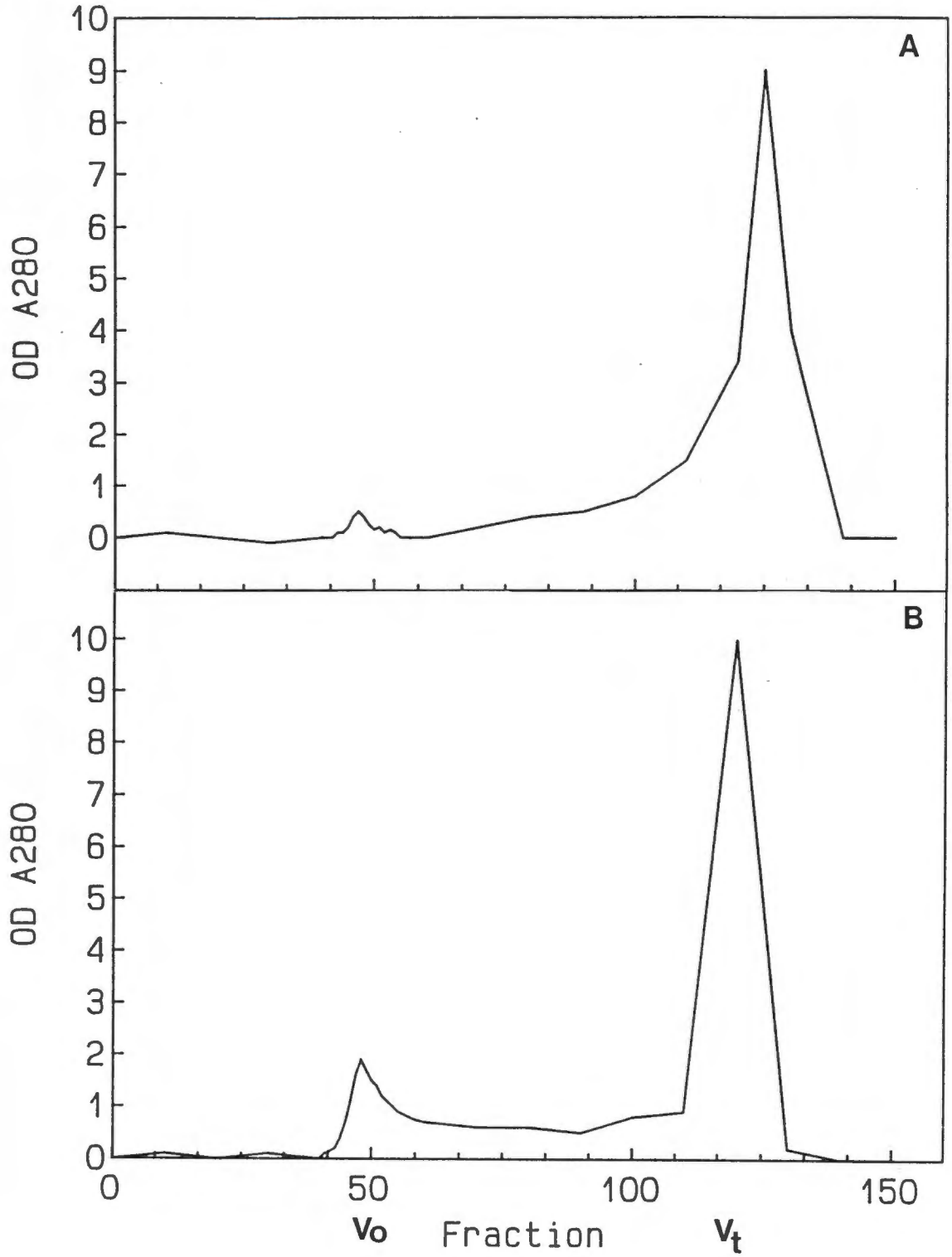

Figure 2.16 Column analysis of VLDL particles secreted by incubated hamster hepatocytes.
Cellular and medium VLDL particles prepared from incubated hamster hepatocytes as described in figure 2.15, were analysed by Sephadex CL4B columns. The absorbance (at 280 nm) of the eluted samples was recorded in order to observe the size of the particles (called VLDL) synthesized (A) and secreted (B) by incubated hamster hepatocytes.

2.3.6 Apo B immunoprecipitation

The quantitative immunoprecipitation of apo B was investigated using OSAN, a commercially obtainable anti-human LDL antibody. An anti-human albumin antibody was also found to be quantitative in its immunoprecipitation (Fig. 2.17). Fig. 2.18 shows the time course of synthesis of apo B over 5 hours: both medium and cellular apo B increased in a linear fashion. In the case of immunoprecipitations of apo B produced by cells from euthyroid hamsters, apo B100 and high molecular weight lytic products of apo B100 were observed but never apo B48 (Fig. 2.19). When hamsters were rendered hyperthyroid by oral administration of a bolus of T3 (1 μg per g hamster mass) the preparation (2 days later) of hepatocytes enabled a test of the apo B species produced under the influence of excess thyroid hormone to be carried out; Apo B48 immunoprecipitated from isolated intestinal cells (gift from Dr M. I Combrinck) was used as a control. The cells from hyperthyroid animals gave a clear band at the position of apo B48 (Fig. 2.19). Thus hamster hepatocytes synthesize and secrete apo B48 only when the donor animals are clearly hyperthyroid. In the same experiment, VLDL was prepared from the media of both cell preparations; the amount of VLDL prepared from the medium isolated from hyperthyroid cells was less than that from the normal euthyroid cells (data not shown).

The effects of azide on apo B secretion was the same as that on the secretion of total proteins, while tunicamycin treatment (which inhibits protein N-glycosylation without interfering with apo B secretion (185)) gave rise to a band which had a higher mobility than that observed in untreated cells (Fig. 2.20).

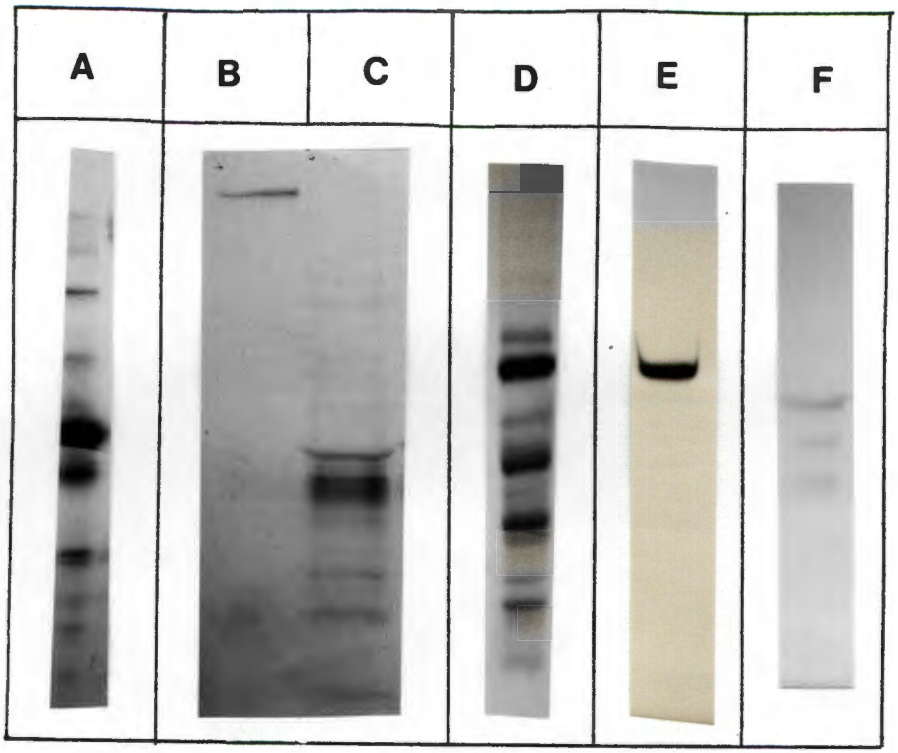

Figure 2.17 The quantitative immunoprecipitation of Apo B and albumin from media samples of incubated hamster hepatocytes.

Hamster hepatocytes were prepared as described in 2.1.3 and 5 x 10^6 cells were placed in a 10 ml stoppered Erlenmeyer flask in 5 ml methionine-free MEM with 50 μCi S^{35}-methionine and incubated for 3 hours. The cells and media were separated and apo B or albumin were immunoprecipitated from 2 ml of each medium sample as described in 2.1.10. SDS-PAGE followed by fluorography was carried out on the medium (A and D), immunoprecipitated apo B (B) or albumin (E) and the proteins remaining after immunoprecipitation with the anti-apo B antibodies (C) or anti-albumin antibodies (F). The quantitation of the procedure was thus demonstrated.

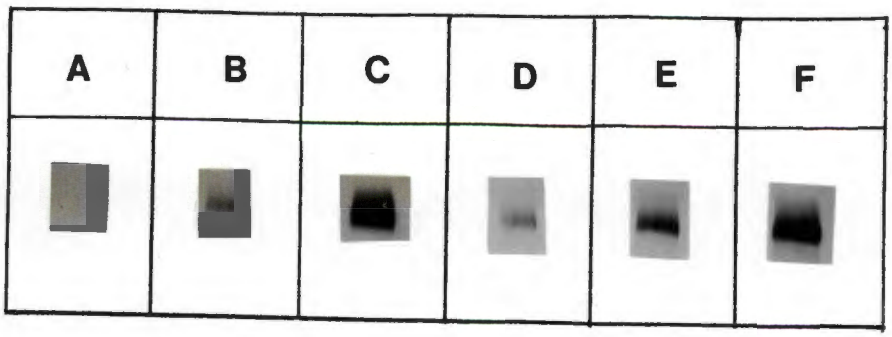

Figure 2.18 A five-hour time course of the synthesis and secretion of apo B by incubated hamster hepatocytes.

Hamster hepatocytes (5 x 10^6), prepared as described in 2.1.3, were incubated for up to 5 hours in 5 ml methionine-free MEM with 50μCi S^{35}-methionine in stoppered 10 ml Erlenmeyer flasks, at 37°C in a shaking water bath. The cells and media were separated at the end of each incubation and apo B was immunoprecipiated from each sample as described in 2.1.10. SDS-PAGE followed by fluorography was carried out on each of the media samples: 1 hour (A), 2.5 hour (B) and 5 hour (C) incubations. The same process was carried out on each of the cellular samples: 1 hour (D), 2.5 hour (E) and 5 hour (F) incubations. The linearity of the synthesis and secretion of apo B over five hours was thus demonstrated,

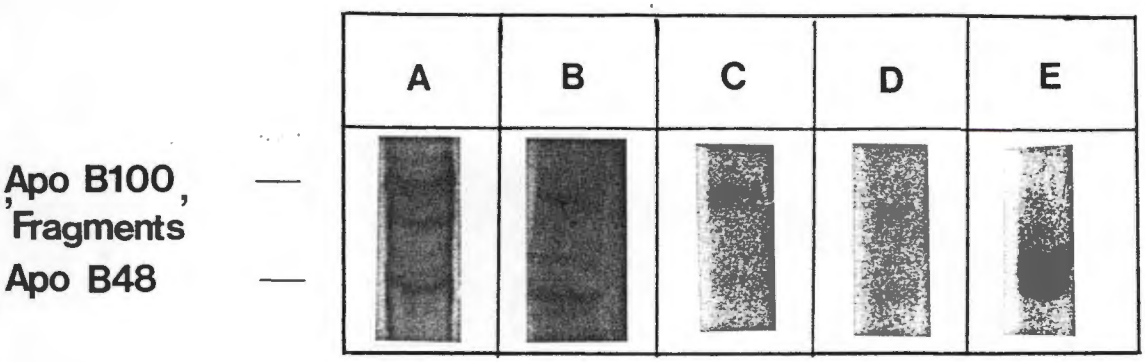

Figure 2.19 The production of apo B48 by incubated hepatocytes prepared from hyperthyroid hamsters.
Hamsters were rendered hyperthyroid by administration of T3 as described in 2.3.5. Two days after the administration, hepatocytes were prepared from both hyperthyroid and normal hamsters as described in 2.1.3. The cells were incubated for 3 hours as described in figure 2.17 and apo B was immunoprecipitated from the cellular extracts and media samples of the euthyroid and hyperthyroid samples as described in 2.1.10. SDS-PAGE and fluorography was performed on the cellular immunoprecipitates from euthyroid (A) and hyperthyroid (B) samples, media of euthyroid (C) and hyperthyroid (D) incubations and media from incubated intestinal cells (E), (gift from Dr M.I. Combrinck).

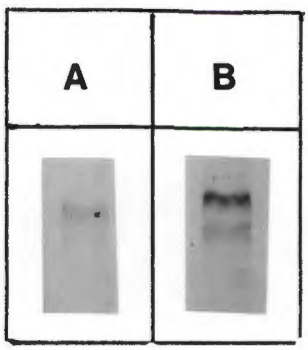

Figure 2.20 The effect of tunicamycin on apo B secreted by incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells were incubated as described in fig 2.18 for 1 hour. The medium was removed from each flask and the cells washed thrice as described in 2.1.7 and fresh MEM was added to the cells, in some incubations tunicamycin (10 μg/ml) was also added. The cells were incubated for a further 90 minutes after which the cells and medium were separated and apo B was immunoprecipitated from the media samples as described in 2.1.10. SDS-PAGE followed by fluorography was carried out on media samples in the absence (A) or presence (B) of tunicamycin thus demonstrating the carbohydrate processing of apo B.

2.3.7 Lipid synthesis

An experiment was carried out to show that lipid synthesis was also carried out by the isolated suspended hepatocytes. Cells were incubated with H^3-glycerol or H^3-oleic acid. Table 2.3 shows the incorporation of these compounds into lipids and their distribution amongst the lipid classes in the cells and in the medium. Lipid synthesis clearly took place in isolated suspended hepatocytes; triglyceride synthesis and secretion were most notable. In medium from cells incubated with H^3-glycerol, the high incorporation into phospholipid may have been a spurious result since phospholipids remain at the origin together with other entities with which the label might have become associated. Apo B immunoprecipitation of lipid-labelled medium samples was carried out in Triton X-100-free solutions. Triglyceride was the most abundant of the lipids associated with the immunoprecipitated protein, since a very small proportion of the medium lipid was immunoprecipitated, the validity of the method is open to question. Cab-O-Sil failed to associate with much lipid label but the proportion of the lipids that it bound was similar to that observed in the case of immunoprecipitated lipids (Table 2.4). The results for the H^3-oleic acid treated cells were very similar to those observed with H^3-glycerol, if the high free fatty acid background was removed (values in brackets). Triglycerides were the lipid class that was associated with the putative particles isolated by both methods.

2.4 Isolated Hepatocytes In Tissue Culture

2.4.1 General culture conditions

The effects of various manipulations and tissue culture flasks was investigated. Attachment of the cells was used to assess

Table 2.3 The incorporation of H^3-glycerol and H^3-oleic acid into lipids by incubated hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells (5×10^6) were incubated in 5 ml MEM in 10 ml stoppered Erlenmeyer flasks and 50 µCi of radiolabelled precursor was added as described in 2.1.12. The cells were incubated for 3 hours at 37 °C in a shaking water bath after which the cells and media were separated. Lipid extraction (2.1.13) and separation by TLC (2.1.14) followed. The total incorporated radioactivity and the proportion in each of the lipid classes was calculated for each of the precursors.

	Glycerol label		Oleate label	
	Cells	Medium	Cells	Medium
Total incorporation [i]	102682	47046	447962	1597687
Phospholipid	1.6%	48%	0.1%	0.2%
Diacylglycerol	4.6%	1%	2.4%	0.4%
Cholesterol	1.5%	0.7%	1.4%	0.4%
Free fatty acid	7.7%	12.8%	25% *	76.7% *
Triglyceride	84.5%	37.3%	70.5%	22.1%
Cholesterol ester	0.1%	0.2%	0.6%	0.2%

i Expressed as dpm per system.
* The proportions are very high in since the fatty acid label was separated with the free fatty acids.

Table 2.4 Lipids associated with apo B immunoprecipitated from medium or with cab-o-sil treated medium in incubated hamster hepatocytes.

Hamster hepatocytes were prepared and incubated with lipid precursors for 3 hours as described in Table 2.3. At the end of the incubations media samples were either immunoprecipitated with OSAN as described in 2.1.10 but no SDS or Triton X-100 was present during the immunoprecipitation, or they were treated with cab-o-sil (183). Lipid was extracted from the immunoprecipitates or cab-o-sil-bound material as described in 2.1.13 and was separated as described in 2.1.14. The total radioactive lipid associated with each sample as well as the proportion of each of the lipid classes was measured. In the case of the oleate label, there was a very high free fatty acid background which when removed (figure in brackets) left the results from each precursor quite comparable.

	Glycerol label		Oleate label			
	Immunoprecipitation	Cab-o-sil	Immunoprecipitation		Cab-o-sil	
Total incorporation[i]	1549	3589	9740	(5375)	248734	(11374)
Phospholipid	9.3%	15%	5.1%	(9.2%)	0.6%	(12.7%)
Diacylglycerol	7.6%	4.7%	2.3%	(4.2%)	0.5%	(11.2%)
Cholesterol	7.6%	3.2%	2.9%	(5.4%)	0.8%	(18%)
Free fatty acid	6.7%	4.5%	48.4%	(6.5%)	95.6%	(4.8%)
Triglyceride	64.2%	71.5%	38.9%	(70.5%)	2.2%	(47.1%)
Cholesterol ester	4.6%	1.1%	2.3%	(4.2%)	0.3%	(6.2%)

i Expressed as dpm per system

their viability under such conditions. It has been reported that culture dishes plated with collagen are the most appropriate for hepatocytes (61). The disadvantage is the time required for the preparation of such plates and the fact that the latter become an added source of infection. Hardly any attachment was obtained on ordinary plastic tissue culture dishes, but attachment was good on plates coated with collagen, and also good (75%) when Primaria tissue culture dishes were used. These commercially available tissue culture dishes have been reported to enhance the growth of freshly isolated cells (185). The commercial plates were used in all further experiments.

A particular batch of FCS was found to be optimal for the attachment of the cells (this had been true for Hep G2 cells cultured by others in our laboratory). A number of batches were tested and the batch resulting in the highest attachment (75%) was retained for use in all further experiments. Attachment ranged from 20% to 75% for the FCS lots tested.

By 4 hours, the attached cells had already begun to be arranged in cord-like structures which were well defined by 24 hours. The effects of removing calcium, upon attachment and cord formation was investigated by the addition of 2 mM EGTA. Few cells attached to the tissue culture dishes in the presence of the chelator and there was no cord formation. After 48 hours in the presence of EGTA very few cells remained. Added calcium and magnesium (final concentrations 5mM) had no effect upon attachment or cord formation. BSA-oleate (1 mM oleate) was added to some cultures in the place of FCS; neither attachment nor protein synthesis, as measured by the incorporation of S^{35}-methionine into total protein, were affected by the addition.

The effects of cycloheximide and chloramphenicol were the same as in the case of cells used as suspensions.

2.4.2 Protein synthesis

The secretion of protein by cultured hepatocytes was linear over a 24-hour time course, but by 12 hours cellular incorporation had begun to plateau (Fig. 2.21). The staining and radiolabelling pattern of the proteins secreted by the cultured cells was similar to that of the proteins secreted by suspended cells, but cellular proteins were very different; albumin was only about 4% of intracellular proteins, and a band with molecular mass of about 40 kdal was predominant (20%) the protein was most likely actin. Fig. 2.22 shows a comparative medium and cellular profile from cultured and suspended cells. VLDL synthesis by cultured cells was also tested (data not shown); the synthesis and secretion of immunoprecipitated apo B is shown in Fig. 2.23. The fluorographic profile shows linear accumulation of apo B in the medium over the 24 hour time period; intracellular apo B appears to be increasing in a linear fashion and does not reach a plateau in the same way that was observed for the synthesis of total proteins shown in Fig. 2.21. Lipid synthesis was not investigated in cultured cells.

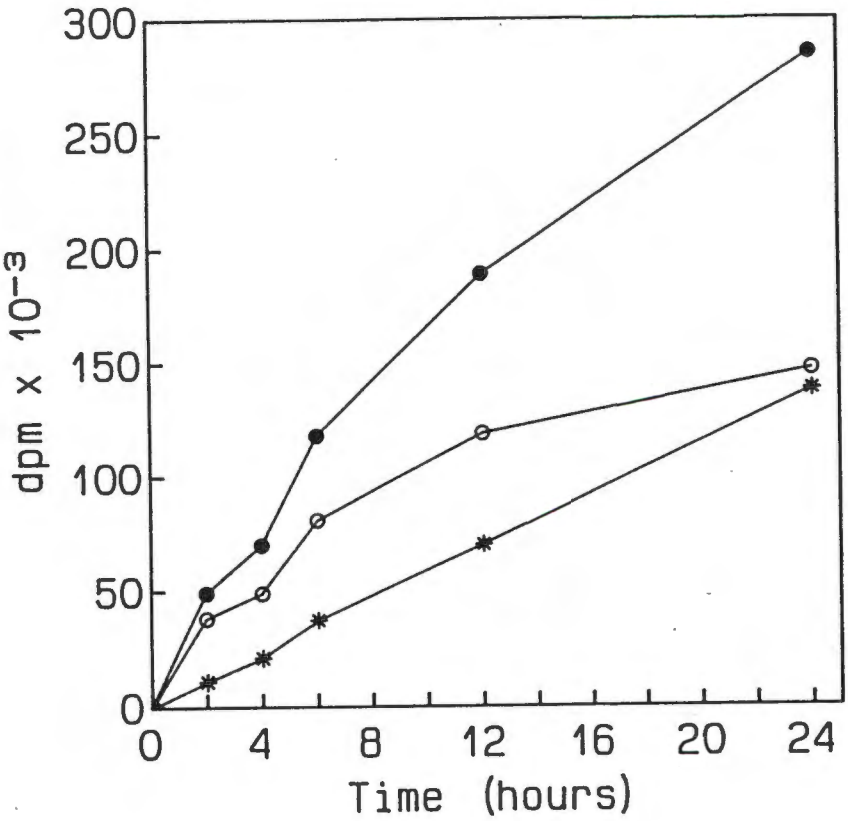

Figure 2.21 The 24-hour time course of protein synthesis and secretion by cultured hamster hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3. The cells were placed into tissue culture as described in 2.1.4 and after 24 hours 2 ml fresh methionine-free MEM and 2 μCi S^{35}-methionine was added to each flask. The cells were placed in the incubator for the indicated times after which the medium was removed from the flasks and the cells were scraped from the flasks in three changes of homogenization buffer. TCA precipitation was carried out on the cellular extracts and medium samples, and a time course for the synthesis of total (•) and cellular (o) proteins and secretion into the medium (*) over a 24 hour time period was obtained. The results are the means of duplicates from a single experiment and are expressed as dpm per flask.

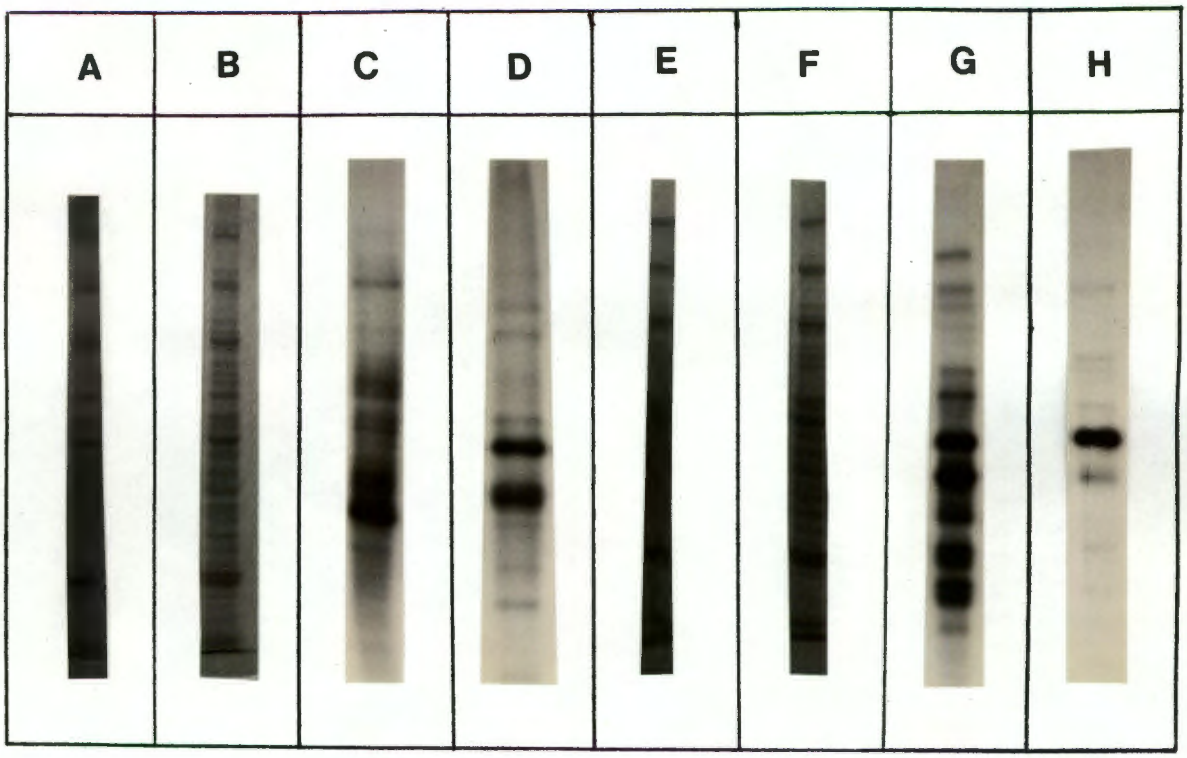

Figure 2.22 The Coomassie-stained proteins and fluorogram of radiolabelled proteins synthesized and secreted by cultured hamster hepatocytes compared to those from incubated hamster hepatocytes

Hamster hepatocytes were prepared as described in 2.1.3 and used in tissue culture (2.1.4) or incubated immediately (2.1.5). The cultured cells were incubated for 24 hours as described in figure 2.21 and radiolabelled cellular and medium samples were subjected to SDS-PAGE and fluourography. The incubated cells were treated as described in figure 2.11 and SDS-PAGE and fluorography were also carried out on those cellular and medium samples. The cellular protein patterns from coomassie blue stained gels prepared from cultured (A) and incubated (B) cells and the cellular radiolabelled patterns from each (C and D) were compared. The medium coomassie blue (E and F) and fluorographic (G and H) were also compared to observe similarities or differences between each preparation.

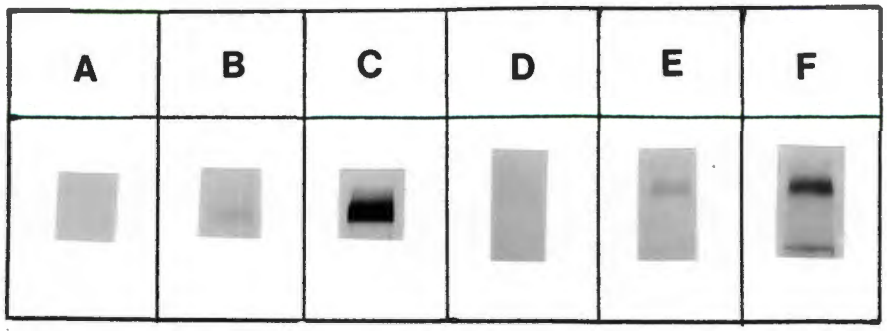

Figure 2.23 The 24-hour time course for the synthesis and secretion of apo B by cultured hamster hepatocytes. Hamster hepatocytes were prepared for tissue culture as described in 2.1.3 and 2.1.4 and after 24 hours fresh methionine-free MEM and 30 μCi S^{35}-methionine was added to each flask. The flasks were incubated for 2.5, 5, or 24 hours after which the medium was removed from each flsk and the cells were scraped with three changes of homogenization buffer. Apo B was immunoprecipitated from the medium and cell samples as described in 2.1.10. The media (A, B and C) and cellular (D, E and F) immunoprecipitates from each time point were subjected to SDS-PAGE and fluorography to observe the time-course of synthesis and secretion of apo B by cultured hamster hepatocytes.

2.5 Discussion

Hamster hepatocytes were characterized in single-cell suspensions and under tissue culture conditions. Their responses to various treatments differed very little from those described in the case of rat liver cells (for reviews see 20,32,36,63). Synthesis and secretion of VLDL apoproteins and lipids took place and conditions for this were optimized. Since this appeared to be the first detailed study of suspended hepatocytes for the study of apo B metabolism, there was no data (except that obtained for cultured cells) with which to compare the results that have been presented. The cells did appear to be most comparable with suspended rat hepatocytes in terms of total protein synthesis and incubation conditions (36).

Although both kinds of hepatocytes appeared to be suitable for detailed studies of VLDL and apo B metabolism, suspended cells were easier to use than the cultures because their preparation did not require stringent sterile procedures and because they could be used immediately after isolation. It was of some concern that the intracellular pattern of radiolabelled proteins in the two systems were so different. This might have been due to differences in transcription and translation events between the two types of cells: the suspended cells, while trying to overcome their preparation were still under the control of recent *in vivo* stimuli while the cultured cells had overcome their preparation but had begun to lose the "memory" of the *in vivo* organ.

Chapter 3: CHARACTERIZATION OF LIVER SLICES

3.1 Materials and Methods

Heparin was purchased from Sigma Chemical Co. (St Louis,USA) and suramin (Germanin) was purchased from Bayer-Miles (Pty) Ltd. (Isando,South Africa).All other materials used for the preparation of liver slices did not differ from those already described for the isolation of hamster hepatocytes.The animals were from the same source and the methods of protein and lipoprotein investigation were also unchanged.

3.1.1 Preparation of the liver slices

Each hamster was anaesthetised with ether and weighed.The liver was removed and placed in a beaker of ice-cold saline (0.9% NaCl) and its mass was recorded before removing the gall bladder.The liver was cut into individual lobes which were stored on ice.Individual lobes were placed on damp blotting paper and a glass microscope slide was placed over them.Using a Thomas tissue slicing blade under the slide,thin slices were cut.The first,last and any thicker than about 0.45 mm was discarded;the rest were placed in a covered petri dish on ice.The slices were weighed and about 100 mg was used per incubation.The slices were placed in 10 ml Erlenmeyer flasks in 2 ml methionine-free MEM and after thorough gassing,were incubated with radiolabelled methionine in a rotating water bath (75 per min) for the required time.

At the end of each incubation,the liver slices were separated from the medium and homogenized in fractionation buffer with 5 strokes of a dounce homogenizer.The debris was removed by centrifugation in the cold in a Beckman centrifuge at 5000 x g for 5 minutes.The debris was re-homogenized and the pooled supernatant fluids were layered onto a 2 M sucrose cushion and

centrifuged in a Beckman ultracentrifuge (100 000 x g for 60 minutes).The supernatant fluid was retained and the microsomal pellet was either treated with sodium carbonate (described before) or suspended in homogenization buffer and sonicated on ice with 2 x 5 second bursts (with the microtip set on 3). TCA-precipitations,VLDL preparation,immunoprecipitation and SDS-PAGE and fluorography were carried out as described before.

3.1.2 Heparin and Suramin

At the end of some incubations,the medium was carefully removed from the liver slices using a pasteur pipette.Some slices were then processed as described above,while others were incubated further in PBS or PBS containing either heparin (0.4% (w/v))or suramin (1.435% (w/v))for a further one hour with shaking at 4°C.The new medium was removed after an hour and retained.The slices were treated as described above and the medium and cell fractions were analysed as required.

3.2 Results

The rates of synthesis and secretion of various proteins by incubated liver slices were systematically investigated and the behaviour of the tissue was observed under various conditions to optimize the system.

3.2.1 Total protein synthesis

Liver slices were incubated for up to 5 hours with S^{35}-methionine to investigate total protein synthesis (Fig. 3.1). Total protein synthesis was linear for the first 3 hours but then reached a plateau.Secretion of radiolabelled proteins into the medium remained linear over the 5 hours but the label in media proteins

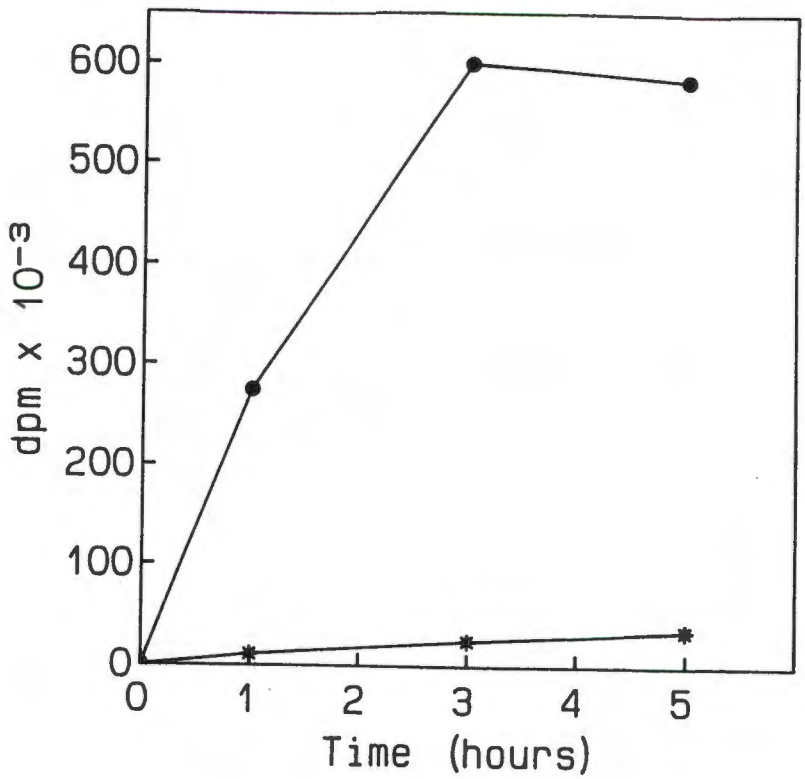

Figure 3.1 A five-hour time course of the incorporation of S^{35}-methionine into protein by incubated hamster liver slices.

Hamster liver slices were prepared as described in 3.1.1. Each slice was placed in 2 ml well-oxygenated methionine-free MEM with 20 µCi S^{35}-methionine in a stoppered 10 ml Erlenmeyer flask and incubated for the incubated times at 37°C in a shaking water bath. After the incubation the tissue and media were separated; the tissue was homogenized in homogenization buffer and samples of the tissue extracts and media were taken for TCA precipitation and protein determination. The time course for incorporation of radiolabelled methionine into the tissue (●) and medium (*) was thus determined. The results are expressed as dpm of incorporated radiolabel per mg slice protein and are the means of duplicate samples from a single such experiment.

expressed as a proportion of the total radioactivity, remained between 5 and 10 %. When H^3-leucine (in leucine-free MEM) was used instead of S^{35}-methionine, protein synthesis was linear for 5 hours and no plateau was observed (Fig. 3.2). This was probably due to differences in the total intracellular pool sizes of leucine and methionine and the rate with which each was metabolized in pathways other that those of protein synthesis. The contribution by the medium to the total TCA-precipitable radioactivity remained 5 - 10% throughout.

The patterns obtained from Coomassie blue-stained gels and fluorograms showed that the protein patterns from both the media and tissue extracts were clearly different (Fig. 3.3). Albumin constituted about 40 - 45% of the secreted medium proteins (on scanning and extraction of the band from the fluorogram) and about 20-25% of the radiolabelled cellular proteins. The proteins in media and tissue extracts from liver slices and incubated hepatocytes were very similar but the cellular pattern from cultured cells was very different (Fig. 3.4).

3.2.2 VLDL synthesis

Liver slices synthesized and secreted VLDL particles (isolated by flotation) that contained apo B100, apo E and some apo AI. TCA-precipitable radioactivity in the medium VLDL was compared with the VLDL present in the tissue in order to define the secretory activity of the cells in the slices. The ratio was 0.29, which was clearly lower than that of 2.25 obtained in the case of isolated hepatocytes. The ratio for albumin was about 0.5 , also lower than that obtained with hepatocytes (data not shown).
The low ratio of medium-to-tissue VLDL suggested that VLDL particles were failing to reach the medium, either due to impaired secretion from the cells or because of trapping in the intercellular spaces of the slices. This was investigated by gently homogenizing the slices in homogenization buffer (as

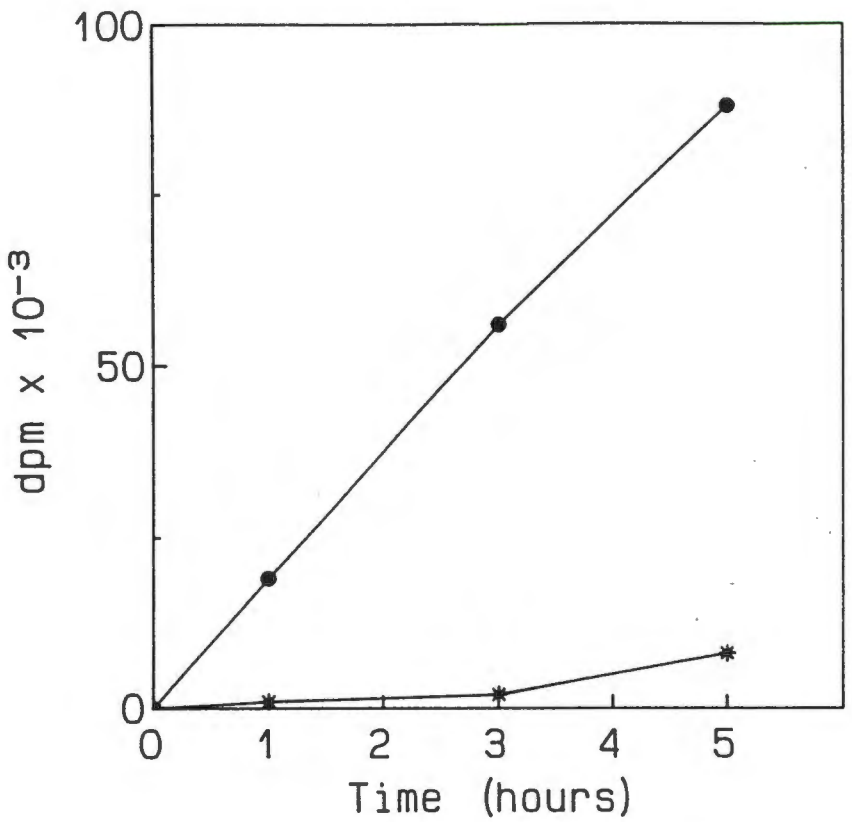

Figure 3.2 A five-hour time course of the incorporation of H^3-leucine into protein by incubated hamster liver slices.
Hamster liver slices were prepared as described in 3.1.1. Each slice was placed in 2 ml well-oxygenated leucine-free MEM with 5 uCi H^3-leucine in a stoppered 10 ml Erlenmeyer flask and incubated for the incubated times at 37°C in a shaking water bath. After the incubation the tissue and media were separated; the tissue was homogenized in homogenization buffer and samples of the tissue extracts and media were taken for TCA precipitation and protein determination. The time course for incorporation of radiolabelled leucine into the tissue (•) and medium (*) was thus determined. The results are expressed as dpm of incorporated radiolabel per mg slice protein and are the means of duplicate samples from a single such experiment.

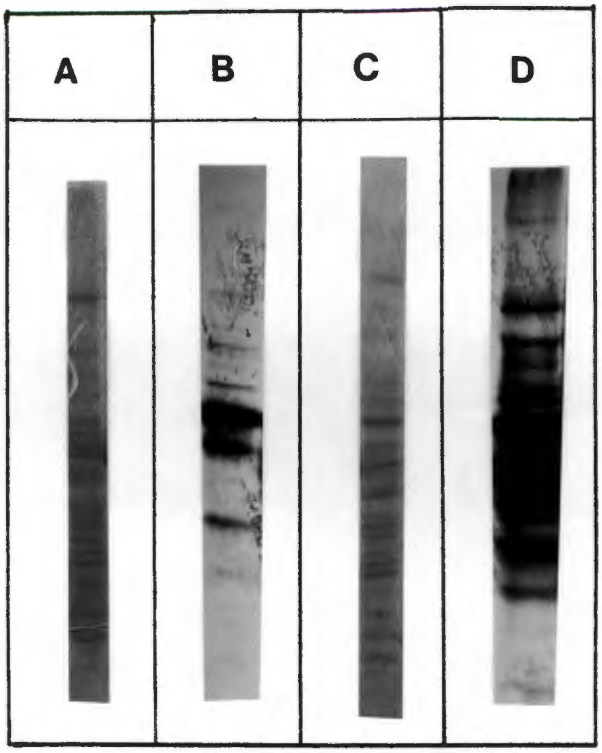

Figure 3.3 The coomassie blue-staining and fluorographic patterns of the proteins synthesized and secreted by hamster liver slices.
Hamster liver slices were prepared as described in 3.1.2. Each slice was placed in 2 ml well-oxygenated methionine-free MEM with 20 μCi S^{35}-methionine in a stoppered 10 ml Erlenmeyer flask. After 3 hours incubation at 37°C in a shaking water bath, the tissue was separated from the medium and samples of each were subjected to SDS-PAGE. The dried gels were stained with coomassie blue and subsequently fluorographs were prepared. The coomassie-stained and fluorographic patterns of the media (A and B) and tissue (C and D) proteins were thus compared.

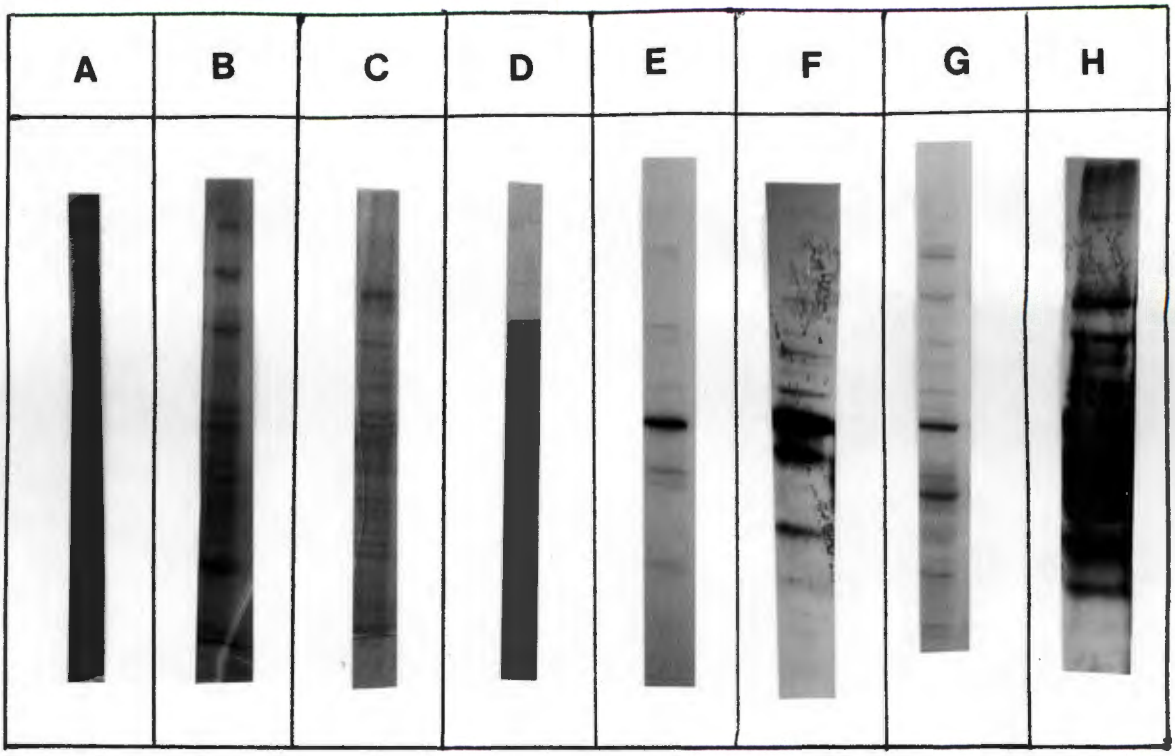

Figure 3.4 A comparison of the proteins in tissue and media from incubated hamster liver slices and freshly isolated hepatocytes.
Hamster hepatocytes were prepared as described in 2.1.3 and liver slices were prepared as described in 3.1.2. The hepatocytes were incubated as described in figure 2.11 and SDS-PAGE and fluorography were performed on samples of the media and cell extracts. The liver slices were treated as described in figure 3.3. The coomassie blue-staining patterns of the media from the cells and slices (A and B) and the tissue from each system (C and D) were compared, as were the fluorographic patterns (media: E and F and tissue: G and H) of the proteins synthesized and secreted by each system.

described above) and,following centrifugation,separating the microsomal pellets from the cytoplasmic and extracellular fluids.The microsomal pellets were extracted with sodium carbonate (as previously described) and VLDL was prepared from both the microsomal extracts and the cytoplasmic and extracellular fluids.The homogenization procedure was designed to ensure that all the cells in the slice were broken while the microsomal membranes remained intact (186).The results showed that of the previously defined tissue VLDL,about 80% was intercellular and only 20% was truly intracellular.

In order to establish whether the VLDL particles were binding to B/E receptors on cells in the slice,slices were incubated at 4°C in PBS,heparin or suramin (described above) because heparin and suramin prevent binding of lipoproteins to receptors.Subsequent preparation of VLDL particles from the solutions showed that about 40% of the intercellular counts could be recovered in either PBS,heparin or suramin.The remaining 60% remained trapped within the slices and it was assumed that their trapping was not due to binding of the particles to B/E receptors,but perhaps to "architectural" changes to the tissue (caused by the preparation of the slices) which hindered the passage,particularly of large particles,from the liver slices.

3.2.3 Pulse-chase experiments

Pulse-chase protocols were used to investigate the kinetic behaviour of the intracellular and trapped extracellular proteins.Slices were pulsed for 60 minutes with S^{35}-methionine and then chased for 2 hours in the presence of cold methionine and either azide,cycloheximide or colchicine.As azide and colchicine prevented protein secretion and cycloheximide inhibited only synthesis but not secretion,it was hoped that

details of the movement of particles from the cells into the intercellular spaces and then into the medium could be elucidated.

The extracellular pool of radioactive albumin remained constant in the chase period in the presence of azide (Fig. 3.5) or colchicine (results not shown) but dropped by nearly 70% in the presence of cycloheximide (Fig. 3.5). Loss from the intercellular pool was observed in all cases but this was most noticeable in azide-treated slices where the pool was not being fed by newly secreted albumin. The gain of albumin in the medium in the presence of cycloheximide was double that in the azide-treated slices because only the synthesis and not the secretion of proteins was inhibited by the agent.

3.2.4 Synthesis and secretion of apo B

Liver slices synthesized and secreted apo B (Fig. 3.6). In slices prepared from animals that had been treated with T3 to make them hyperthyroid, apo B48 synthesis was clearly induced (Fig. 3.7). Apo B from slice tissue appeared to more prone to proteolysis than was the case of isolated hepatocytes, probably because of the greater number of proteolytic enzymes freed during the homogenization of the tissue.

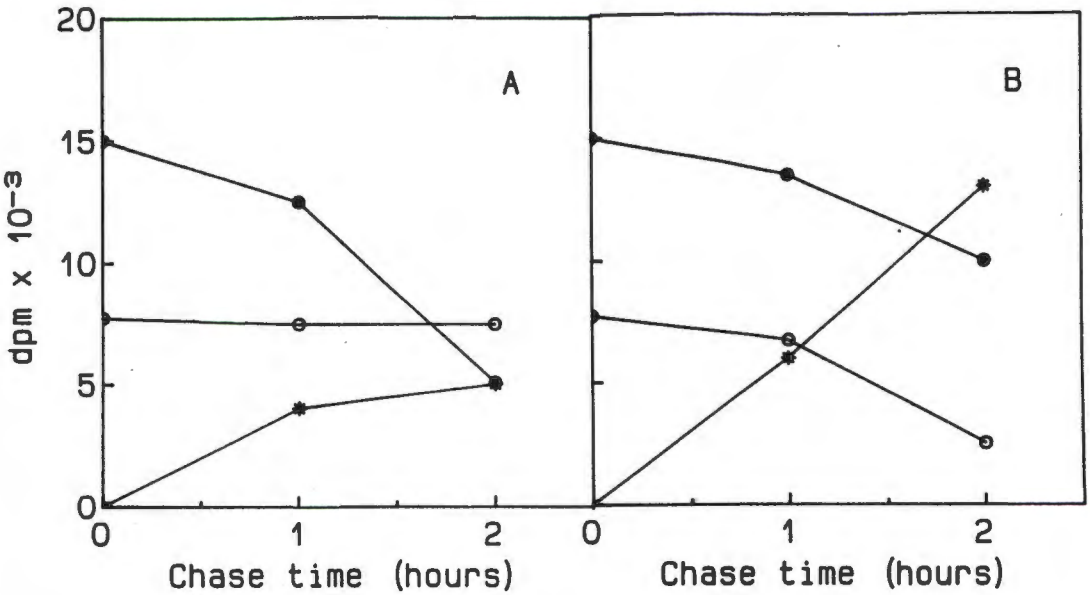

Figure 3.5 A pulse-chase experiment in incubated hamster liver slices to investigate the effects of azide and colchicine on movement of albumin from the cells into the tissue and then into the medium.
Liver slices prepared as described in 3.1.2 were subjected to a pulse-chase protocol as described in 3.2.3. The slices were incubated for 1 hour in methionine-free MEM with 50 μCi S^{35}-methionine in each 2 ml incubation. The medium was removed and fresh MEM with (A) azide (10 mM) or (B) cycloheximide (10 mM) was added and the slices were incubated for a further two hours. At the indicated times flasks were removed and the tissue and medium separated. The tissue was treated as described in 3.1.1 to result in a tissue component that was intracellular and another which was intercellular. Albumin was measured as described in 2.1.8, in fluorograms of SDS-PAGE from samples of each intracellular (●), extracellular (o) fraction (and the medium (*)) to investigate the movement of albumin from the putative tissue compartments and into the medium.

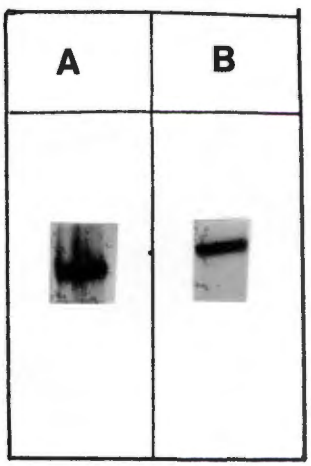

Figure 3.6 Apo B synthesized and secreted by hamster liver slices.

Hamster liver slices were prepared as described in 3.1.2 and were incubated for three hours as described in figure 3.1. The tissue and media were separated after the incubation and apo B immunoprecipitation was carried out (see 2.1.10) on media and tissue extracts prepared by homogenization of the whole slice in homogenization buffer. SDS-PAGE followed by fluorography was performed on the immunoprecipitated apo B from the tissue (A) and medium (B) to demonstrate the synthesis and secretion of apo B by incubated hamster liver slices.

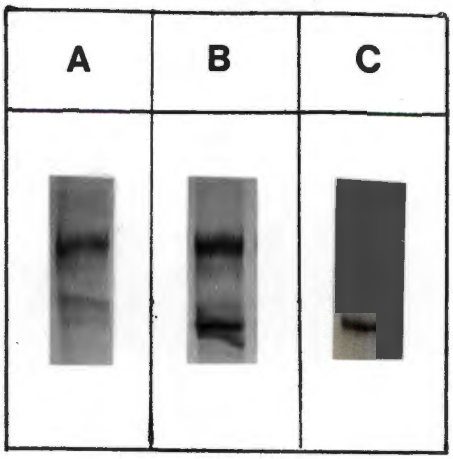

Figure 3.7 The induction of the synthesis of apo B48 by liver slices prepared from hyperthyroid hamsters.
Hamsters were rendered hyperthyroid as described in figure 2.19 and liver slices were prepared from both euthyroid and hyperthyroid animals. The slices were incubated as described in figure 3.1 and after three hour the cells and media were homogenizedd and apo B immunoprecipitation was carried out as described in figure 3.7. SDS-PAGE followed by fluorography was performed on the immunoprecipitated tissue from euthyroid (A) and hyperthyroid (B) hamsters. The induction of the synthesis of apo B48 was shown by comparison with radiolabelled medium secreted by hamster intestinal cells (C) which was known only to contain apo B48 (gift from Dr M. I. Combrinck).

Liver slices synthesize proteins as actively as do isolated cells but they fail to secrete proteins (particularly VLDL particles) as efficiently as isolated hepatocytes. In the liver, proteins secreted by hepatocytes leave the microsomes and are released into the intercellular spaces (spaces of Disse); from there they diffuse to the capillary beds and are removed to other parts of the body by the circulatory system. It was found that in liver slices, proteins (and particularly particle-associated proteins) accumulated within the intercellular spaces of the slices and had difficulty in leaving the tissue. It was assumed that the preparation of the slices caused architectural changes to them which prevented their efficient secretion of molecules. The careful separation of the tissue into intracellular and extracellular entities was found to overcome this problem. Careful homogenization of the slices released unbroken microsomes (containing intracellular secretory proteins) and cytoplasmic and extracellular proteins. Separation of the microsomes from the rest of the tissue enabled the microsomal contents to be released with sodium carbonate.

Thus in the case of apo B, the slices contained particle-bound (those released by sodium carbonate treatment of the microsomes), membrane-associated (those not released by sodium carbonate) and extracellular entities (those trapped in the intercellular spaces and released by homogenization of the slices). Slices were therefore quite suitable for the investigation of lipoprotein synthesis and secretion provided that they were fractionated in the appropriate manner.

Chapter 4: HEPATOCYTES PREPARED FROM DIET-ADAPTED HAMSTERS

4.1 Materials and Methods

Most of the components of the diets (lard,sunflower oil,oats,maize meal,skim milk powder,yeast) were purchased at a local supermerket.Cholesterol was obtained from Sigma Chemical Co. (St.Louis,USA).The Cholesterol kit was from E.Merck A.G. (Darmstadt,West Germany) while the SVR enzymatic triglyceride kit was from Behring Diagnostics (La Jolla,USA).The Vitamin capsules (Gevral) were from Lederle Laboratories (Isando,South Africa).

All other chemicals and reagents were of the best commercial quality available,as described in chapter 2.

4.1.1 Diets

Four dietary groups were investigated;the diets were designed to be calorifically equivalent (Table 4.1).The lipid diets were designed so that lipid constituted 20% of the calorie intake and when added,cholesterol was 0.06% of the mass of food intake;sucrose was 45% of the calorie intake in the sucrose-rich diet.Gas liquid chromatography,carried out by Dr J.Cohen,was used to analyse the saturation of the fatty acids in the commercial sunflower oil and lard used in the preparation of some of the diets.The sunflower oil consisted primarily of unsaturated C18:2 fatty acids while in lard C16:0 and C18:1 predominated (data not shown).Diets were made up on a weekly basis (using a food processor to thoroughly mix all the ingredients) and stored in the cold.Animals were fed for 2 to 3 weeks with ad libitum access to the food and water.Half an hour prior to sacrifice all food was removed from the cages.

Table 4.1 The composition of hamster diets.
Each diet was prepared in bulk and stored in a cold room. A food processor was used to thoroughly mix all the ingredients and 20 g was provided per animal each day.

Ingredient	Control * (g)	Lard/oil (g)	Sucrose (g)
Maize meal	586	298	298
Oats	586	298	298
Skim milk powder	298	298	298
Sugar	-	-	586
Lipid	-	110	-
Yeast	4.2	2.9	4.2
Salt Mix **	21	14.5	21
Cholesterol	-	0.65	-
Vitamins ***	15 caps	10 caps	15 caps

* The fat content of the control diet was about 5% and was mainly unsaturated fat

** The salt mix contained: NaCl (22.36g), Na_2HPO_4 (35.8g)
CaLactate (15.5g), FeCitrate (5.98g)
KI (0.15g), $MgSO_4$ (24.6g)
Ca_3PO_4 (68.8g), K_2HPO_4 (63.6g)

*** The vitamin capsules were emptied of their granular contents.

4.1.2 Incubations

The isolated cells were prepared as described in chapter 2. Incubation of suspended cells was in methionine-free MEM and each suspension volume was 5 ml. S^{35}-methionine (50 µCi) was added to each incubation, following thorough gassing. Unless otherwise stated, incubations lasted 3 hours. In the case of pulse-chase protocols, experiments rarely took more than 5 hours to complete.

4.1.3 Subcellular Fractionation

After incubation, the hepatocytes were separated from the medium by brief centrifugation in a benchtop centrifuge. The cells were placed in fractionation buffer and passed through a cell cracker 10 times to homogenize them (as described in chapter 2). The nuclei and mitochondria were removed by centrifugation at 4°C in a Beckman centrifuge at 10 000 x g for 5 minutes. The pellet was re-extracted and the combined supernatant was layered onto 1 ml of fractionation buffer containing 2 M sucrose and centrifuged in a Beckman ultracentrifuge in the SW 40 rotor at 100 000 x g for 60 minutes. The microsomal pellet at the interface between the two sucrose solutions was resuspended in sodium carbonate (to open the microsomes) giving a final concentration of 100 mM in 10 ml. After 30 minutes on ice, the solution was neutralized with HCl and placed on a 2 M sucrose cushion as described above. The centrifugation (100 000 x g for 60 minutes) in the SW 40 rotor in the Beckman ultracentrifuge yielded a microsomal membrane pellet at the interface between the 2 M sucrose and the sodium carbonate while the microsomal luminal contents were in the supernatant above the pellet. The supernatant was aspirated and retained and the pellet was treated again with sodium carbonate and centrifuged. The supernatant was combined with that from the first spin, and the pellet was sonicated in homogenization buffer with

two 5 second bursts with the microtip set on 3. Both samples were retained for the various analyses described in the previous chapters.

4.1.4 Measurement of liver and plasma lipids

Plamsa and liver triglycerides and total cholesterol were measured making use of commercially available kits. The assays were adjusted for smaller volumes and optimized for use on whole tissue homogenates.

Plasma samples were prepared from blood drawn from each animal half an hour after the last feed: animals were anaesthetized with ether and secured ventral side upwards, the heart was exposed and blood was drawn from it (approximately 5 ml was obtained per hamster). EDTA (4 mM) was immediately added to the blood because it was found that hamster blood rapidly formed clots, the samples were centrifuged for 30 minutes at 2000 rpm in a Beckman centrifuge and the plasma was removed. PMSF (1 mM) and aprotinin (100 U/ml) were added and the samples were stored at 4°C.

Livers were removed from each animal and 100 mg was cut from each liver. The samples were homogenized very well in a dounce homogenizer in 5 ml PBS with PMSF (1 mM), Benzamidine (1 mM) and Aprotinin (100 U/ml). The homogenate was centrifuged for 10 minutes at 10 000 x g in a Beckman centrifuge and the pellet was homogenized again in 5 ml PBS with protease inhibitors. The homogenate was added to the supernatant from the centrifugation and retained for analysis without lipid extraction.

Triglyceride was measured using a test kit (SVR enzymatic triglycerides test kit). This makes use of the quantitative enzymatic hydrolysis of triglycerides to glycerol and fatty acids; glycerol is then measured in a reaction that causes the

absorbance change at 340 nm.

Total cholesterol was also determined enzymatically using a test kit (Cholesterol enzymatic test kit). In this reaction peroxide is formed from the action of cholesterol oxidase, subsequent conversion of iodide to iodine by peroxide is used to measure the cholesterol content photometrically.

In the case of both reactions, volumes were lower than suggested on the kit instructions; photometric measurements were made in 0.5 ml cuvettes. Each sample was determined in duplicate and standards of known triglyceride or total cholesterol content were also measured. Homogenate samples were dilute and thus not turbid.

4.2 Results

4.2.1 Weight gained by hamsters

Weight gained by animals over the 2 to 3 week period on the different diets was difficult to measure accurately, but animals of comparable mass were chosen for each dietary group and after 2 to 3 weeks there was no significant difference in the average mass of animals in each group. Mature hamsters were used for all studies and therefore the active growth-phase was completed; longer periods of feeding would probably be required to observe any significant effects of the diets on weight gain.

4.2.2 Plasma lipids

The plasma lipids were measured in animals after 2 to 3 weeks feeding. Table 4.2 shows that both of the fat-containing diets led to increased plasma triglyceride concentrations. Sucrose feeding caused triglycerides in the plasma to rise in a manner comparable

Table 4.2 Plasma triglyceride and total cholesterol levels in male hamsters fed different diets for 2 weeks.
Male hamsters were fed one of the four diets described in Table 4.1 for 2 weeks. The animals were sacrificed half an hour after the last meal, in the mid-dark phase, and blood was drawn directly from the heart. Plasma was prepared as described in 4.1.3 and commercial kits were used to assess the plasma lipid and total cholesterol. Results were expressed as mmol/l and are the means of three different animals - standard deviations appear in brackets). The turbidity was assessed by eye and used to qualitatively assess the plasma triglyceride content. Plasma from each of the groups was compared to plasma from control animals.

	CONTROL	LARD	OIL	SUCROSE
Triglyceride (mg%)	1.8 (0.5)	8.8 (0.9)	3.7 (0.6)	4.5 (0.7)
Total cholesterol (mg%)*	2.5 (0.4)	8.6 (0.6)	4.1 (0.7)	3.3 (0.5)
Turbidity	+	++++	++	++

* The plasma cholesterol mainly found to be predominantly in the form of cholesterol ester.

to that in animals fed unsaturated fat. Total plasma cholesterol was unchanged in animals fed a sucrose-rich diet, but was increased by both fat-containing diets particularly the saturated fat-rich diet. In none of the dietary groups had the rise in plasma triglyceride reached a plateau after 14 days and in the animals fed fat-containing diets, plasma total cholesterol was also still increasing after 2 week of feeding (data not shown).

4.2.3 Liver morphology

The gross morphology of livers was noted during each hepatocyte preparation. While some animals did not conform, some general trends emerged from the study: liver mass was unaffected by the type of diet (Table 4.3) but the liver mass as a percentage of body mass was significantly increased by oil-feeding and after only 2 weeks on the lard- or oil-rich diets the livers were blotchy and had the appearance of "fatty liver".

Liver lipids were also measured (Table 4.4). Hamster liver triglyceride content was increased by feeding sucrose and saturated or unsaturated fat-containing diets, but total liver cholesterol content was only increased in animals fed the fat-rich diets. Most of the liver cholesterol was present as free cholesterol while in the plasma the majority had been in the form of cholesterol esters.

Table 4.3 Liver and hamster mass in animals fed various diets. Hamsters were fed one of the four diets described in 4.1.1 and half an hour after the last feed the animals were anaesthetized and the mass was recorded. The livers were removed and placed in beakers of ice cold saline and then weighed. The livers were either used for the preparation of liver slices or in the analysis of liver lipids. Statistical analysis was performed using the student,s T test.

	CONTROL			LARD			OIL		
	Hamster (g)	Liver (g)	Ratio %	Hamster (g)	Liver (g)	Ratio %	Hamster (g)	Liver (g)	Ratio %
	225.6	9	4	147.4	7.5	5.1	137.4	7	5.1
	184	9.1	4.9	174.4	8.3	4.8	176.1	10.6	6.1
	174.8	8.4	4.8	152.8	6.9	4.5	189	10.4	5.5
	168	9	5.4	162.1	6.7	4.1	138.9	6.3	4.5
	185.3	8.1	4.4	147	6.8	4.6	189.3	11	5.8
	155.7	7	4.5	184.1	8.6	4.7	156.5	8.6	5.5
	157.3	6.5	4.1						
	154.2	6.6	4.3						
	131.4	4.9	3.7						
	170.3	8.2	4.8						
	141.8	5.9	4.2						
Average	168(25.3)	7.5(1.4)	4.5 (0.5)*	161.3(15.2)	7.5(1)	4.6 (0.3)*	164.5(23.7)	9(2)	5.4 (0.5)*

* $p < 0.05$, thus the data are significantly different.

Table 4.4 Liver triglyceride and total cholesterol levels in hamsters fed various diets.
Hamsters were fed one of four different diets for 2 weeks as described in 4.1.1. The livers were removed, weighed and prepared for lipid determination as described in 4.1.3. Total cholesterol and triglycerides were measured using commercial kits. The results were corrected for total liver mass and are the means of three different animals.

	CONTROL	LARD	OIL	SUCROSE
Triglyceride (mg/liver)	324	451	506	486
Total cholesterol (mg/liver)*	122	255	245	201

* Liver cholesterol was primarily in the free form.

4.2.4 Protein synthesis

The rates of synthesis of total radiolabelled proteins by isolated hepatocytes from hamsters on different diets were rather variable. Table 4.5 shows results obtained in nineteen different experiments. The mean for each diet was very similar, but the large variations made it impossible to document specific effects that diet might have had on the rates of total protein synthesis. In some experiments, a "high methionine" approach was used in an attempt to overcome possible differences in the pools of intracellular methionine in different preparations. Hepatocytes were accordingly incubated with 0.1mM cold methionine and 10 uCi labelled amino acid, but results were also variable (results not shown). Animals were sacrificed at the same time in their light/dark cycle and so this could not have been the reason for variations; also cells were not used unless their viability was above 85% (determined by trypan blue exclusion). Variations probably reflected subtle differences between individual animals and not the effects of particular dietary pre-treatment.

The possibility of diet-induced alterations in albumin synthesis and secretion was investigated in some incubations. There was again a great variation in the total radioactivity of intracellular and medium albumin, but when expressed as a proportion of the total radiolabelled proteins in each system, the results were more consistent. Cells from oil-fed animals synthesized and secreted significantly less albumin than did those from chow-fed individuals; while the synthesis of cellular albumin by hepatocytes from lard-fed hamsters was similar to that in cells from chow-fed animals but the rate of secretion was significantly reduced (Table 4.6).

Table 4.5. The incorporation of radiolabelled methionine into proteins by incubated hamster hepatocytes prepared from animals fed different diets.

Hamster hepatocytes were prepared for incubation half an hour after the last meal, and in the middle of the dark phase, as described in chapter 2. Each 10 ml Erlenmeyer flask contained 5 x 10^6 cells in 5 ml well-oxygenated methionine-free MEM and 10 µCi S^{35}-methionine. The cells were incubated for 1 hour at 37°C in a shaking water bath, after which the cells and media were sonicated as described in chapter 2 and samples were taken for TCA precipitation and protein determination. Results are expressed as dpm of radioactivity inorporated per mg cell protein in one hour. The number in brackets after the average is the standard deviation for the given data.

	CONTROL	LARD	OIL
		dpm/mg	
	742259	366337	228054
	357701	932914	903024
	386377	715910	911940
	201660	356692	286378
		253756	241451
		118660	249632
			544218
			284370
			145955
Average	421999	457378	422780
	(228414)	(305808)	(295204)

Table 4.6 The radioactivity in cellular and medium albumin expressed as a proportion of the total radioactive protein synthesized by incubated hepatocytes prepared from hamsters fed different diets.

Hamster hepatocytes were prepared and incubated for one hour as described in table 4.5. The medium albumin was measured as described in 2.1.8 and values for the cellular albumin were obtained from fluorograms of the SDS-PAGE patterns for the cellular extracts. The medium, cellular and total albumin values were expressed as a proportion of the total incorporated radioactivity in each system. Results are the means and standard deviations for the indicated number of experiments. T-test analysis was used to assess the significance of the data.

	CONTROL n=4	LARD n=4	OIL n=4
Medium albumin	6.62% ±1.5 *	4% ±1.7	3.68% ±0.8 *
cellular albumin	15.86% ±1.8 *	16.58% ±8.3	9.46% ±3.8 *
Total albumin	22.48%	20.58%	13.14%

* $p < 0.02$, thus data are significantly different

4.2.5 VLDL synthesis and secretion

VLDL proteins were measured by TCA-precipitation of floated VLDL from media and intracellular samples. The intracellular and medium VLDL proteins were expressed as a proportion of the total radioactivity. In order to investigate the "secretability" of the VLDL population, in the case of cells from each diet-adapted animal, the ratio of medium to intracellular VLDL radioactivity was calculated. In suspended hepatocytes, VLDL synthesis by cells from chow-fed animals was significantly lower than in the case of cells from either oil- or sucrose-fed animals, the latter having the highest rates of VLDL synthesis (Fig. 4.1). The ratio of medium to intracellular VLDL was lowest in the oil-fed system but lard-feeding also impaired secretion of VLDL (Fig. 4.2). The results for VLDL synthesis were similar in cultured hepatocytes, but the ratios were different; increased tendencies to secrete were observed in the case of lard- and sucrose- feeding of animals. Cultured hepatocytes were incubated for 24 hours with radiolabelled methionine prior to the preparation of VLDL while suspended cells were incubated for only 3 hours - the plateau of radiolabelled amino acid incorporation was reached in cultured cells and thus may explain the differences. VLDL synthesis by suspended, freshly isolated cells was thus increased by both saturated or unsaturated fat feeding of hamsters, but unsaturated fats tended to impair the secretion of VLDL from such cells.

SDS-PAGE analysis of synthesized and secreted VLDL, followed by fluorography, enabled differences in the apoprotein-profile obtained in the case of cells from each type of animal to be recorded. While it is difficult to come to any firm conclusions from the data because of low counts, there were some interesting trends (Fig. 4.3). Albumin contamination was most often observed in media samples, but albumin was never more than 10% of the

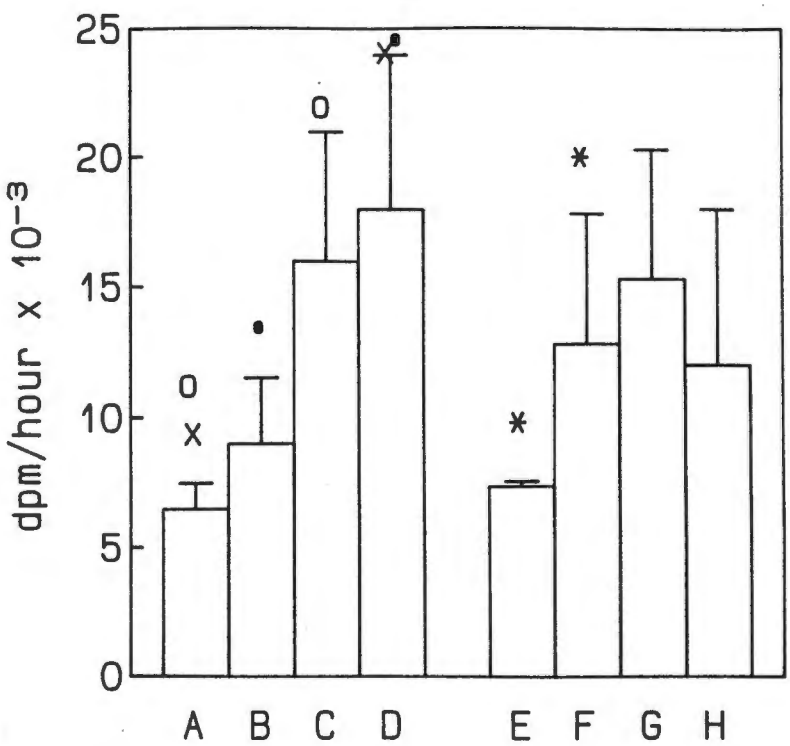

Figure 4.1 VLDL synthesis in incubated hepatocytes prepared from animals fed different diets.
Hamster hepatocytes were prepared from animals adapted to various diets as described in Table 4.5. Each Erlenmeyer flask of cells was incubated for 5 hours with 50 µCi S^{35}-methionine in 5 ml methionine-free MEM while those in tissue culture were cultured for 24 hours in 3 ml methionine-free MEM with 30 µCi radiolabelled amino acid.. VLDL was prepared from media and cell extracts as described in 2.1.9. The radioactivity associated with each sample of floated particles was measured by TCA precipitation; total incorporated cellular radioactivity and protein was also determined. The radioactivity in the VLDL floated from each sample was then expressed as a proportion of 10^{6} dpm of incorporated radioactivity in total cellular proteins in that sample. The results were also corrected for incubated cellular protein content and incubation time and are the means plus standard deviations for each group. Total VLDL from control incubated (A) and cultured cells (E) were compared from those from lard-fed (B and F), oil-fed (C and G) and sucrose-fed (D and H) animals and 4 animals were used per group. Statistical analysis was performed used the student's T analysis.

* $p < 0.05$ thus significantly different.
x, ●, O $p < 0.02$ thus significantly different.

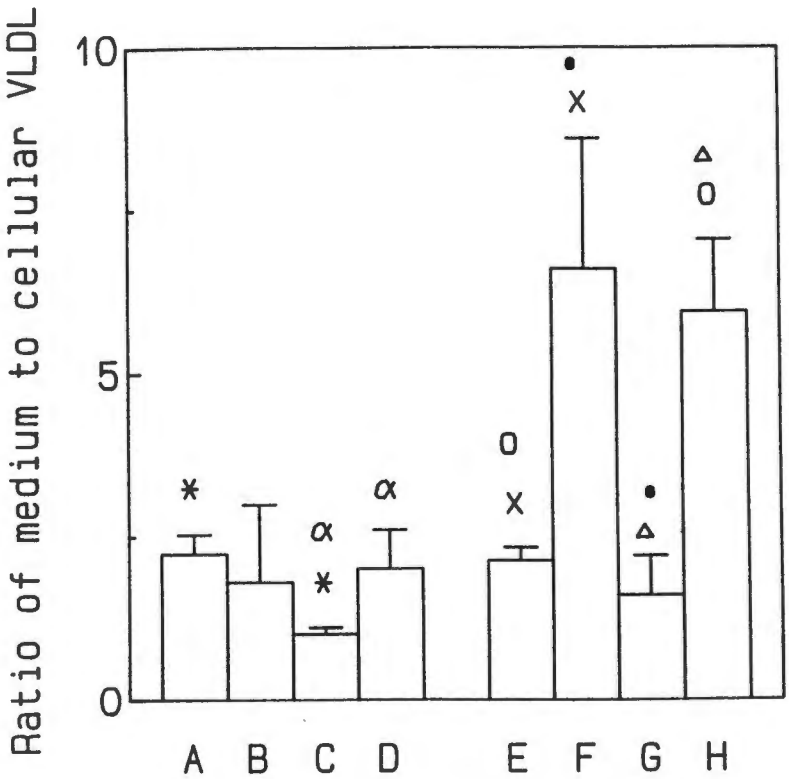

Figure 4.2 The secretability of VLDL synthesized by hepatocytes prepared from hamters fed different diets.
Hamster hepatocytes were prepared from animals adapted to various diets as described in Table 4.5. Each Erlenmeyer flask of cells was incubated for 5 hours with 50 uCi S^{35}-methionine in 5 ml methionine-free MEM while those in tissue culture were cultured for 24 hours in 3 ml methionine-free MEM with 30 μCi radiolabelled amino acid.. VLDL was prepared from media and cell extracts as described in 2.1.9. The radioactivity associated with particles floated from the cells and media was measured by TCA precipitation; total protein was also determined. The radioactivity in the VLDL floated from the cells in each sample was then expressed as a proportion of that in the medium to give a ratio of the two figures thus indicating the "secretability" of the particles. The results are the means standard deviation for each group. The ratios from control incubated (A) and cultured cells (E) were compared from those from lard-fed (B and F), oil-fed (C and G) and sucrose-fed (D and H) animals and 4 animals were used per group. Statistical analysis was performed used the student's T analysis.

*,●,O, , ,x $p < 0.05$ thus significantly different.

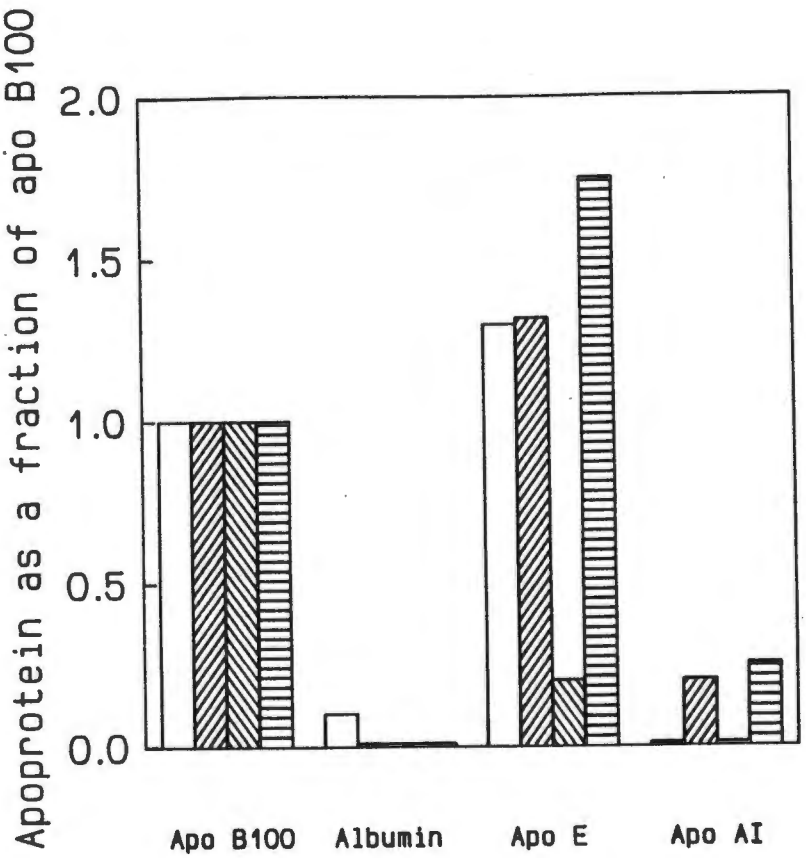

Figure 4.3 Apolipoprotein distribution in cellular VLDL prepared from hamsters fed different diets.
VLDL was prepared from cellular extracts prepared from incubated hamster hepatocytes as described in figure 4.1. Each sample was subjected to SDS-PAGE and fluorography and the lanes on the fluorogram were scanned with an integrating Helena scanner to obtain the proportions of each of the apolipoproteins. Apo B was taken to be 1 and values for each of the other apoproteins were expressed as a proportion of the apo B. The data is from single experiments representative of atleast 4 for each diet. Control (no hatching) samples were compared with those from animals fed lard (▨), oil (▧) or sucrose (▤).

total VLDL radioactivity. Apo B48 was never identified but varying amounts of high molecular weight proteolytic fragments of apo B100 were regularly seen - there was no effect of dietary pre-treatment on this. The ratio of apo E to apo B100 in cellular VLDL prepared from suspended cells was highest in sucrose- and chow-fed hamsters and very much reduced by oil feeding. The media in the case of each diet had a ratio of apo E : apo B100 of about 0.5 : 1. Apo AI synthesis and secretion seemed to be increased by sucrose- and oil-feeding (Fig. 4.4).

4.2.6 Apo B synthesis and secretion

Apo B synthesis and secretion was measured in suspended cells for reasons already discussed (chapter 2). (Sucrose feeding was omitted from this study). Apo B synthesis - expressed as a proportion of total protein synthesis - was highest in cells prepared from lard- and oil-fed animals (Fig. 4.5). When the data were expressed as percentages of albumin synthesis, cells from oil-fed animals clearly synthesized more apo B than did either of the other groups (Fig. 4.6). The data appeared to show that there was reduced secretion of apo B by hepatocytes of oil-fed animals and oil-fed individuals were accordingly chosen for the optimization of a method of successfully preparing membrane-associated and particle-bound apo B from cells.

Sodium carbonate releases VLDL from within microsomal membranes without adversely affecting the particles (99,100,101). Initial attempts to prepare intracellular apo B from membrane-associated and particle-bound pools were made on cells that were ruptured in sodium carbonate with dounce-homogenization, followed by removal of nuclei and mitochondria from the neutralized solution, and preparation of microsomal membranes (by ultracentrifugation for 1 hour at 100 000 x g) from the post-mitochondrial supernatant.

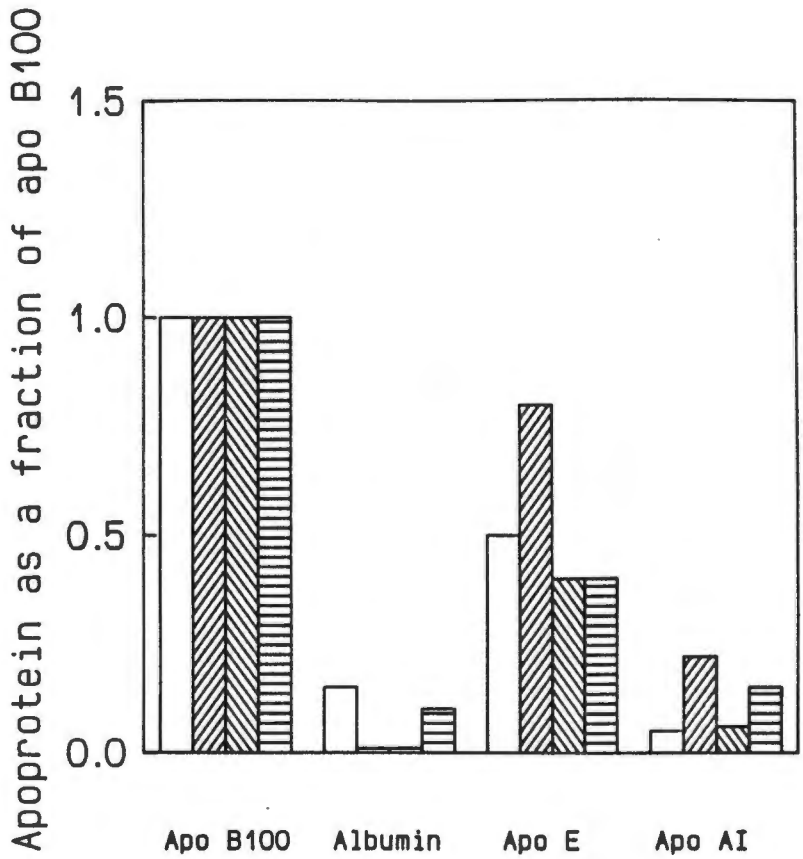

Figure 4.4 Apolipoprotein distribution in secreted VLDL prepared from hamsters fed different diets.
VLDL was prepared from the medium of incubated hamster hepatocytes as described in figure 4.1. Each sample was subjected to SDS-PAGE and fluorography and the lanes on the fluorogram were scanned with an integrating Helena scanner to obtain the proportions of each of the apolipoproteins. Apo B was taken to be 1 and values for each of the other apoproteins were expressed as a proportion of the apo B. The data is from single experiments representative of atleast 4 for each diet. Control (no hatching) samples were compared with those from animals fed lard (▨), oil (▧) or sucrose (▤).

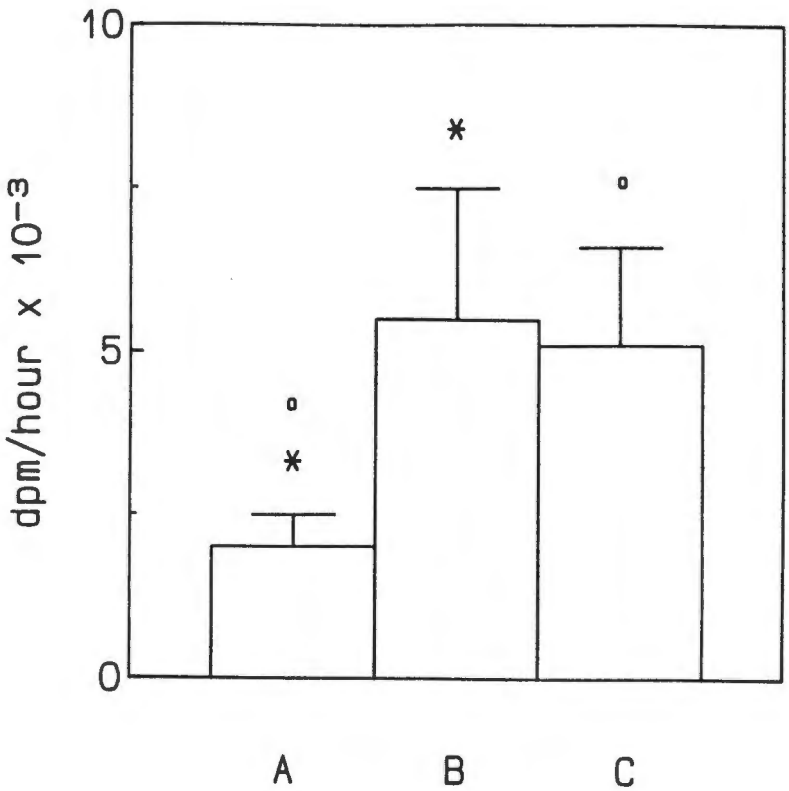

Figure 4.5 Apolipoprotein B synthesis by incubated hepatocytes prepared from hamsters fed different diets.
Hepatocytes were prepared from hamsters fed different diets as described in 2.1.3.The cells (5 x 10^6) were incubated,at 37°C in a shaking water bath, for 3 hours in 5 ml well-oxygenated methionine-free MEM with 50 µCi S^{35}-methionine.After the incubation the cells and media were separated and the cells were sonicated in homogenization buffer.The media and cell extracts were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE.The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8.The radioactivity associated with the combined cellular and media apo B was expressed as a proportion of the total incorporated radioactivity and normalized to 10^6 dpm of total incorporated counts in the same way as described in figure 4.1.Results from control (A),lard-fed (B) and oil-fed (C) animals were compared.The results are the means plus standard deviations for at least five different experiments and the statistical analysis made use of the student´s T test.

o,* $p < 0.05$,thus data were significantly different.

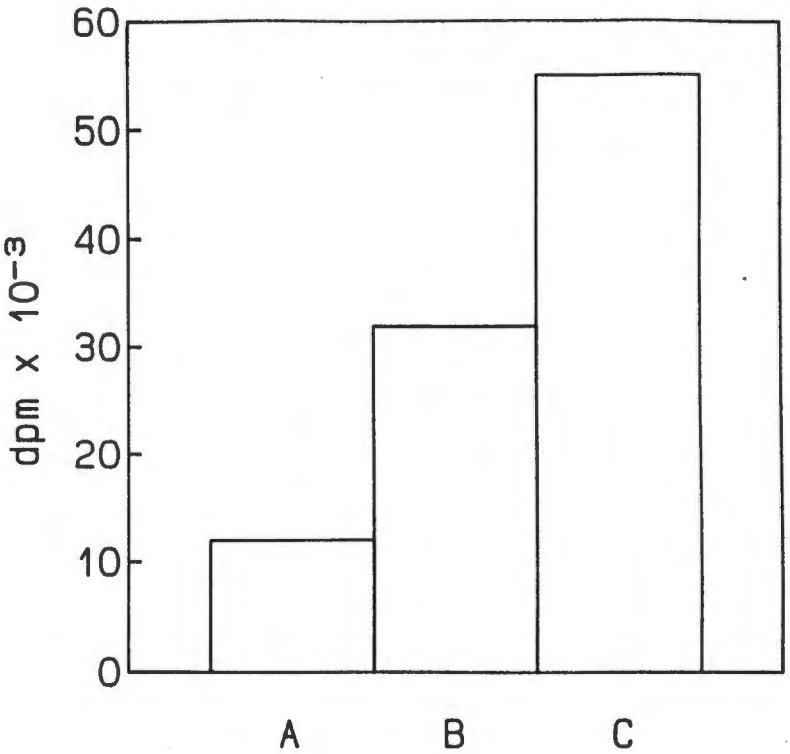

Figure 4.6 Apo B synthesis expressed as a proportion of albumin synthesis in incubated hepatocytes prepared from animals fed different diets.

Hepatocytes were prepared from hamsters fed different diets as described in 2.1.3. The cells (5×10^6) were incubated, at 37°C in a shaking water bath, for 3 hours in 5 ml well-oxygenated methionine-free MEM with 50 µCi S^{35}-methionine. After the incubation the cells and media were separated and the cells were sonicated in homogenization buffer. The media and cell extracts were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B, and cellular and medium albumin, as described in 2.1.8. The radioactivity associated with the combined cellular and media apo B was expressed as a proportion of the total incorporated radioactivity associated with albumin and normalized to 10^6 dpm of total incorporated counts in the same way as described in figure 4.1. Results from control (A), lard-fed (B) and oil-fed (C) animals were compared. The results are from a single experiment representing what was obtained in two others.

"Membrane-associated" apo B was immunoprecipitated from the resuspended pellet and particle-bound protein from the supernatant. Initial data showed that in the case of cells from oil-fed animals, 90% of the apo B100 was luminal. Under pulse-chase conditions, losses from the intracellular pools of apo B were matched by gains in the media (Fig. 4.7). The ratio of membrane- to particle-associated apo B decreased with the chase period, indicating that there was movement of apo B from the membranes to the lumen. In lard-fed animals, the situation was similar except that the luminal apo B was a smaller proportion of intracellular apo B and secretion was evidently more active (Fig. 4.8). When albumin was used as an indicator of microsomal release, being located in the microsomal lumen it should have been observed in the supernatant only of the ultracentrifugal spin, it was found that there was a 20% contamination of the membrane-associated proteins (Fig. 4.9). An alternative method was accordingly derived (see above methods): a cell cracker was used in the preparation of microsomes, and albumin contamination of the pelleted membranes was very low (less then 5%); in Fig. 4.10 the fluorographic patterns are shown. Several factors were found to be important in the preparation: the nuclei and mitochondria had to be re-extracted twice prior to preparation of the microsomes; pellets had to be collected on a 2 M sucrose cushion and resuspended in large volumes of sodium carbonate (5 ml final volume); and after sodium carbonate treatment, the pelleted microsomal membranes had to be treated again with sodium carbonate and pelleted for the second time in the presence of 0.5% BSA. The combined supernatants were used for the isolation of luminal proteins, and the membranes for membrane-bound proteins.

The distribution of apo B between intracellular compartments (membrane and luminal subfractions) and the medium was different in the case of cells from animals exposed to each diet.

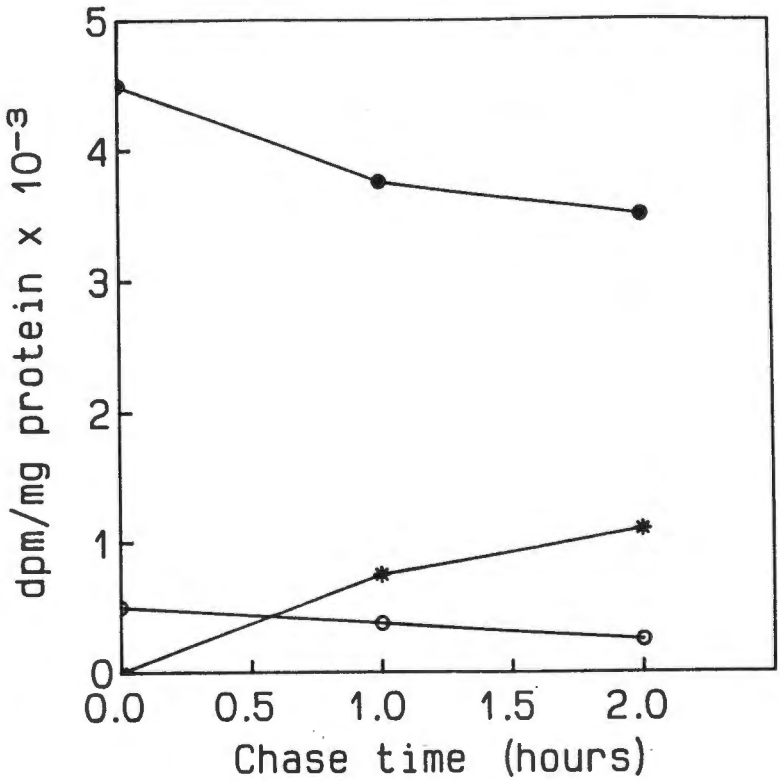

Figure 4.7 Apo B movement through intracellular compartments during a pulse-chase experiment involving hepatocytes from from oil-fed hamsters.
Hepatocytes were prepared from hamsters fed the oil-rich diet (4.1.1) as described in 2.1.3. The cells (5×10^6) were incubated, at 37°C in a shaking water bath, for 1 hour in 5 ml well-oxygenated methionine-free MEM with 100 µCi S^{35}-methionine, the medium was removed and the washed cells were incubated for a further 2 hours in MEM. At the indicated times the cells and media were separated and subcellular fractions were prepared from the cells as described in 4.2.6. The media and cell fractions were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8. The decline of radioactivity associated with intracellular membrane-associated (●) or particle-bound (o) apo B was contrasted with increasing secreted apo B (*). The results are from a single experiment representing what was obtained in two others.

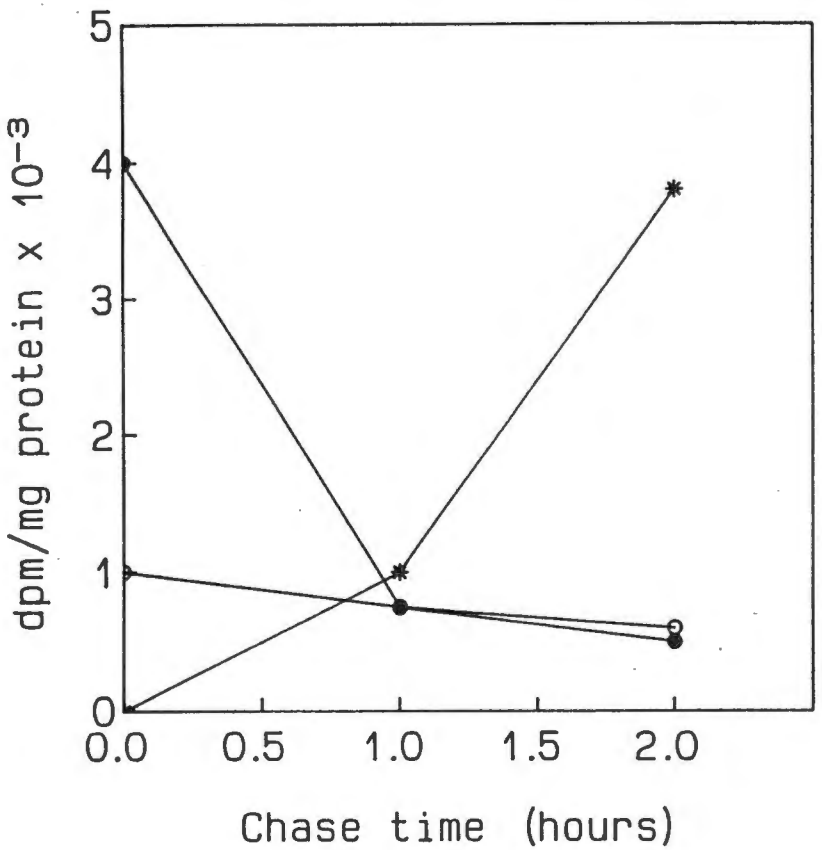

Figure 4.8 Apo B movement through intracellular compartments during a pulse-chase experiment involving cells from lard-fed hamsters.

Hepatocytes were prepared from hamsters fed the lard-rich diet (4.1.1) as described in 2.1.3. The cells (5 x 10^6) were incubated, at 37°C in a shaking water bath, for 1 hour in 5 ml well-oxygenated methionine-free MEM with 100 uCi S^{35}-methionine, the medium was removed and the washed cells were incubated for a further 2 hours in MEM. At the indicated times the cells and media were separated and subcellular fractions were prepared from the cells as described in 4.2.6. The media and cell fractions were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8. The decline of radioactivity associated with intracellular membrane-associated (o) or particle-bound (o) apo B was contrasted with increasing secreted apo B (*). The results are from a single experiment representing what was obtained in two others.

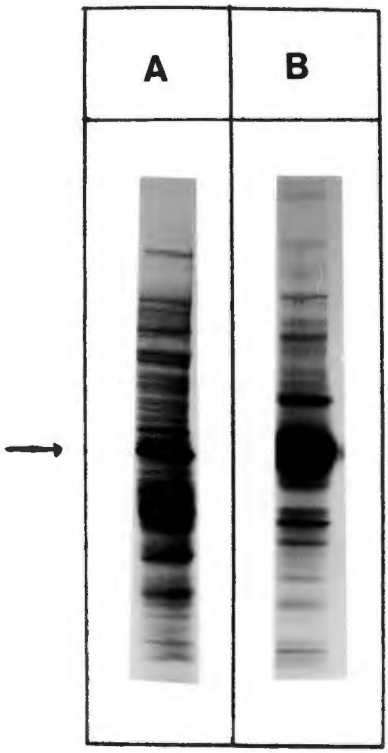

Figure 4.9 An assessment of the subcellular fractionation proceedure.
Subcellular fractions were prepared from incubated hepatocyes (see table 4.5) from oil-fed hamsters as described in 4.2.4. The intracellular luminal proteins (A) and those from the pelleted membranes (B) were subjected to SDS-PAGE and the fluorographic patterns were inspected to assess the contamination by luminal proteins, of the membranes. Albumin (indicated) was used as a marker.

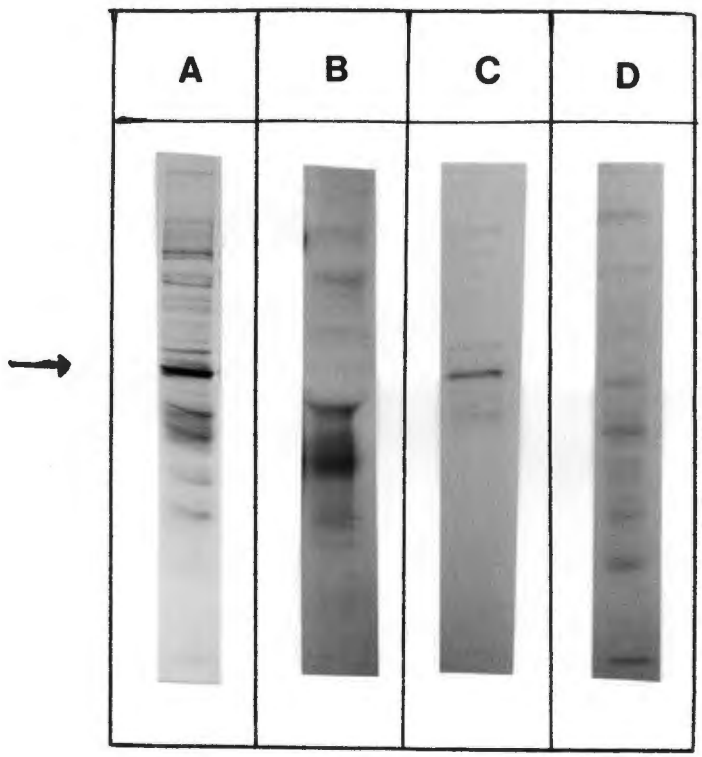

Figure 4.10 Assessment of a second method for preparing subcellular fractions from incubated hepatocytes prepared from oil-fed hamsters.
Oil-fed hamsters were used for the preparation of isolated hepatocytes (see 2.1.3). The cells were incubated as described in table 4.5 and subcellular fractions were prepared as described in 4.1.2. Samples of the subcellular fractions were subjected to SDS-PAGE and the fluorographic patterns were assessed. The presence of albumin (indicated) in membrane fractions was used to assess the contamination of microsomal membrane samples with luminal proteins. Luminal patterns were obtained for the samples subfractionated with by direct homogenization in sodium carbonate (A) as described an 4.2.4 or making use of the cell cracker (B) as in 4.1.2; membrane patterns (C and D respectively) were also obtained.

Membrane-associated apo B was corrected for luminal contamination by calculating the proportion of contaminating albumin,using this figure to obtain the amount of apo B that was truely membrane-associated.The "secretability" of apo B was similar to that of VLDL in the case of cells from hamsters on all three diets (Table 4.7).Hepatocytes from oil-fed hamsters secreted apo B least well,while cells from the chow-fed animals were apparently the most active in terms of apo B secretion.The "inhibition" of secretion of apo B in the fat-fed diets was more pronounced than that observed in the case of albumin and thus probably reflected a selective phenomenon.The "passage" of apo B through hepatocytes has been reviewed;apo B probably moves from a membrane-associated form to a luminal or particle-associated state,before secretion occurs.After a three-hour pulse-labelling,the contribution of the various "compartments" to total apo B was measured (Table 4.8).Apo B in hepatocytes from oil-fed animals was found predominantly in the luminal compartment (probably particle-bound);while in the lard- and chow-fed situations much of the apo B had been secreted.The cells from chow-fed animals were the only ones in which there was a greater proportion of membrane-associated intracellular apo B than particle-bound apo B.

Microsomes were prepared and exposed to trypsin prior to sodium carbonate treatment.In hepatocytes from oil-fed animals,trypsin treatment caused no significant alteration in the patterns of radiolabelled proteins in either the membrane or luminal compartments;immunoprecipitated apo B from both compartments showed a laddering of bands that indicated some degradation,but this was not limited to one particular compartment (Fig. 4.11).Trypsin treatment has since been used by Davis and co-workers (87) in the separation of apo B into two distinct ER pools:one that is secreted and the other that is degraded.In that study,the action of trypsin on the microsomes was completely

Table 4.7 An expression of the "secretability" of apo B synthesized by incubated hepatocytes from hamsters fed different diets.

Incubated hepatocytes from animals fed different diets were prepared for immunoprecipitation as described in figure 4.5. The immunoprecipitated apo B from cellular and medium samples was subjected to SDS-PAGE and the fluorographs were used for the calculation of the radioactivity in each sample, as described in 2.1.8. A ratio of the medium to cellular apo B was calculated for each immunoprecipitation from samples of animals fed each of the diets. The results are the means standard deviations for at least 3 different animals and statistical analysis was carried out making use of the t-test.

	Ratio of medium to cellular apo B
CONTROL	1 : 2.2 ±0.32*
LARD	1 : 1.18 ±0.27 *
OIL	1 : 0.32 ±0.2 *

* $p < 0.05$, thus data are significantly different.

Table 4.8 Apo B distribution between the intracellular fractions and the medium in incubated hepatocytes prepared from hamsters fed different diets.

Apo B was immunoprecipitated from intracellular membrane and luminal fractions (prepared as described in 4.1.2) from hepatocytes, incubated as described in fig 4.5 and prepared from animals fed different diets. The contribution of each fraction to the total immunoprecipitated apo B was calculated. The results are from single experiments, although they represent what was obtained in 3 similar experiments for each diet.

	CONTROL	LARD	OIL
Membranes	2%	21%	27%
Lumen	12%	32%	55%
Media	68%	47%	18%

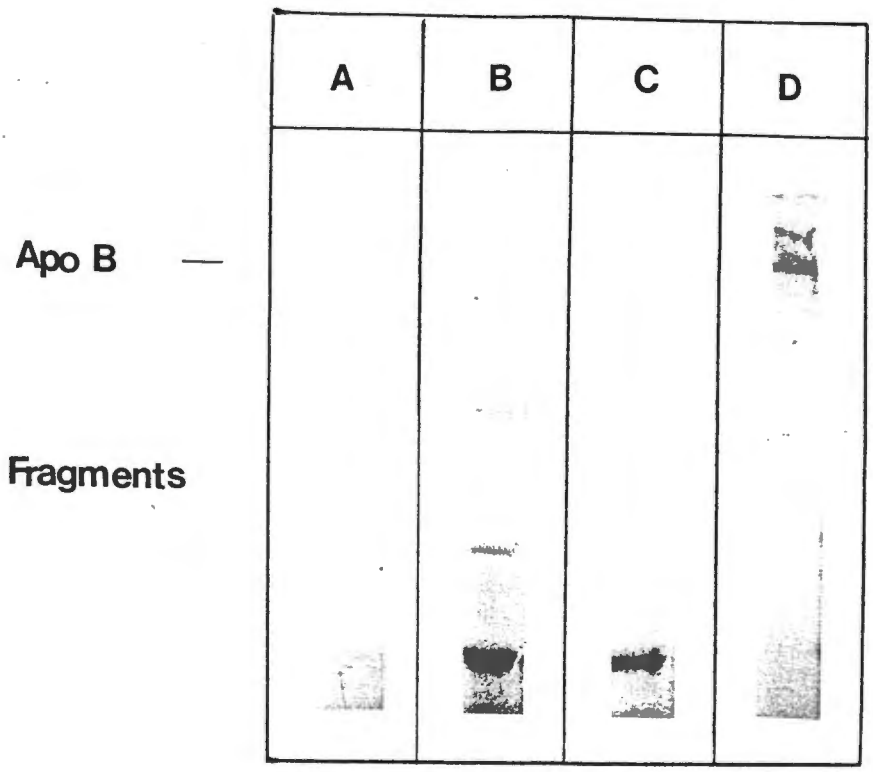

Figure 4.11 The effect of trypsin treatment of microsomes on the apo B immunoprecipitated from the intracellular fractions of incubated hepatocytes from oil-fed hamsters.

Microsomes were prepared from incubated hamster hepatocyets after homogenization with a cell cracker. After the removal of the nuclei and mitochondria the microsomes were pelleted in a centrifugation spin for 1 hour at 100 000 x g. The pelletted microsomes were exposed to trypsin (0.05% (w/v)) for 20 minutes, excess FCS was added to halt the reaction. The microsomal membranes and liminal contents were prepared by sodium carbonate treatment as described in 4.2.2. Apo B was immunoprecipitated (as described in 2.1.10) from membrane fractions treated (A) or not treated with (B) trypsin and from the luminal samples (C and D, respectively).

halted by the addition of soybean trypsin inhibitor,but the present study made use of FCS.There is the possibility that the trypsin was not inhibited completely and that residual cleavage of apo B took place causing apparent proteolysis in both the microsomal compartments.

4.2.7 Apo B degradation

The degradation of apo B in cells from animals from the three dietary groups was assessed in pulse-chase studies since it appeared that degradation might be the reason for the differences in secretion patterns and intracellular distribution observed in the case of the different diets.Cells were pulse-labelled for 1 hour and then washed twice,and chased for up to three hour in the presence of excess cold methionine;cycloheximide was added in some experiments and not in others.There was no evidence of significant apo B degradation in cells from any of the dietary groups under any conditions (Figs. 4.12,4.13 and 4.14). Degradation of total proteins was never more than 6% per hour,the figure for albumin was 3% and that for apo B was always less than 6% (data not shown).Cycloheximide addition to the chase medium clearly inhibited secretion of apo B (Fig. 4.15) without giving rise to a significant increase in its degradation.

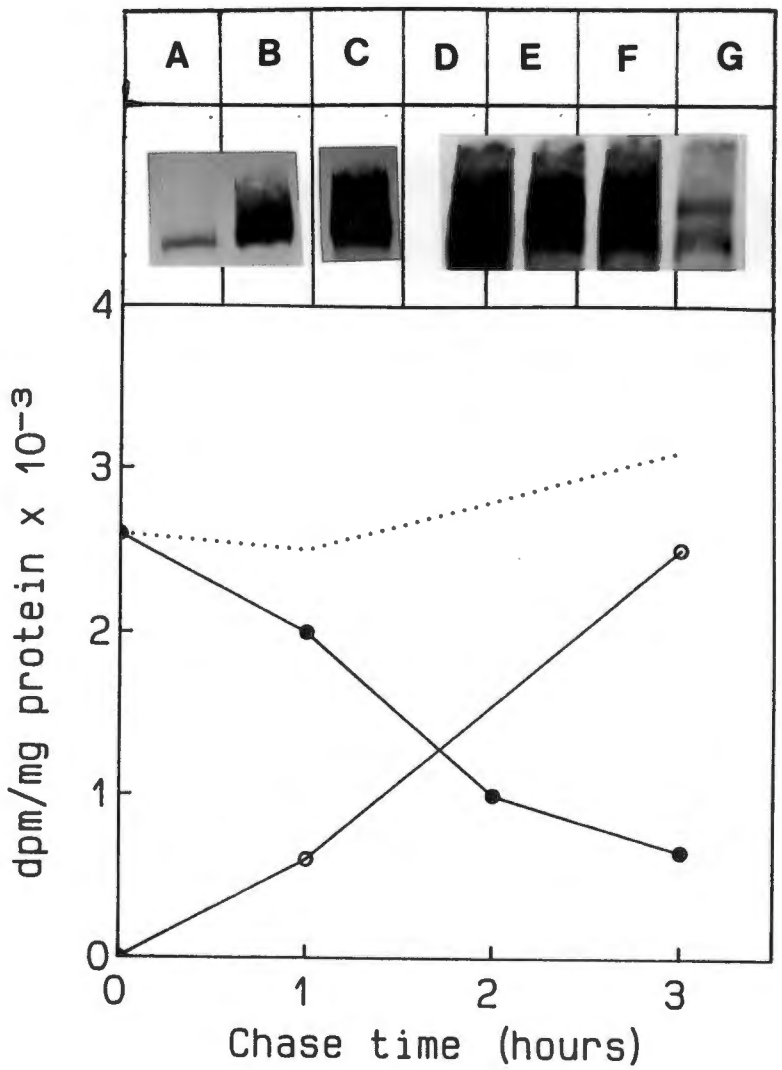

Figure 4.12 Pulse-chase experimentation to investigate apo B degradation in hepatocytes from control hamsters. Hepatocytes were prepared from control hamsters (4.1.1) as described in 2.1.3. The cells (5 x 10^6) were incubated, at 37°C in a shaking water bath, for 1 hour in 5 ml well-oxygenated methionine-free MEM with 100 µCi S^{35}-methionine, the medium was removed and the washed cells were incubated for a further 2 hours in MEM. At the indicated times the cells and media were separated. The media and cell samples were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8. The decline of radioactivity associated with the cellular (●) apo B was contrasted with increasing secreted apo B (o), the sum of these figures is indicated by the dotted line. Inset are the fluorographic patterns obtained in the immunoprecipitations of media (A - C) and cells (D - G) at the indicated times. The results are from a single experiment representing what was obtained in two others.

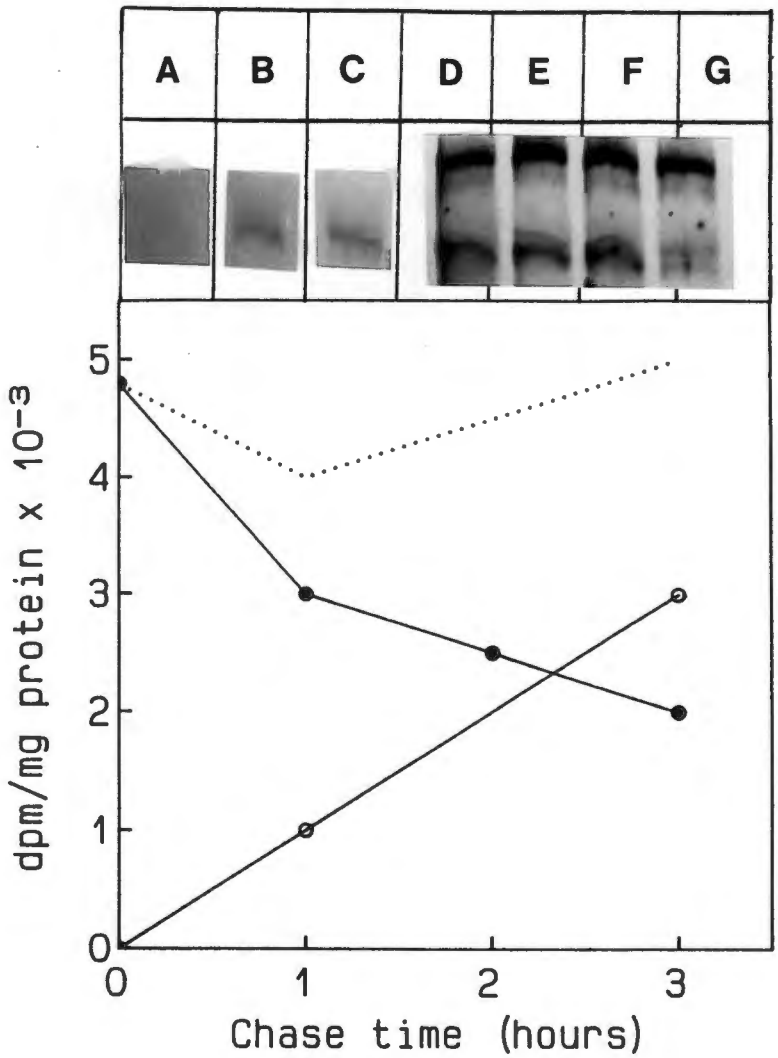

Figure 4.13 Pulse-chase experimentation to investigate apo B degradation in hepatocytes from lard-fed hamsters. Hepatocytes were prepared from lard-fed hamsters (4.1.1) as described in 2.1.3. The cells (5 x 10^6) were incubated, at 37°C in a shaking water bath, for 1 hour in 5 ml well-oxygenated methionine-free MEM with 100 µCi S^{35}-methionine, the medium was removed and the washed cells were incubated for a further 2 hours in MEM. At the indicated times the cells and media were separated. The media and cell samples were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8. The decline of radioactivity associated with the cellular (●) apo B was contrasted with increasing secreted apo B (o), the sum of these figures is indicated by the dotted line. Inset are the fluorographic patterns obtained in the immunoprecipitations of media (A - C) and cells (D - G) at the indicated times. The results are from a single experiment representing what was obtained in two others.

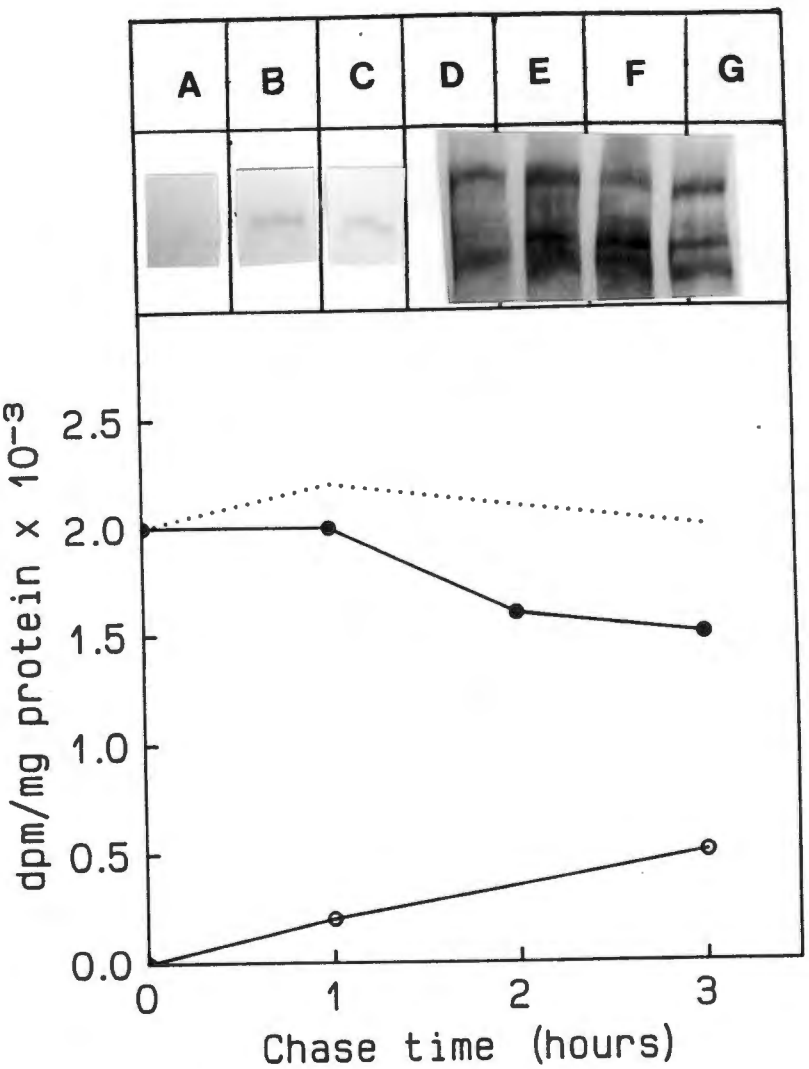

Figure 4.14 Pulse-chase experimentation to investigate apo B degradation in hepatocytes from oil-fed hamsters. Hepatocytes were prepared from oil-fed hamsters (4.1.1) as described in 2.1.3. The cells (5 x 10^6) were incubated, at 37°C in a shaking water bath, for 1 hour in 5 ml well-oxygenated methionine-free MEM with 100 µCi S^{35}-methionine, the medium was removed and the washed cells were incubated for a further 2 hours in MEM. At the indicated times the cells and media were separated. The media and cell samples were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8. The decline of radioactivity associated with the cellular (●) apo B was contrasted with increasing secreted apo B (o), the sum of these figures is indicated by the dotted line. Inset are the fluorographic patterns obtained in the immunoprecipitations of media (A - C) and cells (D - G) at the indicated times. The results are from a single experiment representing what was obtained in two others.

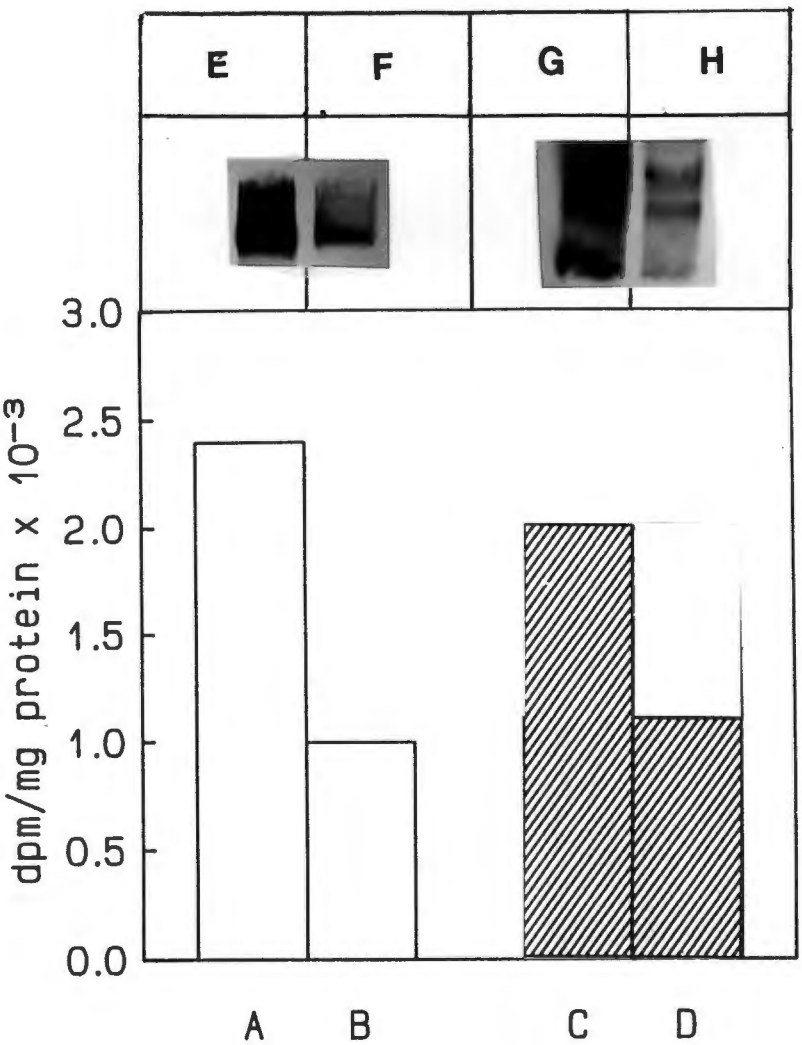

Figure 4.15 Pulse-chase experimentation to investigate the effect of cycloheximide on apo B degradation in hepatocytes from control hamsters.

Hepatocytes were prepared from control hamsters (4.1.1) as described in 2.1.3. The cells (5 x 10^6) were incubated, at 37°C in a shaking water bath, for 1 hour in 5 ml well-oxygenated methionine-free MEM with 100 µCi S^{35}-methionine, the medium was removed and the washed cells were incubated for a further 2 hours in MEM in the presence or absence of cycloheximide (10 mM). After 2 hours the cells and media were separated. Media and cell samples were immunoprecipitated as described in 2.1.10 and the immunoprecipitated proteins were separated by SDS-PAGE. The fluorograms prepared from the dried gels were used to determine the radioactivity associated with the immunoprecipitated apo B as described in 2.1.8. The increase of apo B in the medium in the absence (A) or presence of cycloheximide was compared with the decline of radioactivity associated with the cellular apo B under both conditions (C and D respectively). Inset are the fluorographic patterns obtained in the immunoprecipitations of media (E and F) and cells (G and H) under both conditions. The results are from a single experiment representing what was obtained in two others.

4.3 Discussion

There is little evidence in the literature to suggest that the *in vivo* ingestion of different fats or carbohydrates alters the synthesis or secretion of total proteins (or albumin) by isolated hepatocytes. Fasting causes reduced synthesis and secretion of a number of hepatic proteins, including albumin and some of the apoproteins, and re-feeding stimulates their synthesis and secretion (112, 188). In one study, hepatocytes isolated from rats fed saturated fat and sucrose were shown to synthesize more protein than those from animals fed unsaturated fat and starch (129); further investigation suggested that increased albumin synthesis might have accounted for this and that it was a result of fat saturation and not the carbohydrate source (N. M. Friedlander - Hayward - unpublished data).

In the present study, the incorporation of S^{35}-methionine into total protein by isolated hepatocytes was variable and no significant effects of different prior dietary intake were observed. Albumin synthesis and secretion was equally variable, but when expressed as a proportion of the total protein synthesis by the cells, dietary variations were in fact evident. Hepatocytes isolated from hamsters fed a diet rich in unsaturated fat, synthesized and secreted significantly less albumin than did cells isolated from animals fed chow or saturated fat-rich diets. These findings agree with observations in cells obtained from rats (129) but why this should happen remains unclear. Rates of protein synthesis are under the control of a number of factors: DNA transcription, mRNA translation and the complex processes of amino acid activation, peptide chain initiation, elongation, termination, folding and processing (66). Hormonal, nutritional and nervous stimuli may alter any of these steps and thus affect the synthesis or secretion rate of a

particular protein. The effects of the saturation of incoming lipid on albumin synthesis would thus probably not be a direct one. Albumin does play a role in transporting free fatty acids from the intestine to hepatocytes in the liver, but there is no evidence for differences in binding affinities between albumin and saturated or unsaturated fatty acid (189). Albumin inhibits VLDL secretion in cultured rat hepatocytes (190); whether the reduced albumin synthesis was the reflection of increased VLDL secretion from the liver *in vivo* in the oil-fed hamsters, is unclear. While there was no evidence of alterations in body mass, liver mass as a proportion of body mass tended to be increased by unsaturated-fat feeding. The increased liver mass was accompanied by the macroscopic appearance of "fatty liver", but whether this had any effect upon the synthesis and secretion of albumin is not certain.

Plasma total cholesterol and triglyceride concentrations were increased in hamsters fed diets rich in both saturated or unsaturated fat. Liver cholesterol and triglycerides were also increased; the effects were most marked in the case of animals fed saturated fat and cholesterol. In the plasma, most of the cholesterol was free while in the liver it was mainly in the form of cholesterol esters. Dietschy and co-workers fed hamsters saturated fat (hydrogenated coconut oil) or unsaturated fat (safflower oil) and cholesterol and after 4 weeks, hepatic cholesterol ester content was significantly higher in both fat-fed groups than in control animals, while the plasma LDL-cholesterol was significantly higher only in the saturated fat-fed animals (140). In that study, liver cholesterol ester contents were higher in animals fed unsaturated fats and cholesterol than in those fed saturated fat and cholesterol, while raised hepatic LDL clearance ensured that plasma LDL-cholesterol was kept low in animals fed unsaturated fat and cholesterol. Values for plasma or hepatic triglycerides were not

reported. Therefore, while the results differ somewhat from those obtained by Dietschy, the trends are similar and failure to correspond entirely might be due to different sources of saturated and unsaturated fats, different periods of time on the diet or sacrifice of the animals at different times after the last meal. [Dr A.D Marais in this laboratory, has evidence for a biphasic response, in the 12 hours after ingestion of saturated fat and cholesterol, of plasma cholesterol concentrations (data not shown)]. It is clear that the feeding patterns of the hamster are associated with fluctuations in enzymes, hormones and stimuli and that it is important that each animal in a series of experiments is sacrificed at precisely the same time after the last meal (157).

The study of VLDL synthesis and secretion was made difficult by the variations in total protein (and thus VLDL) synthesis, therefore VLDL synthesis was expressed as a proportion of total protein synthesis. The ratio of secreted VLDL to intracellular VLDL was then used to express the "secretability" of the VLDL in each incubation. Rates of synthesis of VLDL were similar when cultured cells were compared with suspended cells, but the ratios of secreted to intracellular VLDL were higher in cultured cells. This was probably because the cultured cells had reached the plateau of incorporation of labelled amino acid into proteins, while incubated cells were still in the linear phase; data obtained from the cultured cells were thus biased towards accumulation of VLDL in the medium. The effect of increasing medium VLDL on cellular VLDL synthesis and secretion would be expected to have no effect since Davis has reported that there is no uptake of VLDL by cultured hepatocytes (96).

The consequences of sucrose-feeding on VLDL synthesis and secretion by cultured rat hepatocytes are well documented (112, 114). In this study in cultured hamster hepatocytes, the rate

of VLDL synthesis was not significantly different from that in control cells,while in the suspended cells there was a significant difference. In suspended cells,the ratio of medium to intracellular VLDL was similar to that of control cells,but in cultured cells this ratio was significantly higher. Intracellular VLDL from suspended hepatocytes contained more apo E and apo AI than did the control particles,concurring with data obtained in cultured hepatocytes prepared from sucrose-fed rats (117,120).Since hepatocytes from sucrose-fed hamsters were similar to cells prepared from rats in terms of their responses to the dietary pre-treatment,it was assumed that hamster hepatocytes could be used to investigate VLDL and apo B metabolism.

Suspended hepatocytes prepared from oil-fed hamsters synthesized significantly more VLDL than did those prepared from control animals but in oil-fed cells there was evidence for decreased secretion of the particles.Similar results were obtained in the case of cultured cells.Suspended cells from lard-fed animals did not differ significantly from control cells but in culture,VLDL synthesis was significantly higher and so too was the ratio of medium to cellular VLDL.VLDL composition was also altered by dietary fat:intracellular apo E synthesis was reduced in cells from lard-fed animals,but was unchanged in cells from animals fed oil,while oil-feeding caused increases in intracellular VLDL-band apo AI and in secreted particles enriched in apo E and apo AI. It has been suggested that cholesterol plays a role in regulating apo E secretion (68) but these data suggest that fat saturation might also be important in this regard.

To assess the significance of the effects of dietary pre-treatment on the synthesis and secretion of VLDL in suspended hepatocytes,the effects on apo B alone were considered. Apo B synthesis was significantly increased in hepatocytes from animals

fed both saturated and unsaturated fat,and yet apo B secretion was significantly reduced,particularly in the oil-fed system. Apo B was not degraded in cells prepared from animals fed any of the diets. Thus under control conditions over a 3 hour incubation period, about two thirds of the synthesized VLDL and apo B was secreted and there was no significant degradation of apo B. Saturated fat and cholesterol caused a two-fold increase in apo B synthesis without a significant change in VLDL apoprotein synthesis. The proportion of VLDL secreted was unchanged,but only half of the synthesized apo B was secreted;there was no degradation of apo B. Therefore the increased apo B synthesis was not accompanied by increased secretion of VLDL particles;since no degradation of the apo B took place it could be assumed that there was some accumulation of apo B intracellularly. Feeding of unsaturated fat and cholesterol resulted in a two-fold increase in both VLDL and apo B synthesis,with one-half of the VLDL being secreted and only one-fifth of the apo B being secreted;since no intracellular degradation of apo B was detected,it appeared as if substantial intracellular accumulation of apo B took place.

In order to substantiate the intracellular accumulation of apo B, the cells were fractionated into membrane-associated and particle-bound or luminal compartments by two different methods. It was shown that under control conditions there was more apo B associated with the membranes than was luminal,but much apo B was secreted. Saturated fat feeding resulted in an increase in the luminal apo B and so too did unsaturated fats,only this was more pronounced. Therefore it appeared as if the accumulating apo B was present in a form that was capable of existing free from the microsomal membranes and thus was associated with some lipid.

While there is some controversy about the intracellular

degradation of apo B in liver cells,the process does seem to be regulated by insulin,and in studies in which apo B degradation has been investigated, hepatocytes have been cultured in the presence of varying amounts of insulin (96,97,108). In this study insulin was omitted from the incubation medium,and no degradation of apo B was detected.This result contrasts with that in Hep G2 cells,where apo B mRNA was very stable with a long half life which led to the conclusion that fluctuations in apo B synthesis and secretion were regulated by apo B degradation (76,103).Hepatoma cell lines are transformed and thus might not be the best model on which to base theories;never the less,in rats there is evidence for apo B degradation while in chickens there is not,and species differences may thus account for the discrepancy. It is possible that cycloheximide alters the rate of apo B degradation because of the absence of apo B degradation in chick hepatocytes where cycloheximide was added to the chase medium (97),and in cultured rat hepatocytes apo B degradation was detected in the absence of added cycloheximide (96).

A number of studies have been reported in which the membrane and luminal forms of apo B have been separated;Hep G2 cells or chow-fed animals were usually used.Classic electromicrographic studies showed that nascent VLDL particles were only observed in the smooth ER and Golgi complex.Thus most of the membrane-associated apo B is probably in the rough ER.Wong and Pino found in whole rat liver that 90% of the intracellular apo B was membrane-associated,while 50% of the intracellular apo B was in the ER and the remainder in the Golgi;40% of the apo B was lost in the fractionation procedure (102).Lane and co-workers found in chick hepatocytes that 40% of the intracellular apo B was membrane-associated,mostly in the ER,but that 30% of apo B in the Golgi was also membrane-bound (97). In rat hepatocytes,Davis did not investigate the contribution of membrane-bound apo B to the total,but only 10% of the total apo B was found in the Golgi

subfraction (96). In Hep G2 cells, an assembly compartment has been suggested to be the place after apo B leaves the ER, where association into VLDL particles takes place (89,98), while no losses of apo B were experienced during preparation of the vraious fractions. Gibbons has explained these conflicting data by suggesting that apo B associates and dissociates from the microsomal membrane all the way along the secretory pathway, a mechanism which allows for lipid addition and modifications of the particle (104). For the present study, the published data were interpreted in terms of the available techniques of subcellular fractionation; it was decided that the whole microsomal fraction would be divided into membrane and luminal forms (with albumin as a marker), removing the necessity for subfractionation of the ER and Golgi fractions. Membrane-associated apo B was operationally defined as being found in the rough ER and luminal apo B in the Golgi and smooth ER. Bostrom and co-workers have suggested some precautions necessary for the sodium carbonate treatment if all the luminal proteins are to be freed; these have been taken into account in the present study (90).

In control cells, the membrane-associated fraction of intracellular apo B was about 60%; in between the figures obtained in rat-liver cells and chick hepatocytes. Fat-feeding reduced this to 40% (saturated fat) and 30% (unsaturated fat). Fat feeding thus significantly changed the intracellular distribution, and the diet of experimental animals is clearly a further factor that may account for many variations. It is generally accepted, because of data obtained in Hep G2 cells and intestinal cells, that the rate of apo B transcription and translation is constitutive, and unlikely to be responsible for the large changes in apo B synthesis and secretion rates that are observed under various conditions (72,96,191). The translocation of apo B across the ER membrane and its degradation have not been demonstrated in

hamster hepatocytes and there is a possibility that changes in the intracellular distribution shown to occur with altered diets,may play a role in controlling the rate of secretion of apo B;what mechanisms might be responsible for this is unclear.There is the further possibility that apo B48 might be a key factor in the process of apo B degradation (and the mechanisms by which the two pools of ER apo B are separated in the translocation process).Since,under euthyroid conditions,hamster hepatocyes synthesise no apo B48,the control mechanisms might differ from the rat where apo B48 is present.

Various diets have been shown to effect VLDL-triglyceride secretion (68),either in terms of the number of particles secreted,or in that of the size of the particles.In this study,changes in the rate of apo B synthesis have been demonstrated.It is probable that in hamsters,incoming lipid,whether saturated or unsaturated,in the presence of some cholesterol,stimulates increased apo B biosynthesis by a mechanism that is not clear.That the rate of apo B and VLDL secretion dropped,in the case of unsaturated fat,could imply that the incoming lipid is not repackaged and secreted,but oxidised (as unsaturated fats have been suggested to be) or that larger particles (not requiring additional apo B) were secreted.Kalopissis and co-workers have found that hepatocytes prepared from rats previously fed saturated fat showed a 40% decrease in VLDL secretion:they suggested that incoming free fatty acids do not stimulate VLDL secretion as expected and also inhibit hepatic lipogenesis (68).If the incoming saturated and unsaturated fats had such an effect in hamsters,the results could be explained.

Dietary fat saturation has been shown to affect receptor-dependant uptake of LDL in hamsters (139),and it appears

also to affect the synthesis and secretion of apo B and VLDL. A model derived from the present data could be that increased incoming fat, in the presence of cholesterol, caused increased synthesis of apo B and VLDL-proteins, especially if the lipid was unsaturated. Inhibition of the secretion of apo B occurred by some mechanism, probably involving the effects of the incoming lipids and concomitant fatty acid oxidation, so that apo B accumulated in the hepatocyes without being degraded. Unsaturated fats caused greater inhibition of apo B secretion than did saturated fats, but under both conditions plasma triglycerides and cholesterol levels increased; suggesting that a diet of unsaturated fat caused the secretion of fewer, lipid-laden VLDL particles. Receptor-dependent LDL uptake is diminished in saturated fat-fed animals, but not in those fed unsaturated fat. Thus saturated fats and cholesterol clearly have the potential to be atherogenic in the male hamster, while unsaturated fats do not.

The study has therefore successfully investigated the effects of four different diets upon VLDL and apo B synthesis, secretion, intracellular distribution and degradation in both freshly suspended and cultured hamster hepatocytes. The atherogenic effects of saturated fats have been demonstrated, but unsaturated fats have also been shown to affect lipoprotein metabolism, although it appears to be in a manner more favourable to the long term prospects of the animals, than in the case of dietary saturated fats. The intracellular processing of VLDL under such conditions remains an area of great potential as well as a detailed study of apo B degradation. The objectives for this section of the work have thus been successfully fulfilled.

Chapter 5: LIVER SLICES FROM DIET-ADAPTED HAMSTERS

5.1 Materials and Methods

All materials,diets and methods were the same as those already described in chapters 2,3 and 4.

5.2 Results

5.2.1 Protein synthesis

The rate of total protein synthesis was assessed in liver slices prepared from hamsters previously fed control or fat-rich diets for 2 - 3 weeks by measuring the incorporation of S^{35}-methionine into TCA-precipitable proteins over three hours;a time chosen in order to remain within the linear range in which rates of protein synthesis were known to occur.The rate of incorporation of radiolabel into total protein (expressed as dpm/mg cell protein) was very similar in all dietary groups (Table 5.1) which suggests that the variations previously observed in isolated cells were a specific feature of that particular system,with its very different preparatory steps.In three experiments where the livers from fat-fed animals were recorded to be particularly fatty,incorporation of label into total protein was 3-fold greater than the average and these experiments were discarded.

Liver slices were fractionated after incubation into a number of compartments:extracellular and cytoplasmic (medium) intracellular luminal contents;nuclei and debris;and intracellular membranes.The distribution of protein and albumin between these compartments was determined and the radioactivity in each compartment expressed as a proportion of the total incorporated radioactivity (Table 5.2).There were no apparent differences between livers from different dietary treatments in respect of

Table 5.1 The incorporation of S^{35}-methionine into total proteins by liver slices prepared from hasmters fed different diets.

Liver slices were prepared from hamsters fed different diets (4.1.1) as described in 3.1.2. Each slice was placed in 2 ml well-oxygenated methionine-free MEM with 20 μCi S^{35}-methionine and incubated for 1 hour in a shaking water bath set at 37°C. At the end of the incubation the tissue and medium were sonicated as described in 3.1.1. Samples were taken for TCA precipitation and protein determinations were also carried out. The results are expressed as dpm of incorporated radioactivity per mg tissue protein. The value in brackets represents the standard deviation on the presented averages.

	CONTROL	LARD	OIL
	343037	141658	191921
	286836	359128	242502
	174202	304740	156791
	202669	122845	130248
	273688	155790	
Average	256086	216832	180366
	(67776)	(107456)	(48518)

Table 5.2 The distribution of total radioactive proteins in tissue subfractions and medium of liver slices from hamsters fed different diets.

Liver slices were prepared from hamsters fed different diets as described in 3.1.1. Each slice was incubated (as described in table 5.1) for 3 hours after which the tissue and media were separated and the tissue was subjected to fractionation as described in 3.1.1. Total radioactivity associated with proteins in each fraction (extracellular, intracellular luminal and membrane associated and debris; lost count were also calculated) was measured as well as that in the medium. The radioactivity in each fraction is expressed as a proportion of total radioactivity (cells + medium) prior to the fractionation proceedure.

	CONTROL	LARD	OIL
Medium	11.5%	9.9%	9.2%
Extracellular	25%	25.3%	24.7%
Intracellular luminal	6%	6.2%	6.1%
Intracellular membranes	4.6%	4.6%	6.2%
Debris	42.1%	42%	42.6%
"Lost" counts	10.8%	12%	11.2%

the distribution of radioactivity in these intracellular compartments,except that in slices from oil-fed hamsters,intracellular luminal contents and membrane-associated radioactivity was similar,while in the slices from lard-fed and control animals,the luminal contents contained relatively more radioactivity.Albumin was determined in order to observe this effect further (Table 5.3).In the case of each of the diets, overall albumin contamination of the membranes was low,but the figure was highest in livers from oil-fed animals.It is thus possible that in the latter case,contamination of the medium fraction with greater amounts of luminal proteins than in the other systems was responsible for the differences seen.It was unlikely that oil-feeding could have caused albumin to be more membrane-associated since albumin is known to be a free secretory protein in the intracellular pathway (87).The rate of albumin secretion by liver slices from oil-fed animals was higher than in slices prepared from hamsters fed either lard or chow.

5.2.2 VLDL synthesis and secretion

VLDL synthesis was assessed by isolating VLDL particles from the medium,and from the intracellular and extracellular tissue components.In earlier experiments,where only medium and tissue extracts were prepared,70-80% of the total VLDL was found to be present in the tissue samples,irrespective of diet (Fig. 5.1).When the tissue was divided into extracellular and intracellular compartments (as described above) these proportions were altered;since,as previously discussed,both medium and extracellular particles were defined as having been secreted,the intracellular VLDL awaiting secretion was inside membranes;when the "secretability" ratio was calculated in the case of slices from animals fed the different diets,marked differences were evident (Fig. 5.2).The amount of total VLDL protein synthesized

Table 5.3 The distribution of radioactive albumin in tissue subfractions and medium of liver slices from hamsters fed different diets.

Liver slices were prepared from hamsters fed different diets as described in 3.1.1. Each slice was incubated (as described in table 5.1) for 3 hours after which the tissue and media were separated and the tissue was subjected to fractionation as described in 3.1.1. Samples from each fraction were subjected to SDS-PAGE followed by fluorography and the albumin in each sample was calculated as described in 2.1.8. Total radioactivity associated with albumin in each fraction (extracellular, intracellular luminal and membrane associated) was measured as well as that in the medium. The albumin in each fraction is expressed as a proportion of total radioactivity associated with albumin (cells + medium) prior to the fractionation proceedure.

	CONTROL	LARD	OIL
Medium	29.6%	32.1%	38.9%
Extracellular	31.4%	38,9%	30.8%
Intracellular luminal	36.4%	24.3%	24.2%
Intracellular membrane	2.6%	4.7%	6.1%

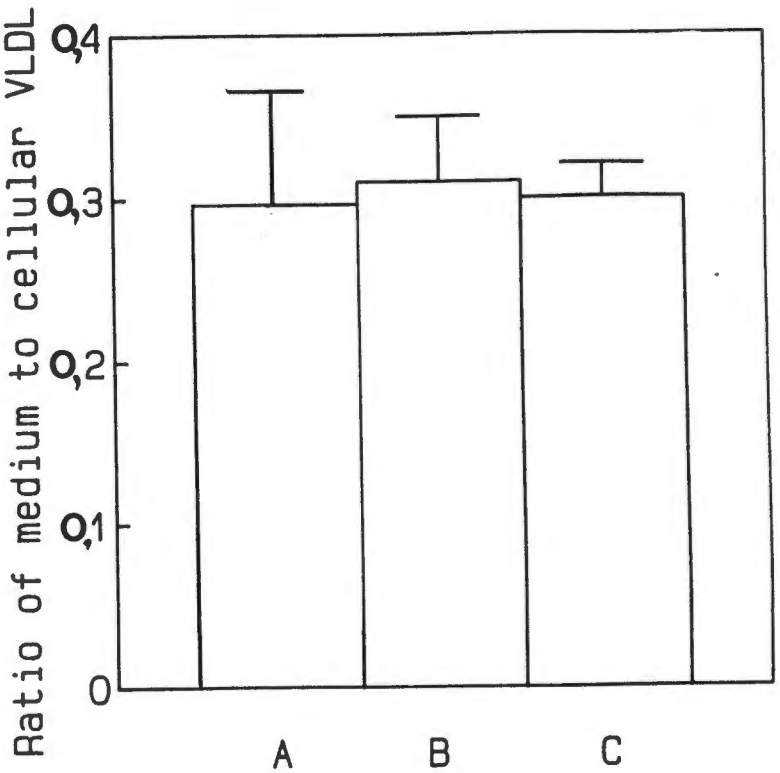

Figure 5.1 The relationship between medium VLDL and that in the tissue in liver slices prepared from hamsters fed different diets.
Liver slices prepared from animals fed different diets (see 3.1.1) were incubated for three hours as described in table 5.1. At the end of each incubation the medium was removed from the tissue. VLDL was prepared from the medium and the whole slice extract as described in 3.1.2. The ratio of the medium and tissue VLDL was calculated in three experiments per diet (control (A), lard (B) and oil (C)) to assess the "secretability" of the particles in each case.

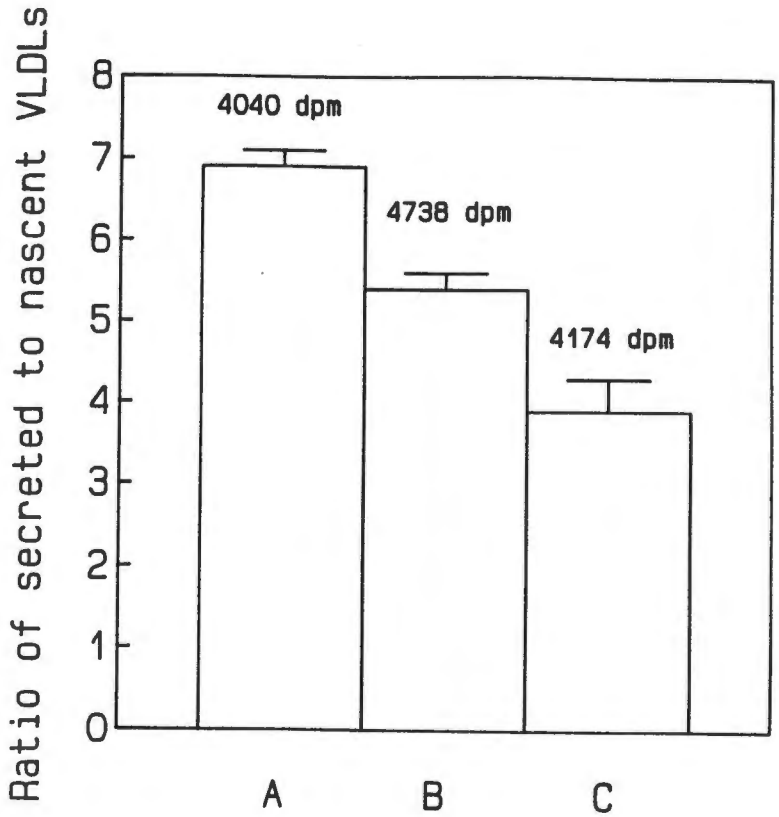

Figure 5.2 The relationship between secreted VLDL and nascent intracellular particles in liver slices prepared from hamsters fed different diets.
Liver slices prepared from animals fed different diets (see 3.1.1) were incubated for three hours as described in table 5.1. At the end of each incubation the medium was removed from the tissue. VLDL was prepared from the medium and the extracellular and intracellular fractions of the slice (prepared as described in 3.1.1) as described in 3.1.2. The ratio of the secreted (medium + intercellular) and tissue (nascent intracellular) VLDL was calculated in three experiments per diet (control (A), lard (B) and oil (C)) to assess the "secretability" of the particles in each case. The total VLDL radioactivity as well as that associated with total proteins was measured, thus the radioactivity associated with VLDL was expressed as a proportion of 10^6 dpm of total incorporated counts.

was similar in the case of each dietary modification,and lower than had been found in isolated hepatocytes (Chapter 4).The secretability ratio in each case was higher than in the case of isolated hepatocytes,but oil-feeding again caused a "block" in secretion relative to chow or lard. Isolated hepatocytes and liver slices thus displayed similar behaviour in terms of how readily VLDL was secreted.

5.2.3 Apo B synthesis and secretion

Apo B synthesis was highest in slices from oil-fed animals,those from chow and lard-fed animals being quite similar (Fig. 5.3).Slices from oil-fed animals secreted apo B most readily,while the chow and lard-fed systems were very similar (Table 5.4).This result contrasted significantly with what had been observed in isolated hepatocytes.The distribution of VLDL in the various compartments was also different from that in isolated hepatocytes.The secreted apo B was considered to be that found in the medium and in "extracellular" fractions:there was an interesting variation in the distribution in relation to the preceding diet (Table 5.5):"control" slices had 70% of the apo B in the medium,while 30% was trapped in the slice;in slices from lard-fed animals there was 50% in each compartment,and in slices from oil-fed hamsters there was only 30% of apo B in the medium and 70% in the extracellular spaces of the slice.None of the diets was associated with a specific accumulation of apo B in the membrane-bound form,but in the slices from chow-fed animals,the apo B that was not secreted did accumulate in the luminal compartment (Table 5.6).

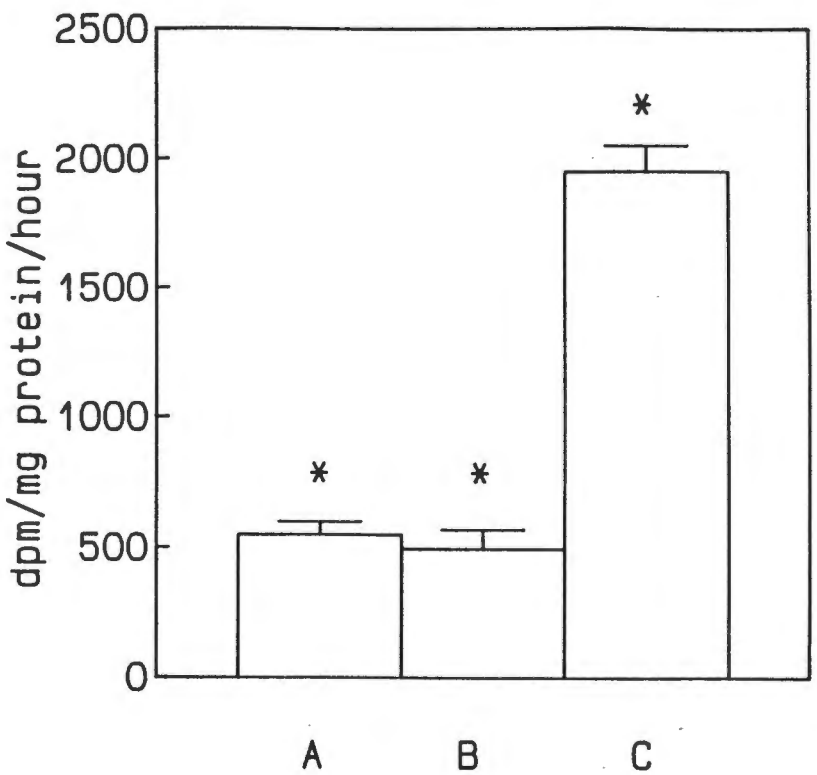

Figure 5.3 Apo B synthesis in liver slices prepared from hamsters fed different diets.
Liver slices prepared from animals fed different diets (see 3.1.1) were incubated for three hours as described in table 5.1. At the end of each incubation the medium and tissue were sonicated as described in 3.1.1 and apo B was immunoprecipitated from each sample as descibed in 2.1.10. SDS-PAGE and fluorography fascilitated the calculation of the radioactivity associated with the immunoprecipitated proteins. Total incorpotrated radioactivity was also measured by TCA precipitation and then the apo B was expressed as a proportion of 10^6 dpm of radioactivity incorporated into total proteins in each sample. The results are the means of three experiments for each of the diets (control (A), lard (B) and oil (C)). Statistical analysis was performed with the student's T test.

* $p < 0.05$ thus data significantly different.

Table 5.4 An expression of the "secretability" of apo B prepared from liver slices from hamsters fed different diets.

Apo B was prepared from liver slices prepared from animals fed different diets and incubated for 3 hours as described in table 5.1. After each incubation the tissue and media were separated and the tissue fractionated into intracellular and intercellular components as described in 3.1.1. Apo B was immunoprecipitated from each fraction and the medium; SDS-PAGE and fluorography allowed the quantification of the apo B. The ratio of the secreted (intercellular + medium) and intracellular apo B was calculated to assess the "secretability" of the apo B. The results are the means of two experiments from each of the dietary groups.

	Ratio of secreted to intracellular apoB (n=2)
Control	0.9 : 1
Lard	1.3 : 1
Oil	2.1 : 1

Table 5.5 Distribution of secreted apo B between the medium and intercellular compartment in liver slices hamsters fed different diets.

Apo B was prepared from liver slices prepared from animals fed different diets and incubated for 3 hours as described in table 5.1. After each incubation the tissue and media were separated and the tissue fractionated into intracellular and intercellular components as described in 3.1.1. Apo B was immunoprecipitated from each fraction and the medium; SDS-PAGE and fluorography allowed the quantification of the apo B. The distribution of secreted apo B (intercellular + medium) between the two compartments was calculated for samples of slices from each of the diets. The results are the means of two experiments from each of the dietary groups and are expressed as percentages of the total secreted apo B.

	Apo B propoportion	
	Medium	Cellular
Control	70	30
Lard	50	50
oil	30	70

Table 5.6 The contribution of the various tissue components to the total intracellular and secreted apo B in liver slices from hamsters fed different diets.

Apo B was prepared from liver slices prepared from animals fed different diets and incubated for 3 hours as described in table 5.1. After each incubation the tissue and media were separated and the tissue fractionated into intracellular and intercellular components as described in 3.1.1. Apo B was immunoprecipitated from each fraction and the medium; SDS-PAGE and fluorography allowed the quantification of the apo B. The proportion of apo B in each of the components was calculated to assess each contribution to the total apo B in each system. The results are the means of two experiments from each of the dietary groups and are expressed as radioactivity associated with apo B in each fraction as a percentage of that associated with apo B in the whole system.

	CONTROL (n=2)	LARD (n=2)	OIL (n=3)
Membrane-associated	4.7 ±0.5	5.3 ±1	8.9 ±5
Luminal	46.8 ±6	37.4 ±3	23.4 ±10
Secreted	48.5 ±7	57.3 ±7	67.7 ±20

5.3 Discussion

The use of liver slices to investigate apo B and VLDL metabolism has not been reported in the literature, and the only basis for comparison is the study of isolated hepatocytes or perfused livers. It is important to note that the rates of VLDL synthesis and secretion, and of apo B synthesis, were rather similar to those obtained with isolated hepatocytes, as this confirms that slices were viable in terms of all the cells contained therein. The most significant features of the comparison is the way in which the slices and hepatocytes differed from each other in respect to apo B secretion and intracellular distribution.

When the total amounts of extracellular radioactive apo B (medium plus the material present in the extracellular spaces of the slices) were compared with those that were intracellular, the slices from oil-fed animals appeared to secrete apo B more readily than those from either the control or lard-fed animals; this was in complete contrast with the data for VLDL in slices and apo B and VLDL in isolated hepatocytes. When the distribution of extracellular apo B between medium and slice was investigated however, it appeared that only 30% of the extracellular apo B was isolated from the medium, while in the control animals the figure was 70%. The implication of this result was either that increased numbers of receptors on the surfaces of cells from oil-fed hamsters were binding apo B, causing its retention in the slices, or that an intracellular cytoplasmic stored form of apo B was being isolated with the extracellular extract. In the light of the data obtained with isolated hepatocytes, the latter appears to be the most likely explanation. (If the former were correct, then similar data would have been expected for VLDL particles.)

VLDL synthesis rates were lower in liver slices than in isolated

hepatocytes,and there were no noticeable differences with respect to the dietary modifications.Reasons for the lower rates of synthesis probably relate to the nature of the slice system: if particles had difficulty leaving the slices and accumulated in the extracellular spaces,some sort of feed-back inhibition might have caused lowered VLDL synthesis rates.Such feed-back inhibition could explain why the VLDL synthesis rates of slices from animals on all the diets was similar.The rates of total protein synthesis were also somewhat lower than in the case of isolated cells,which might have been related to poor oxygenation of cells in the slices,and the difficulty that some nutrients might have had in getting into the slice.

Liver slices have thus been shown to synthesize and secrete VLDL and apo B.Some of the differences detected between slices and isolated hepatocytes probably had their origin in known short-comings of liver slices,and it is possible that they are best suited to the measurement of synthesis rates over short periods.Many samples can be prepared,and at the same time blood and whole-liver samples can be collected for the analysis of various metabolites,thus providing data from the whole animal.The procedure for hepatocyte isolation,on the other hand,precludes the collection of blood or preparation of whole liver extracts.Slices have been considered by Clayton and Darnell to be a good model system for the study of liver metabolism (45-48),but the data obtained in this study shows that slices differ very little from isolated hepatocytes and that results from isolated cells are easier to assess and to interpret.

6.1 Discussion

The aims of this study were threefold-

1. To study lipoprotein metabolism in male hamsters fed diets considered to be atherogenic in humans.
2. To compare isolated hamster hepatocytes in suspension with those maintained under tissue culture conditions.
3. To compare isolated hamster hepatocytes with liver slices prepared from hamsters.

While most of the original project proposals have more than been fulfilled, the area of lipoprotein metabolism has advanced so rapidly that there are many new questions to be answered. Numerous "grey areas" require clarification before a full understanding of the field is reached.

Unfortunately, many studies of VLDL assembly (reviewed in chapter 1) lack consistency in terms of the model in which investigations took place, as well as encompassing variations in conditions of incubations, methods of subcellular fractionation and other experimental techniques. In many of the studies it seems as if investigators have tended to drift from the original motivation of this field of research: to obtain a fuller understanding of VLDL assembly in a model system comparable to humans, so that the biogenesis of human atherosclerosis might be better understood.

Rats have been widely studied, but this species is not only highly resistant to the development of atherosclerosis (155) but apo B48 is synthesized in both the liver and intestine (68,72). Chicks treated with oestrogen, have been used to study VLDL biogenesis but avian apoprotein species are different from those in

humans,and oestrogen treatment stimulates VLDL synthesis significantly (80,81) thus questioning the physiological validity of the system.Human Hep G2 cells secrete apo B100 in association with particles in the density range of human plasma LDL and because the cells are transformed,some genetic alterations would be expected to have taken place (40-43).Therefore,in each of the systems extensively used for the study of VLDL biogenesis,there are factors which make comparisons with humans difficult.Cultured cells been used most frequently:either freshly isolated hepatocytes maintained under tissue culture conditions,or immortalized cultured cell lines.The physiological validity of cultured cells has been questioned (45-48) and this point becomes very important when superimposed on the species problems already discussed.It was for these reasons that the present study was undertaken in hamsters (a small rodent known to be susceptible to atherogenesis (155)),that hepatocytes were prepared for use under tissue culture conditions and immediate incubation,and that liver slices were also investigated.

A full understanding of VLDL assembly would require the clarification of,amongst others,the following questions:

1. Is apo B mRNA constitutively expressed in all species?
2. What role does formation of apo B48 play in apo B100 degradation?
3. Does apo B100 or apo B48 phosphorylation play a role in apo B degradation?
4. How important,therefore,is thyroid hormone in regulating VLDL assembly and secretion?
5. What is the exact role of insulin in terms of regulating both apo B degradation and the secretion of VLDL particles?
6. What is the mechanism of phospholipid addition to VLDL in the Golgi complex?
7. Does the nascent VLDL particle associate with microsomal

membranes,and how?

8. How does incoming dietary lipid affect the above processes?

Of course the likelihood of further questions being raised by the solution of these questions is very great.

In the present study,both apo B synthesis and secretion by hamster hepatocytes were shown to be altered by dietary manipulation of the donor animals;degradation of the protein was insignificant and unaffected by the dietary adaptations.The possibility exists that in hamsters,altered apo B transcription rates may explain the observed changes in apo B synthesis and secretion. Insulin was omitted from all incubations in this study because of the controversy concerning its effect upon VLDL synthesis and secretion (68).The recent report that apo B degradation is stimulated by insulin in rat hepatocytes (108) raises the question of what effect the hormone might have in hamster cells,while apo B degradation in hamster liver slices could also be studied (although,as reported,accurate pulse-chase studies have proved difficult in liver slices).Until subcellular fractionation techniques become more precise,the transport of apo B through the secretory pathway may be very difficult to determine in detail in any system.

Hamster livers synthesized no apo B48 under euthyroid conditions but apo B48 synthesis was stimulated by rendering the animals hyperthyroid.This kind of rodent may therefore be a better model in which to study the effects of apo B48 upon the synthesis,secretion and degradation of apo B100 and the effects in turn of phosphorylation and insulin on these processes.Superimposition of diet on other adaptations may also turn out to be very illuminating.

There were some still unexplained discrepancies between cultured cells,incubated hepatocytes and liver slices in terms of the

synthesis and secretion of apo B100 in fat-fed animals, but the synthesis of total proteins was quite similar. Therefore, the response of hepatocytes and liver slices from fat-fed hamsters, in terms of apo B secretion, requires some further clarification. While some of the differences may be artifacts of the architectural properties of liver slices and the behaviour of receptors within the tissue, there may be important phenomena behind the observations. Liver slices are not ideal for the study of lipoprotein secretion, but they may still be a very useful tool for studying liver metabolism. Freshly incubated hepatocytes lack some of the conveniences of cultured cells (long-term viability, availability of multiplicates and the ease with which pulse-chase protocols may be carried out), but they are probably more reliable in terms of their physiological relevance.

The field of lipoprotein metabolism holds many unsolved mysteries, but this study has shown that not only is the hamster a suitable small animal model in which to investigate this field, but it is probably a closer reflection of human liver metabolism than are some of the systems reported elsewhere. Hepatocytes, freshly incubated, can be successfully used to investigate aspects of lipoprotein metabolism and so can liver slices. Because all studies of this nature are undertaken in the hope of a better understanding of human disease, investigators in the field of lipoprotein metabolism may be able to learn much from hamsters, a fact already brought to the fore by Dietschy and co-workers in their detailed *in vivo* studies, for which the present studies have provided a partial *in vitro* counterpart.

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