APOLIPOPROTEIN BIOSYNTHESIS AND TURNOVER IN MAMMALIAN SMALL INTESTINE

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CERTIFICATE OF SUPERVISOR

In terms of Paragraph GP8 of "General rules for the degree of PhD", I, as supervisor of the candidate, Marc Irwin Combrinck, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

Signed by candidate

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CONTENTS

ABBREVIATIONS USED IN TEXT

A	Ångström unt (1 x 10^{-10} metre)
apo	apolipoprotein/apoprotein
ATP	adenosine triphosphate
ß	beta (radiation)
DAG	di-acylglycerol
dpm	disintegrations per minute
EDTA	ethylenediamine tetra-acetate
EGTA	ethylene glycol bis (ß amino ethyl ether)-N,N,N',N'-tetra-acetate
g	acceleration due to gravity (9.8 m/sec^2)
HDL	high density lipoprotein
HEPES	4-(2 hydroxyethyl)-1-piperazine-ethane sulphonic acid
IP	immuno-precipitation/immuno-precipitated
kcal	kilocalorie (1 kcal = 4.185 MJ/megajoules)
kDa	kilo dalton (unit of molecular weight)
KRB	Krebs-Ringer bicarbonate (solution)
LDL	low density lipoprotein
M	molar (unit of concentration)
mM	millimolar (1 x 10^{-3} M)
MAG	mono-acylglycerol
MCT	medium chain tri-acylglycerol/triglyceride
mRNA	messenger ribonucleic acid
MW	molecular weight (mass)
μCi	microcurie (2.2 x 10^6 dpm)
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
nm	nanometer (1 x 10^{-9} metre)

PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
ρ	density (g/ml)
rpm	revolutions per minute
S_f	Svedberg (flotation) units
SDS	sodium dodecyl suphate
TAG	tri-acylglycerol
TCA	tri-chloro-acetic acid
VLDL	very low density lipoprotein
v/v	volume per volume
w/w	weight per weight

ABSTRACT

The mammalian small intestine is a major site (second in total activity only to the liver) for the synthesis and secretion of plasma apolipoproteins, and contributes significantly to overall whole-body lipid dynamics.

A prominent feature of the small intestine is its exposure to periodic loads of meals often containing dramatically varying amounts or types of food components, including lipids such as tri-acylglycerols, cholesterol and cholesteryl esters. Since the trans-epithelial transport of most of these latter materials requires the elaboration of particles partially covered by apolipoproteins, the regulation of the biosynthesis or, more correctly, the availability of these proteins is an important and as yet little-understood problem.

Previous studies have been conducted on systems which, for one or the other reason, have not permitted the following questions to be satisfactorily or coherently answered: Does the ingestion of fat-containing meals, either acutely or chronically, increase the rate of biosynthesis of intestinal apolipoproteins such as apo B-48, and is this the principal method of matching the "demand" with the supply of this "packaging material" needed for fat transport across the intestinal epithelial cells? Alternatively, does the maintenance of a large steady-state intracellular pool in the face of variations in intracellular apolipoprotein degradation, controlled by acute or chronic lipid ingestion, produce the required "match" between supply and demand for these proteins (as has recently been suggested in studies on liver cells)?

An *in vitro* system was therefore devised whereby sheets of intestinal epithelial cells (enterocytes) were freshly isolated from the jejuna of adult male Syrian golden hamsters and incubated for several hours in a medium supporting steady-state protein synthesis, in a manner which was assumed to be similar to the activity just before the killing of the

donor animals. (Hamsters appear on various grounds to be a better small-animal model of human lipoprotein metabolism than the more commonly studied rats). The isolated epithelial cell sheets produced primary apolipoprotein products that could be extracted from the cells or detected in the incubation media, free from the subsequent modifications that they are known to undergo *in vivo*.

Hamsters maintained on a low-fat chow were either studied as such or subjected to a variety of dietary treatments designed to maximize (over short or long time periods) intracellular apolipoprotein requirements for the "packaging" of tri-acylglycerol-rich lipoproteins, especially chylomicrons: acute bolus administration of lipid into the gut; overnight feeding of fat-enriched food; and chronic (six week) fat feeding.

Using specific antisera and immuno-precipitation techniques, apo B-48 and two other principal intestinal apolipoproteins were shown to be synthesized in the steady state by intestinal cell sheets derived from control animals and from those subjected to acute or chronic fat-containing diets. Secretion took place, however, only when prior fat exposure of the donor intestines had occurred.

Pulse-chase labelling was used to compare the rates of apolipoprotein synthesis, degradation and secretion in the same cell sheet preparations. The rates of apolipoprotein B-48 synthesis did not vary significantly under conditions of low or high trans-epithelial lipid flux, supporting findings derived from *in vivo* experimental systems. In contrast with data from other systems, however, the biosynthesis of apolipoprotein A-IV was not reproducibly increased on fat challenge. The rates of apo B-48 degradation varied significantly and were markedly reduced under conditions of fat feeding. The experiments permit a choice between the two alternatives mentioned above:
Ingestion of fatty foods, either acutely or over long periods of time, does not increase the rates of biosynthesis of apolipoproteins such as apo B-48; but variations in the rate of

intracellular degradation of this and probably other apolipoproteins allows the intestinal cells to match their requirements for lipid-transporting molecules to the demands of any given situation, relying in each case on a large steady-state intracellular pool maintained by "constitutive" biosynthesis. Importantly, there seems also to be a specific, possibly related effect of fat feeding on the secretion of lipoproteins into the intestinal extracellular fluid.

These conclusions coincide with those obtained by other workers from studies of apolipoprotein B dynamics in isolated hepatocytes and in the hepatoma-derived liver cell line, Hep G2. The mechanisms underlying these phenomena are as yet unresolved.

CHAPTER ONE

THE INTESTINE AND LIPOPROTEIN METABOLISM

1.1 INTRODUCTION: LIPIDS AND LIPOPROTEINS IN HEALTH AND DISEASE

Lipids are a heterogeneous group of organic compounds usually defined in terms of their common property of being insoluble or poorly soluble in aqueous solutions but soluble in non-polar, organic solvents. Lipids such as long-chain fatty acids, tri-acylglycerols and waxes (in some animals) serve as important energy sources, being ultimately oxidised to provide adenosine triphosphate. Tri-acylglycerols, which consist of various long-chain fatty acids esterified with the hydroxyl groups on the three carbon atoms of glycerol, are both a direct source of energy and a potential source when stored as fat in adipose tissue.

A second major function of lipids is as structural components of cell membranes. These contain phospholipids and compound glycerophospholipids, sphingophospholipids and glycosphingolipids. Cholesterol, a sterol compound, is also a major structural component of cell membranes, and serves as a substrate in the liver for the synthesis of bile acids which, in turn, assist in the processes of digestion and absorption of other lipids in the bowel. Cholesterol is also the precursor molecule from which steroid hormones are synthesized in certain tissues. Vitamin D is another sterol derivative with functions in regulating calcium and phosphorus metabolism and bone calcification.

Finally, the terpenes constitute another important class of lipid compounds. They include the dolichols and the lipid-soluble vitamins A, E and K with functions in glycoprotein synthesis, as well as vision and epithelial integrity, anti-oxidant activity and blood coagulation, respectively (Montgomery *et al.* 1977, Mayes 1988).

In order to perform their functions, however, lipids as relatively water-insoluble compounds have to be transported in an aqueous environment - the blood plasma - from one tissue to another. This problem of lipid transport is generally overcome by the formation of a group of water-soluble lipid-transport macromolecules called lipoproteins.

Lipoproteins are particles consisting of non-polar lipids (tri-acylglycerols and cholesteryl esters) in a hydrophobic core, surrounded by a surface layer of amphipathic lipids (free cholesterol and phospholipids) and proteins, with the polar aspects of these molecules orientated towards the aqueous environment. The protein moieties of lipoproteins are known as apolipoproteins or apoproteins. The phospholipids are mainly phosphatidylcholine (lecithin) and sphingomyelin. The larger lipoproteins serve principally to transport exogenous dietary lipid (especially tri-acylglycerols) from the intestine to various body tissues and to export endogenously synthesized tri-acylglycerols from the liver to various tissues including adipose tissue. The smaller lipoproteins have more complex and diverse functions. (The transport of free fatty acids mobilized from tri-acylglycerol stores in adipose tissue during periods of fasting is facilitated by albumin, a soluble protein which binds free fatty acids with high affinity, while ketone bodies, which arise from the partial oxidation of free fatty acids in the liver, are readily soluble in plasma).

Circulating plasma lipoproteins have been separated and classified into four main classes, depending on their density (a function of the lipid/protein ratio) or their electrophoretic mobility (dependent on the apoprotein composition) (Fig. 1.1). These lipoprotein classes are respectively called chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). A further group of intermediate density lipoproteins (IDL) has also been defined (Table 1.1). Of the major groups, the chylomicrons and very low density lipoproteins (VLDL) are quantitatively the most important in the transport of tri-acylglycerols. Chylomicrons transport dietary fat from the intestine while VLDLs transport tri-acylglycerols from the liver to extra-hepatic tissues. IDL's are formed in the plasma by the action of the enzyme, Lipoprotein Lipase, on tri-acylglycerol-rich lipoproteins. High density lipoproteins

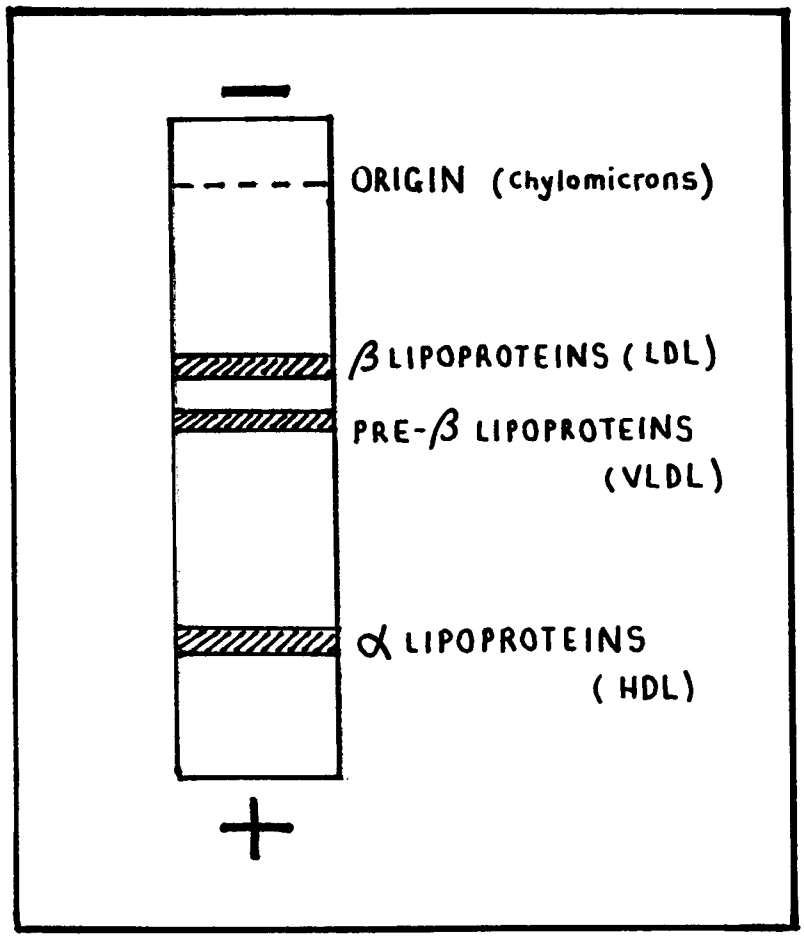


Fig. 1.1. Separation of plasma lipoproteins by electrophoresis.

CLASS	DENSITY (g/ml)	ELECTROPHORETIC MOBILITY	DIAMETER (nm)	MOLECULAR WEIGHT
CHYLOMICRONS	0.93	remain at origin	75-1200	50-1000 $\times 10^6$
VLDL	0.93-1.006	pre-β-lipoproteins	30-80	10-80 $\times 10^6$
IDL	1.006-1.019	slow pre-β-lipoproteins	25-35	5-10 $\times 10^6$
LDL	1.019-1.063	β-lipoproteins	18-25	2 300 000
HDL_2	1.063-1.125	α-lipoproteins	9-12	360 000
HDL_3	1.125-1.210	α-lipoproteins	5-9	175 000

Table 1.1. Physical properties of human plasma lipoprotein classes. (Ref.: Kane & Havel 1989).

(HDL) are involved inter alia in the catabolism of VLDL and chylomicrons and the transport of cholesterol from peripheral tissues to the liver.

The medical interest in lipid transport and metabolism is derived from epidemiological and experimental evidence linking atherosclerotic cardiovascular disease to abnormalities of plasma lipids. There is evidence that the prevalence and incidence of atherosclerosis and its clinical manifestations (coronary heart disease, peripheral vascular disease and cerebro-vascular disease) are increased in societies with a high dietary intake of total fat, saturated fat and cholesterol. The serum cholesterol concentrations in these populations are also generally higher than those of people whose diet is low in these constituents and who also have less atherosclerotic cardiovascular disease.

Experiments in non-human primates and other mammals have shown that chronic, diet-induced hypercholesterolaemia is associated with the development of atherosclerosis (Faggiotto & Ross 1984). The association between premature atherosclerosis and the genetic condition of Familial Hypercholesterolaemia (Type IIa Hyperlipidaemia) in which a deficiency, defect or absence of low density lipoprotein receptors (LDL receptors) results in a failure of normal LDL catabolism and a consequent accumulation of (cholesterol-rich) LDL particles in the plasma, provides further support to the argument linking lipids (especially LDL-cholesterol) to atherogenesis (Steinberg 1983, Goldstein & Brown 1989).

Cholesterol and other plasma lipid or lipoprotein components characteristically accumulate in atheromatous plaques. In the general population, however, who do not have Familial Hypercholesterolaemia and where the majority of cases of atherosclerosis-related disease occurs, atherosclerosis and its clinical manifestations appear to be multifactorial in origin, with cigarette smoking, diet, obesity, hypertension, heredity, diabetes, lack of exercise and stress all playing potential roles in the aetiology of the disease. In addition to the positive correlation between LDL-cholesterol levels and the

risk of coronary heart disease, a negative correlation exists between HDL cholesterol levels and atherosclerotic disease, i.e. low HDL cholesterol levels are associated with an increased risk of disease. Furthermore, it is the reduced cholesterol level of a subfraction of HDL, HDL_2 (as opposed to HDL_3), which correlates even better with the increased risk (Gordon *et al.* 1977, Eder & Gidez 1982).

Post-prandial lipaemia has been postulated to be a factor in atherogenesis (Patsch 1987). Some researchers have demonstrated a strong inverse association between the magnitude of post-prandial lipaemia (fat tolerance) and HDL_2 cholesterol levels. Post-prandial lipaemia could affect the atherosclerotic process directly through the appearance of post-prandially occurring lipoproteins (e.g. chylomicron remnants that may, because of their prolonged circulation in the plasma, interact with endothelial arterial walls), or indirectly, via effects on other lipoproteins such as LDL or HDL.

The serum levels of the apolipoproteins have also been studied in relation to heart disease. Elevated levels of apolipoprotein B (an apolipoprotein found on the LDL particle) and reduced levels of Apolipoprotein A-I (found on HDL) have been found in subjects with coronary heart disease (Kreisberg 1983). More recent epidemiological evidence has suggested that high plasma levels of another lipoprotein, Lipoprotein (a) or Lp(a) correlate with an increased risk of coronary artery disease. Lp(a) consists of an LDL-like particle containing the apolipoprotein B-100 (see later), linked by disulphide bonding to a high molecular weight glycoprotein, apolipoprotein (a), which is a translation product of a gene closely related to the plasminogen structural gene (Lawn 1992) (see Fig. 1.2). While the links between abnormal serum lipids and atherosclerotic disease have been emphasized, abnormalities of lipoproteins are also found in other disease states, the commonest of which is diabetes mellitus. Hypothyroidism and renal disease are also associated with lipoprotein abnormalities and there are a number of less common conditions of inherited defects in lipoprotein metabolism or

dyslipoproteinaemias resulting in either hypolipoproteinaemia (e.g. Abetalipoproteinaemia, Tangiers disease and Familial Hypobetalipoproteinaemia) or hyperlipoproteinaemia/hyperlipidaemia, such as the various types of Familial Hyperlipidaemic disorders (Types I-V).

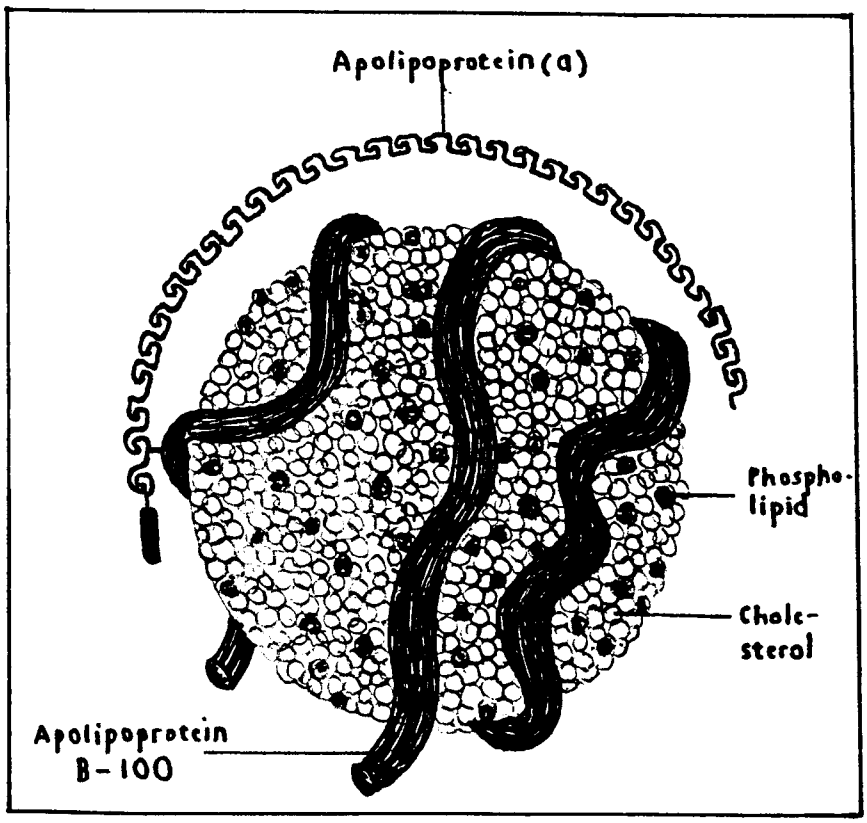


Fig. 1.2. A schematic representation of lipoprotein (a). The apolipoprotein (a) molecule is linked by disulphide bridging to the apolipoprotein B-100 moiety of the LDL particle. (Ref. Lawn 1992).

1.2 DIGESTION AND ABSORPTION OF LIPIDS

The intestinally produced chylomicrons, which consist largely of exogenous diet-derived tri-acylglycerol (TAG), are synthesized and assembled in the endoplasmic reticulum of the intestinal columnar epithelial cells or enterocytes, whence they are transported to the Golgi apparatus of the cells, before being released by exocytosis into the local extracellular spaces. The hepatocytes of the liver make TAG-rich very low density lipoproteins (VLDL) which are synthesized and secreted in a manner analogous to that of the enterocyte.

Fat constitutes about 40% of the kilojoules in an average diet of the industrialised countries, the daily "western" intake averaging about 100 grams a day. Most of this is in the form of TAGs; a small fraction (0.5 - 1 g/day) is in the form of cholesteryl esters, and about 10-30 g of phospholipids (derived from membranes of unprocessed food and from endogenous biliary secretions) are processed by the adult human gut daily (Bisgaier & Glickman 1983, Patsch 1987). The TAGs contain fatty acids mostly with chain lengths greater than 14 carbon atoms, and which may be saturated e.g. palmitic acid (C16:0) and stearic acid (C18:0), or unsaturated e.g. oleic acid (C18:1) and linoleic acid (C18:2) (Table 1.2). A small amount of cholesterol is also derived from salivary, gastric and biliary secretions as well as sloughed intestinal cells. While the absorption of dietary TAG is virtually complete (>95%), cholesterol is usually absorbed at a level equal to between 20-50% of dietary intake (Bisgaier & Glickman 1983).

The processes of lipid digestion and absorption have been comprehensively reviewed by Shiau (1987), Patsch (1987), Thomson & Dietschy (1981) and Westergaard & Dietschy (1986). A summary of the most relevant facts will be presented here.

Dispersion of bulk fat into crudely emulsified particles begins in the stomach. Enzymatic lipolysis does, however, also occur in the stomach as a result of the acid-

UNSATURATED STRAIGHT CHAIN MONOCARBOXYLIC ACIDS						
TRIVIAL NAME	TYPE OF UNSAT. FATTY ACID	FAMILY	FORMULA	STRUCTURE	SYSTEMATIC NAME	NUMERICAL SYMBOL
OLEIC ACID	Mono-enoic/ olefinic/ unsaturated	$\omega 9$	$C_{18}H_{34}O_2$	$CH(CH_2)_7COOH$ ‖ $CH(CH_2)_7CH_3$	cis $\Delta 9$ octa-decenoic acid	C18:1(9)
LINOLEIC ACID	di- enoic / polyenoic/ olefinic / unsaturated	$\omega 6$	$C_{18}H_{32}O_2$	$CH_3(CH_2)_4CH$ ‖ $CHCH_2CH$ ‖ $CH(CH_2)_7COOH$	cis-cis $\Delta 9,12$ octa-deca-dienoic acid	C18:2(9,12)
SATURATED STRAIGHT CHAIN MONOCARBOXYLIC ACIDS						
PALMITIC			$C_{16}H_{32}O_2$	$CH_3(CH_2)_{14}COOH$	hexa-decanoic acid	C16:0
STEARIC			$C_{18}H_{36}O_2$	$CH_3(CH_2)_{16}COOH$	octa-decanoic acid	C18:0

Table 1.2. Examples of common unsaturated and saturated fatty acids.

resistant lingual lipase secreted from the serous glands of Van Ebner on the dorsal surface of the tongue. Lingual lipase works at the Sn-3 position in preference to the Sn-1 position of the TAG molecule (Fig. 1.3) and the major products are di-acylglycerols (DAG) and fatty acids which are ejected into the duodenum.

The upper small intestine (duodenum and jejunum) are the major sites of lipid digestion and absorption. As fat enters the duodenum, the hormones enterogastrone, secretin and pancreozymin-cholecystokinin are released from duodenal mucosal cells. Enterogastrone acts locally and reduces intestinal motility while secretin and pancreozymin-cholecystokinin enter the blood stream and reach the gall bladder and pancreas, thus stimulating the release of bile and pancreatic juice which mix with the stomach-derived emulsion. The enzyme, pancreatic lipase, hydrolyses TAGs in the presence of a co-factor protein, co-lipase, which is also found in pancreatic secretions and appears to assist pancreatic lipase in binding to the TAG and DAG close to the Sn-1 and Sn-3 positions of the acylglycerol molecules. The hydrolysis of TAGs is a step-wise procedure, with one of the fatty acids being removed at a time, so that first a DAG then a Sn-2-monoacylglycerol (MAG) are formed sequentially (Fig. 1.3). The bile salts together with phospholipids (lecithin) and the MAGs produced early on enable emulsification to proceed. The bile salts on their own appear to be poor emulsifying agents but as lipolysis proceeds, more polar lipids are produced and further emulsification occurs. Dietary cholesterol in the form of cholesteryl esters is hydrolysed by a pancreatic cholesteryl esterase to produce free cholesterol and fatty acids. The enzyme phospholipase A_2 hydrolyses phospholipids to yield lysophospholipids. The end products of lipid digestion are therefore fatty acids, glycerol, MAG, (very few TAG and DAGs), cholesterol, and lysophospholipids.

The bile salts released from the gall bladder are present in concentrations in the small bowel exceeding the critical micellar concentration (CMC) so that water-soluble micelles

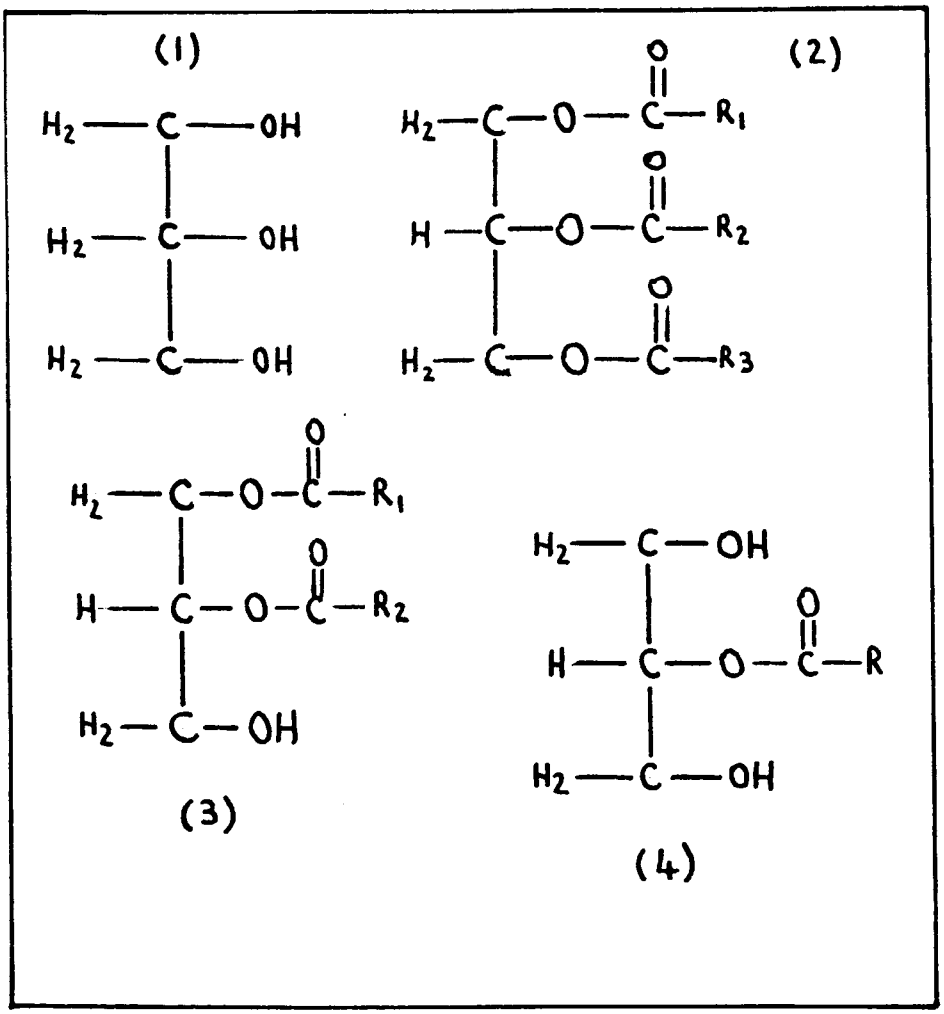


Fig. 1.3. Structures of: (1) glycerol, (2) a tri-acylglycerol (or triglyceride), (3) a sn-1,2-di-acylglycerol and (4) a sn-2 mono-acylglycerol. R represents a fatty acid residue.

are formed. These consist of negatively charged spherical aggregates with the polar hydroxyl and amino acid groups facing the aqueous environment and the non-polar steroid portions forming a hydrophobic core. The fatty acids, MAGs and cholesterol enter these bile salt micelles to form mixed micelles and in this form are able to traverse the unstirred water layer or aqueous layer immediately adjacent to the microvillar brush border of the enterocytes. (This layer is not in equilibrium with the bulk aqueous intestinal fluid contents).

The precise mechanisms of absorption of the constituent lipid molecules from the mixed micelles (once they reach the vicinity of the aqueous-membrane interface) is controversial: the constituent lipids may partition into the cell membrane during direct interaction or collision between the micelles and the cell membrane or absorption may only take place through the monomer phase of the lipid molecules (the monomer phase being present in the unstirred water layer in equilibrium with the lipids in the micelles) (Westergaard & Dietschy 1986). Uptake of cholesterol by the small intestinal brush border membrane may be protein mediated (Thurnhofer & Hauser, 1990). The bile salt micelles are not absorbed in the upper small bowel but proceed to the terminal ileum where absorption takes place and repeated enterohepatic circulation cycles continue. A schematic representation of the luminal events of lipid digestion and absorption is presented in Figure 1.4.

Only a small fraction of the dietary lipid ($<2\%$) reaches the colon. The faecal lipids are mainly fatty acids or their salts and are derived largely from bacteria or shed colonic epithelial cells. These lipids are later chemically transformed by colonic bacteria and although the quantities involved are small, it has been suggested that these processes may be significant in the production of carcinogens and the aetiology of large bowel cancer.

Returning to the upper small bowel, the products of lipid digestion enter the columnar epithelial cells of the small intestinal villi by passive diffusion across the microvillar membranes. Eventually they are all released on the serosal border by complex

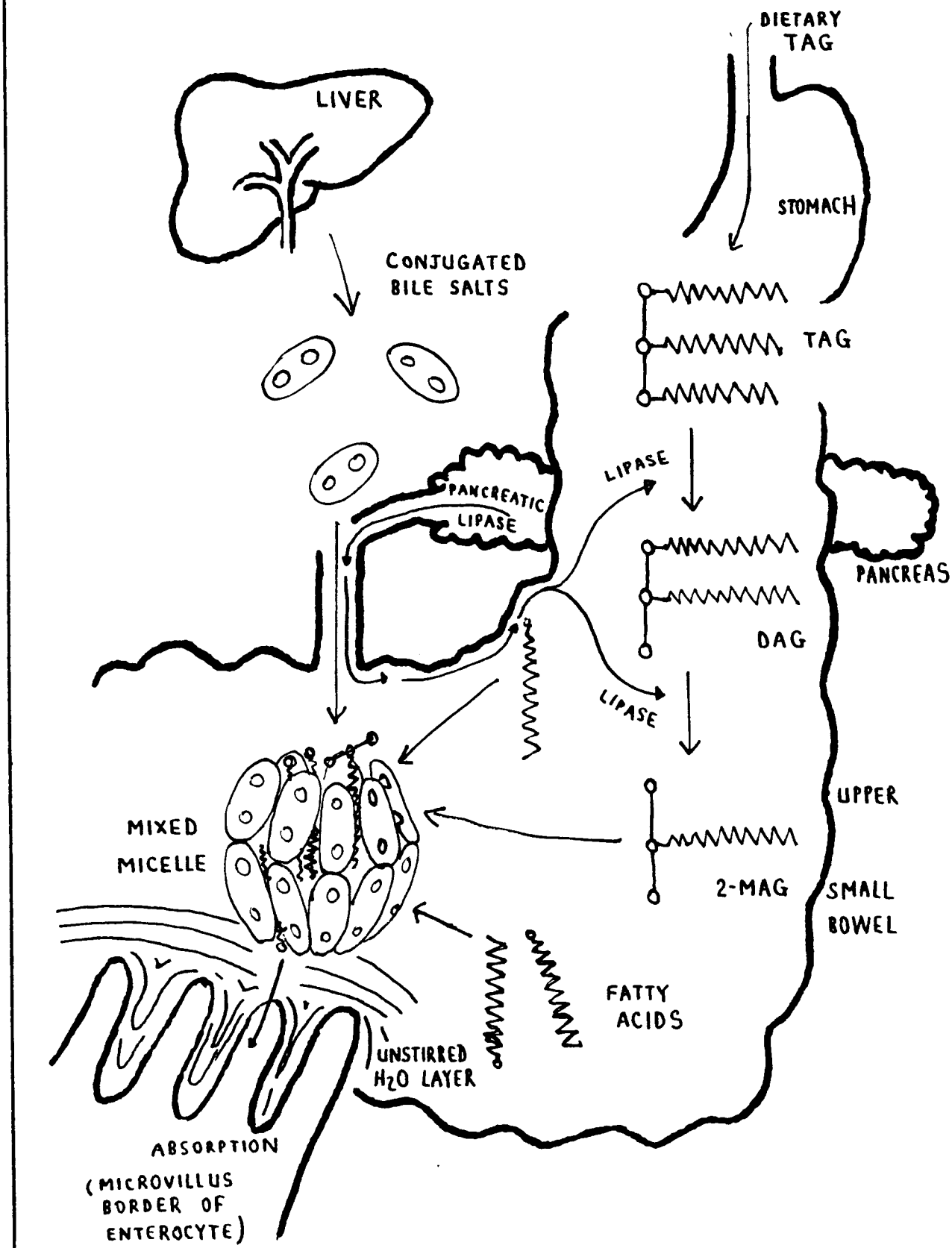


Fig. 1.4. A schematic representation of the intraluminal events of fat digestion. TAG = tri-acylglycerol, DAG = di-acylglycerol, 2-MAG = sn2-mono-acylglycerol.

mechanisms depending on the type of digestive product. Thus, short and medium chain fatty acids (less than 10-12 carbon atoms e.g. fatty acids of milk TAG) pass directly through the mucosal cells into the tissue spaces and are transported as free fatty acids bound to albumin in the portal system to the liver and other tissues. Free glycerol is readily soluble in water and is also transported by the portal blood. Absorbed MAG, cholesterol and fatty acids greater than 12 carbon atoms in length are re-esterified in the enterocyte to form TAGs and cholesteryl esters which are packaged using a monolayer of phospholipids, some free cholesterol and apolipoproteins as surface coating materials, to form chylomicrons.

During the above processes, the free fatty acids first bind to one or more specific fatty acid binding protein(s) (FABP) (MW 15 and 14 kDa, respectively) which have a particularly high affinity for long chain unsaturated fatty acids (Bass *et al.* 1985). These proteins probably enable the fatty acids to be transported from the brush-border membrane to the smooth endoplasmic reticulum (ER) where esterification takes place. The fatty acids are first activated to their co-enzyme A derivative by the enzyme long chain fatty acid: Co-enzyme A ligase (LCFA:CoA ligase). The acyl co-enzyme A is then esterified by enzyme systems on the ER using 2 alternative metabolic pathways - the glycerol-3-phosphate pathway and the MAG pathway (Fig. 1.5). Of the two, the latter is quantitatively by far the most important and involves acylation of the Sn-2 MAGs (derived from the action of pancreatic lipase in the gut lumen) to Sn-1,2 diacylglycerols or Sn-2,3 DAGs and then to TAG (Johnston 1978). The enzymes of TAG biosynthesis, LCFA:CoA ligase, MAG transferase and DAG transferase are situated on the inner membrane of the smooth endoplasmic reticulum.

Phospholipids constitute most of the surface monolayer of the chylomicrons but while the fatty acids of the TAG core reflect the fatty acid composition of ingested TAGs, the fatty acids of the phospholipid monolayer do not, and remain more or less of constant

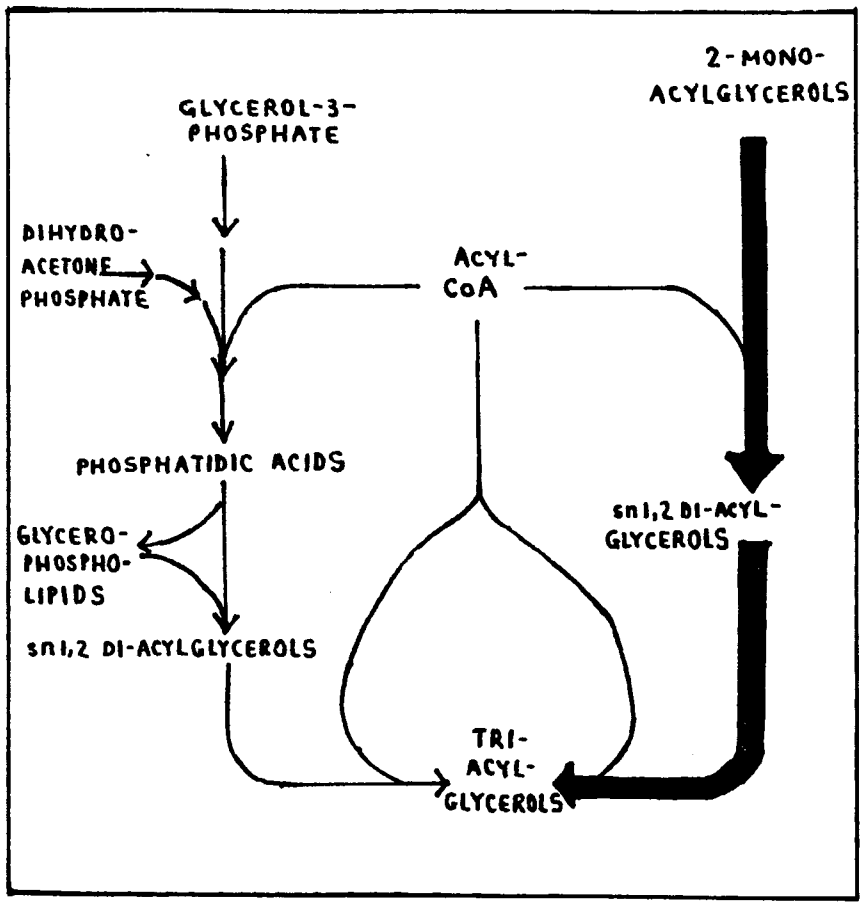


Fig. 1.5. The two pathways of tri-acylglycerol biosynthesis in the enterocyte. The major pathway is denoted by the heavy arrows. (Ref.: Johnston 1978).

composition, irrespective of diet. The phospholipid in the monolayer is largely phosphatidylcholine (lecithin), with small amounts of sphingomyelin and phosphatidylethanolamine. Phosphatidyl-choline may be synthesized *de novo* within the enterocyte from DAG and cytidine diphosphocholine. The DAG is formed via phosphatidic acid from the glycerol-3-phosphate pathway while the choline is derived from the intestinal lumen through dietary intake and biliary secretion. However, phosphatidylcholine may also be formed by the acylation of lysophosphatidylcholine (produced in the lumen by the action of phospholipase A_2 on phospholipids) through the ER enzyme, lysophosphatidylcholine acyltransferase. As with TAG biosynthesis in the enterocyte, the relative importance of each pathway is determined by the substrate availability. In states of dietary lipid deprivation, for example, the glycerol-3-phosphate pathway for TAG synthesis is thought to become important, while in phospholipid biosynthesis, the lyso pathway would be expected to be quantitatively more important in choline-deficiency states.

While the intestine has the capacity for *de novo* cholesterol biosynthesis, this appears to occur mainly in the crypt cells of the intestinal villus (Fig. 1.6) where the activity of the cholesterol biosynthetic enzyme 3-hydroxy 3-methylglutaryl-co-enzyme A (HMG CoA) reductase has been found to be highest and where the cholesterol requirement for rapidly dividing cells is greatest (Stange & Dietschy 1985). Lumen-derived cholesterol is thought to be mostly re-esterified within the enterocyte and "packaged" into the chylomicron core. The mechanisms for cholesterol re-esterification may be via the pancreatic enzyme, cholesteryl esterase, working "in reverse" inside the epithelial cell, or via the enzyme, acyl-CoA:cholesterol acyl tranferase (ACAT). The activity of this enzyme reveals a gradient opposite to that of HMG CoA reductase with respect to the villus tip-crypt axis: ACAT activity is highest in the cells of the villus tip, where most absorption takes place, and is lowest in the rapidly dividing crypt cells at the villus base, where HMG CoA reductase activity is greatest (Stange & Dietschy 1985).

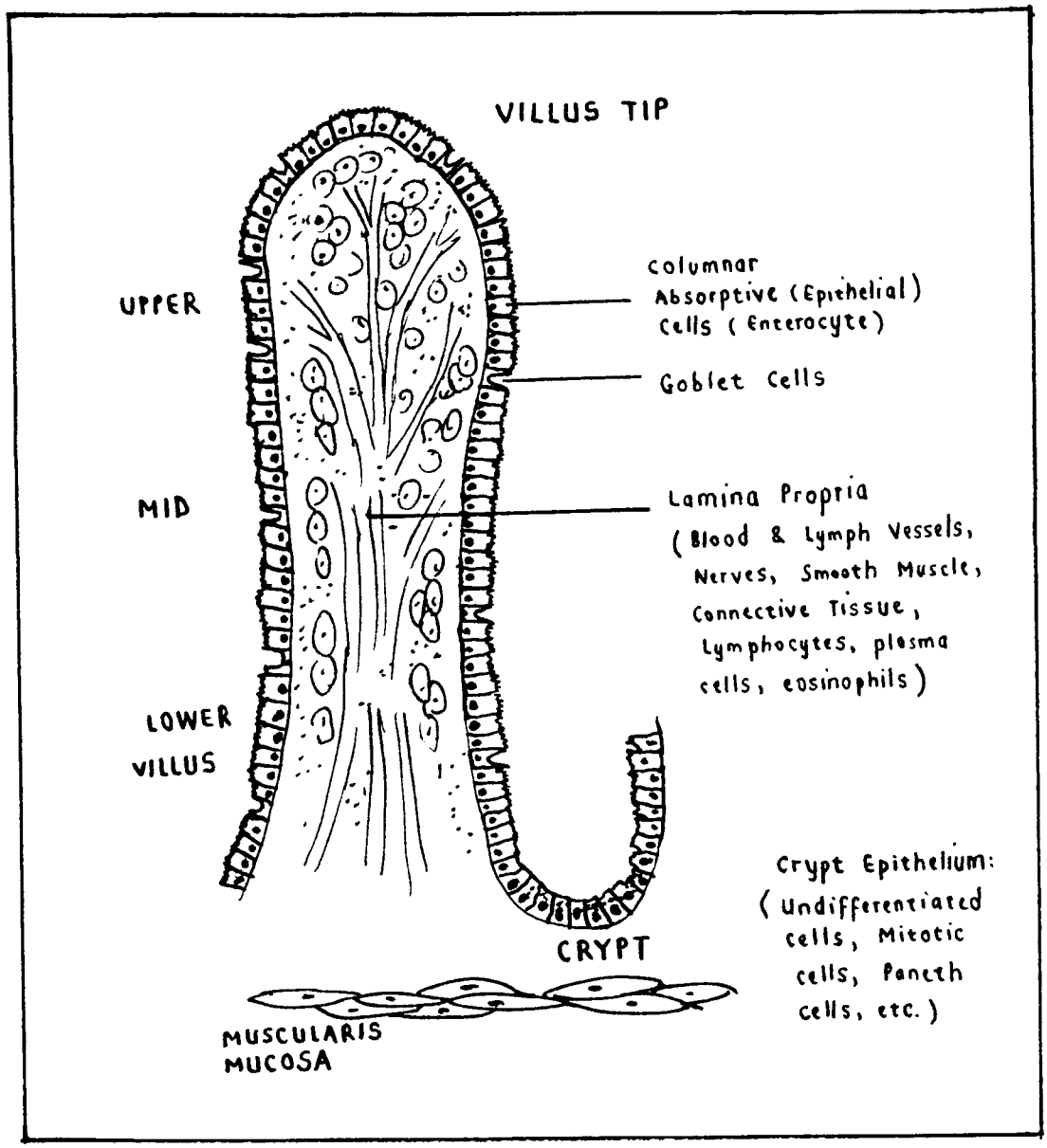


Fig. 1.6. The intestinal villus in longitudinal section. The immature, mitotically active undifferentiated cells are found in the crypt; as they migrate up the villus they become progressively more mature and achieve full differentiation when they reach the upper one third of the villus. The cells of the tip degenerate and are sloughed into the bowel lumen.

The apolipoproteins are synthesized by the enterocytes on the rough ER. From there they appear to be transferred to the smooth ER and incorporated with the resynthesized TAG to form nascent chylomicrons. The chylomicrons then move to the Golgi cisternae and vesicles before they are released via exocytosis ("reverse pinocytosis") from secretory vesicles at the basolateral surface of the cell membrane (Fig. 1.7). The three principal apolipoproteins synthesized by the enterocyte are Apolipoproteins B-48, A-IV and A-I (see later for a fuller review).

The chylomicrons filter from the extracellular spaces into intestinal lacteals of intestinal lymph and eventually drain into the thoracic duct which empties into the subclavian vein. In so doing they give rise to the phenomenon of postprandial lipaemia.

The digestion and absorption of short and medium-chain tri-acylglycerols (C4-12) (fatty acids of milk triglyceride) are of importance in infant nutrition and have become increasingly significant as a result of their clinical use in certain therapeutic diets. Two mechanisms have been proposed for the digestion and absorption of medium chain tri-acylglycerols (MCT):

(i) The MCTs are emulsified and then absorbed intact into the mucosal epithelial cells without prior luminal hydrolysis. Once inside the enterocytes a mucosal cell microsomal lipase completely hydrolyzes the MCTs to free fatty acids and glycerol. These products are readily soluble in aqueous environments and are partitioned into the venous blood where they are carried via the portal vein to the liver as free, ionised fatty acids, partly bound to albumin.

(ii) MCTs may also undergo hydrolysis by pancreatic lipase in the lumen of the upper small bowel. Pancreatic lipase is known to be more effective in the hydrolysis of MCT than of the more abundant dietary long chain fatty acid-containing tri-acylglycerols and complete hydrolysis to free fatty acids and glycerol may occur. However, even in

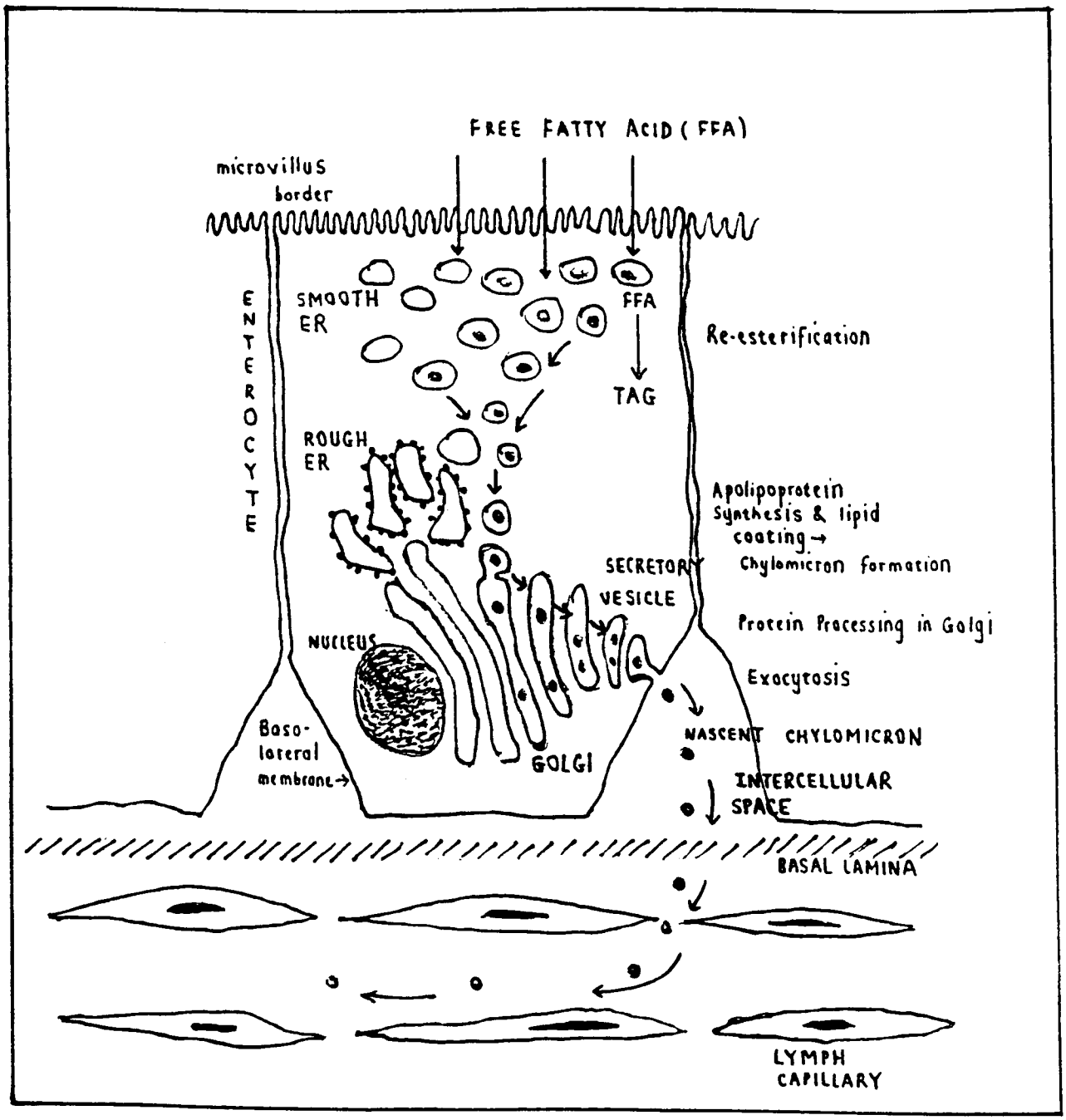


Fig. 1.7. A schematic representation of the events of fat absorption and chylomicron formation by the intestinal mucosal cell (enterocyte).
FFA = free fatty acid, ER = endoplasmic reticulum, TAG = tri-acylglycerol.

the absence of pancreatic lipase, the acid-resistant lingual (or gastric) lipase (secreted by the tongue and found in the stomach) is able to hydrolyse MCTs. This enzyme has a pH optimum of 4 and preferentially hydrolyzes MCT at rates 5-8 times faster than long chain TAGs. It is inhibited by the presence of bile salt micelles in the duodenum. In the neonatal period, when pancreatic function is immature, lingual lipase is thought to account for the digestion of 30 to 50% of ingested fat (Hamosh 1979). Breast milk also contains a milk lipase which is activated by bile salts in the duodenum and this enzyme is also of importance in the digestion of MCTs in the suckling infant. The milk lipase is able to hydrolyze fatty acids from all 3 positions on the TAG molecule and is therefore also able to completely hydrolyse 2 mono-acylglycerols derived by the action of pancreatic lipase (Hernell & Blackberg 1982). Milk lipase therefore complements pancreatic enzyme digestion in the newborn. The products of MCT digestion, viz. glycerol, free fatty acids and, perhaps, some MAG molecules as well, are readily soluble in water and easily partition into the unstirred water layer without the need for bile salt micelles. Their absorption rates are also higher than for long-chain fatty acid mono-acylglycerols. Once in the intestinal epithelial cells they are readily transported to the serosal border of the cells whence they partition into the venous blood and to the liver.

MCT absorption is thus largely independent of bile, pancreatic function and factors that impair apolipoprotein synthesis or obstruct lymphatic transport from the intestine. Synthetically prepared MCTs (C8-C10) are today commonly used as nutritional (caloric) supplementation in patients lacking the mechanisms for the normal absorption of long-chain fatty acid TAGs, e.g. patients with chronic pancreatic insufficiency, parenchymal liver disease with reduced bile salt synthesis, abetalipoproteinaemia, chylomicron retention disease, and intestinal lymphangiectasia.

The subsequent cellular metabolism of medium-chain fatty acids is also different from that of long-chain fatty acids. In general medium chain fatty acids are rapidly oxidised

to CO_2 and H_2O) in the liver and, unlike long chain fatty acids, are incorporated into adipose tissue to a limited extent only.

Little will be said here about the mechanisms underlying the absorption of the four fat-soluble vitamins, A, D, E and K, respectively. Vitamin A may be ingested either as pre-formed vit. A or as carotene, its precursor. It appears that carotene absorption is independent of bile salt concentration and is enhanced by lowering the luminal pH. However, only 20-30% of carotene in a typical diet is believed to be absorbed unchanged. Most of it is oxidatively split into 2 molecules of retinal, which, in turn, is converted to retinol (Vit. A) and then esterified within the intestinal cells (Goodman *et al.* 1966). The retinyl ester is then incorporated into the chylomicron core, along with re-synthesized tri-acylglycerol and cholesteryl esters. Ingested retinyl ester is thought to be hydrolyzed by pancreatic and brush border esterases prior to absorption. Most of the absorbed vitamin A and D is carried via the lymphatics with only small quantities of both vitamins being transported via the portal system (Thomson 1971). Vit E is also transported via the lymphatics although portal transport has been demonstrated. Vit K is carried by lipoproteins in the lymphatics.

In summary:

The digestion and absorption of lipids differs from that of other macromolecules e.g. carbohydrates and protein, in that most of the material leaving the intestine at the end of the process is in the same form as that which entered the gastro-intestinal tract at the outset, i.e. as tri-acylglycerol. (Carbohydrates and protein are absorbed mainly in the form of their digestive end-products, the constituent monosaccharides and amino acids, respectively). Since tri-acylglycerol absorption is virtually complete under normal circumstances, the overall process may be viewed as essentially one of bulk fat transfer (Fig. 1.8). The lingual, gastric and pancreatico-biliary secretions provide the materials for the luminal digestive phase of the process while the intestinal epithelial cells provide

the mechanisms for tri-acylglycerol re-synthesis and packaging into water-miscible lipoprotein particles which eventually enter the circulation. Because of the complexity and scale of the process the intestinal epithelial cells may be expected to contribute a significant proportion of their overall metabolic activity to lipid absorption alone.

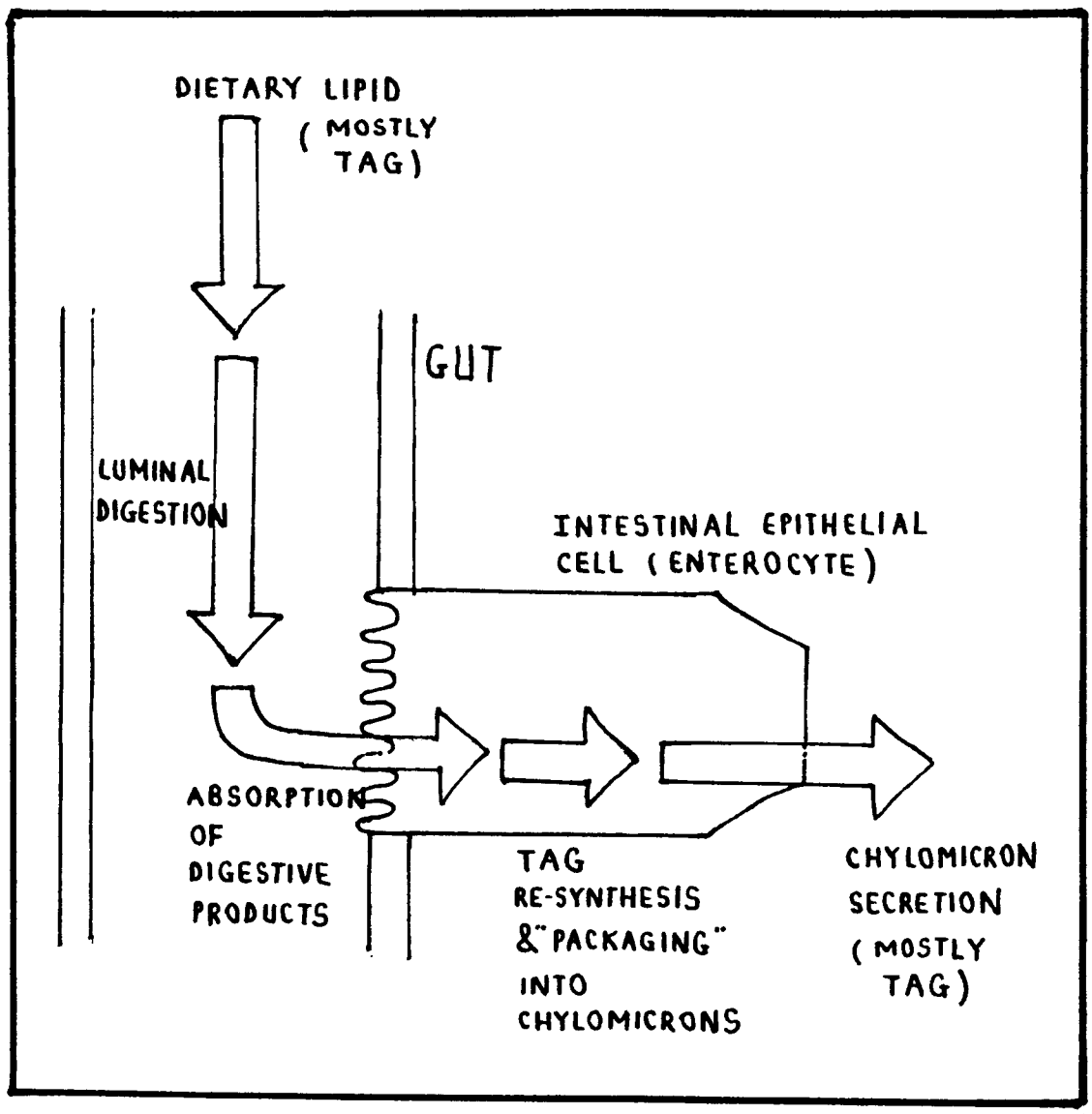


Fig. 1.8. The process of lipid digestion and absorption is essentially one of bulk transfer: most of the material entering the gastro-intestinal tract is tri-acylglycerol (TAG); most of the material leaving the intestine is also tri-acylglycerol.

1.3 MORPHOLOGIC STUDIES OF LIPID ABSORPTION

A number of studies of the intracellular events of fat absorption and chylomicron formation using time-sequence electron micrographs have been reported in the literature (Jersild 1966, Strauss 1976, Sabesin & Frase 1977). Under fasting conditions the endoplasmic reticulum, Golgi apparatus and intercellular spaces are unremarkable structures. During fat absorption, however, smooth endoplasmic reticulum (SER) elements in the apices of the enterocytes distend with fat droplets as a result of the re-synthesis of tri-acylglycerol from the newly absorbed fatty acids (Fig. 1.7). Soon afterwards lipid droplets appear within vesiculated channels of the SER and the Golgi cisternae proliferate and distend with lipid droplets of chylomicron size in the supra-nuclear region of the cell. Secretory vesicles form from the Golgi and these migrate to the basolateral surface of the cell. The secretory vesicles contain discrete vesicle membranes similar to coated pits. At these sites the secretory vesicles become incorporated into the lateral cell membrane and nascent chylomicrons are discharged into the intercellular space by reverse pinocytosis. The chylomicrons penetrate through the basement membrane and the lamina propria and enter the lumen of the lymphatics via gaps which form between the endothelial cells during lipid absorption. (During fasting the endothelial cells closely interdigitate with one another to form a continuous lining) (Fig. 1.7).

The factors responsible for the directional intracellular movement of tri-acylglycerol and lipoproteins are not clear although there is some evidence to support the role of microtubules in the process. The exact mechanisms of the packaging of the intracellular lipid droplets with apolipoproteins, phopholipid and cholesterol to form the chylomicrons are not known.

1.4 LIPOPROTEINS PRODUCED BY THE INTESTINE

Experimental evidence for the intestinal synthesis and secretion of chylomicrons, intestinal very low density lipoproteins (VLDL) and high density lipoproteins (HDL) has been obtained from the following sources:

(1) Collections of intestinal lymph from cannulated mesenteric or thoracic ducts of rats;

(2) Isolation of intracellular lipoproteins from subcellular organelles of intestinal mucosa or isolated epithelial cells (usually of rats);

(3) Cultures of the human colonic carcinoma cell line (CaCO-2) which when confluent possess some properties of intestinal epithelial cells;

(4) Chylous pleural effusions, urine, ascites and thoracic duct collections from humans, and

(5) Postprandial plasma lipoproteins.

1.4 (i) Chylomicrons

Chylomicrons are a heterogeneous group of tri-acylglycerol-rich lipoproteins formed in the intestine during lipid absorption. They are the largest of the lipoprotein classes, with a size range of 750-6000 Å (75-600 nm) in diameter, a mean diameter of 1200 Å (120 nm), and a density of around 0.93 g/ml (S_f 400-10 000) (Lossow *et al.* 1969, Fraser 1970). The size of the chylomicrons depends on the lipid flux through the intestinal cells: under conditions of low lipid flux (e.g. at the start of lipid absorption), they are small; at peak lipid absorption they attain their largest size (Fig. 1.9). With increased luminal lipid load the production rate and size of the chylomicrons in intestinal lymph increases; however, the apolipoprotein content of the larger chylomicrons does not increase proportionally, suggesting a reduction in, or saving of, surface coating materials relative to lipid content (Green & Glickman 1981, Hayashi *et al.* 1990). Compositional analysis of chylomicrons derived from cannulated mesenteric ducts of fat-fed rats have

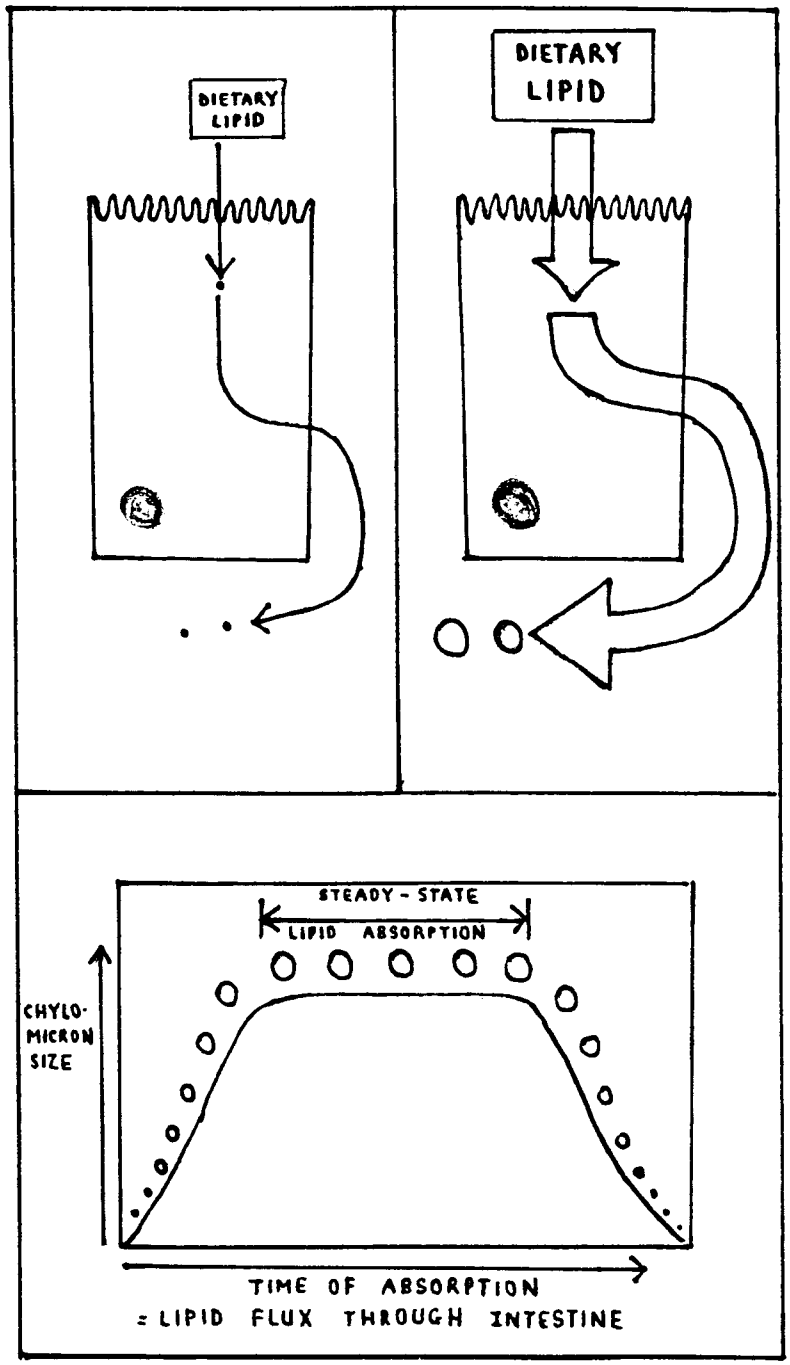


Fig. 1.9. Chylomicron size is dependent on the lipid flux through the intestinal cells.

revealed the following (figures are expressed as % of the total particle mass): surface: phospholipid 6-8%, protein 1-1.5%; core: tri-acylglycerol 86-92%, cholesteryl ester 0.8-1.4%; core and surface: free cholesterol 0.8-1.6%, of which 30% is contained in the core and 70% on the surface (Fig. 1.10).

The fatty acids of the TAG reflect the fatty acid composition of the ingested lipid (Kayden *et al.* 1963). There is, however, evidence that some endogenous lipid is also transported by chylomicrons. The composition of the phospholipid monolayer which coats the particle bears little resemblance to that found in the diet and remains relatively constant despite variations in dietary fatty acid composition (Green & Glickman 1981). Lecithin (phosphatidylcholine) comprises 70 to near 100% of chylomicron phospholipid with smaller amounts of sphingomyelin and phosphatidylethanolamine sometimes present. The bulk of the TAG and cholesteryl ester is contained within the oily core of the particle together with 30% of the free cholesterol. The shell or surface (coat) of the particle consists of a surface monolayer of phospholipids, the apolipoproteins, and 70% of the free cholesterol. It has been estimated that the phospholipids cover 80-90% of the surface area with the apolipoproteins covering 10-20%. Chylomicron composition varies according to the site of isolation: nascent intracellular chylomicrons contain more free fatty acid, free cholesterol and protein but less phospholipid than chylomicrons from lymph; plasma chylomicrons contain more protein and less phospholipid compared with lymph chylomicrons (Green & Glickman 1981).

The apolipoproteins (apoproteins) of rat lymph chylomicrons comprise apolipoprotein A-I (MW ~28 000) (38-50% of chylomicron protein), apolipoprotein A-IV (MW ~46 000) (7-13% of chylomicron protein), apolipoprotein B-48 (apo B-48 or low molecular weight Apo B, B_L) (MW ~240 000) (10% of protein), apolipoprotein C (C-II and C-III) (40%), and apolipoprotein E (5%) (Imaizumi *et al.* 1978). However, not all the apolipoproteins found on the chylomicrons in lymph are necessarily synthesized by the intestinal mucosa

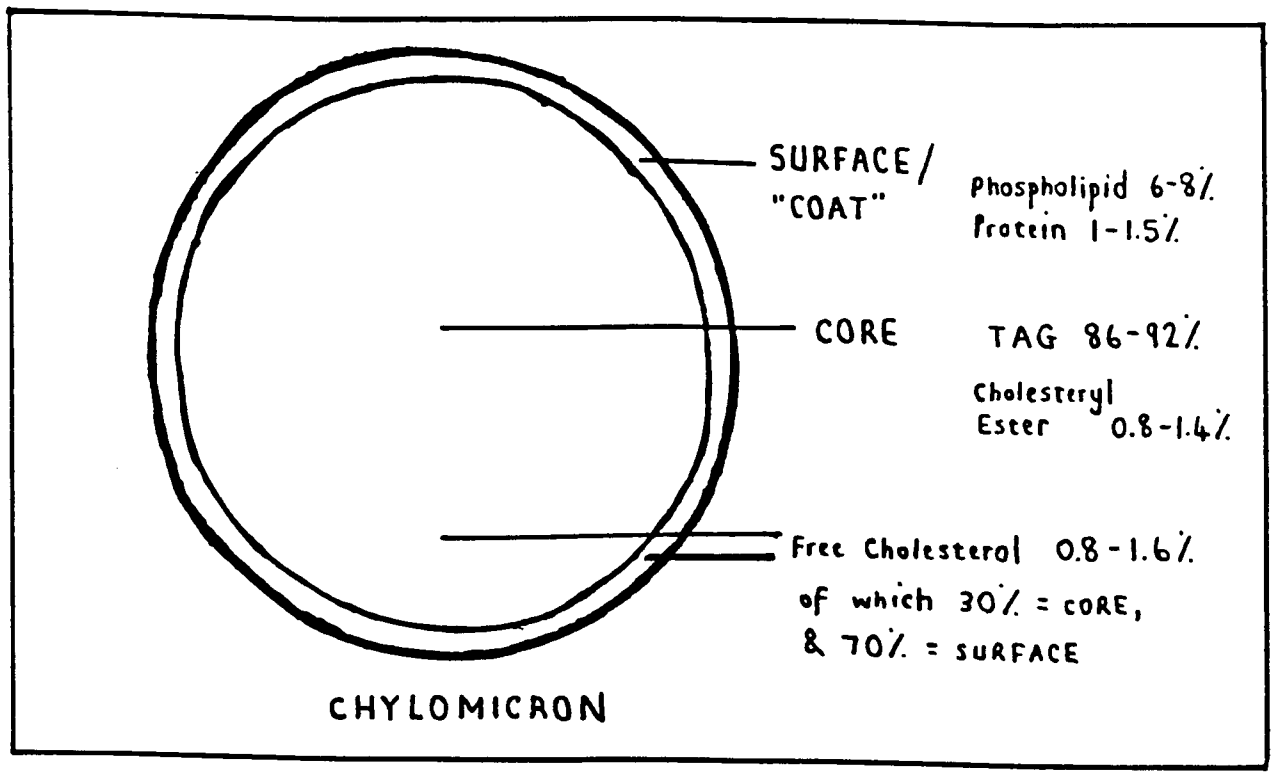


Fig. 1.10. Compositional analysis of chylomicrons from mesenteric ducts of fat-fed rats. Figures are expressed as a % of the total particle mass (Ref.: Green & Glickman, 1981).

and may be acquired by the particle through exchanges of material with other particles e.g. HDL. Radio-isotope labelling studies have shown active intestinal synthesis of apolipoproteins B-48, A-I, A-IV and, to a lesser extent, apo C's (C-II and C-III (Imaizumi *et al.* 1978, Glickman & Green 1977, Wu & Windmueller 1978).

Studies of human thoracic duct chylomicrons and chylous urine have revealed the following distribution of protein: apo B (3.4%), A-I (15%), E (4.4%), A-IV (10%), and A-II and C's, (47.3%) (Green *et al.* 1979).

Soon after leaving the intestinal cells and especially upon entering the plasma from the thoracic duct, chylomicrons acquire apolipoproteins E and C from HDL and other circulating lipoproteins. Apolipoprotein C activates the enzyme lipoprotein lipase and also inhibits chylomicron uptake by the liver so that TAG hydrolysis may occur in the circulation. Most of this breakdown takes place in the capillaries of fat and muscle tissue through the action of the endothelial-bound lipoprotein lipase. The mono- and di-acylglycerols thus formed may be taken up by tissue or are further hydrolyzed in the plasma, while the free fatty acids are either taken up by tissues for oxidation or enter adipocytes where they are re-incorporated into TAG and stored as fat. Some of the released fatty acids may also be removed by binding to albumin which transports them to various tissues, including the liver.

The shrinkage of the TAG core of the chylomicron particles as a result of lipolysis results in excess surface constituents. The apolipoproteins A-I and A-IV are transferred to HDL and in the process the intestine contributes significantly to the HDL apolipoprotein pool (epecially Apo A-I). Chylomicron phospholipid is also transferred to HDL and calculations suggest that even a small fraction of chylomicron phospholipid entering HDL could significantly raise the HDL phospholipid pool (Green & Glickman 1981). Eventually the chylomicron remnant is formed. This small spherical particle

comprises only 4% of the original chylomicron mass and is considerably depleted in TAG but relatively more enriched in cholesterol ester, phospholipid and protein (as proportions of the total particle mass). With the transfer of Apo C back to HDL, Apo B_L and Apo E are now the major protein constituents (Havel & Kane 1989, Tall 1986). Apo C removal results in rapid clearance by the liver of the chylomicron remnants via one or more as yet incompletely characterized specific "remnant" receptors which recognise a binding domain on Apolipoprotein E. Intestinal apo B_L (B-48) is thought not to participate in chylomicron remnant uptake by the liver and lacks the receptor binding domain of apo B-100 for the LDL receptor (Scott 1989). Virtually all dietary cholesterol and the residual tri-acylglycerols are then delivered to the hepatocytes by endocytosis and the components of the particle are hydrolyzed in lysosomes. The cholesterol molecules released from hepatocyte lysosomes may undergo various fates: they may be used in the synthesis of bile acids, they may be secreted directly into bile as free biliary cholesterol, they may be incorporated into nascent hepatic lipoproteins (especially VLDL), or they may be esterified with long chain fatty acids and stored within the hepatocytes in lipid droplets (Tall 1986, Tall *et al.* 1979, Havel & Kane 1989). A schematic representation of the events of chylomicron metabolism is shown in Fig. 1.11.

1.4 (ii) Intestinal Very Low Density Lipoproteins (VLDL)

Intestinal VLDL (ρ < 1.006, 280-750 Å = 28-75 nm in diameter, S_f 20-400) is the major lipoprotein of the intestine found during fasting states and is believed to transport mainly endogenously derived (biliary) lipids (Ockner *et al.* 1969, Ockner & Jones 1970). Biliary diversion or cholestyramine administration in the rat results in reduced intestinal VLDL production (below that of simple fasting) due to decreased biliary lipid flux. However, with lipid infusion into the bowel, luminal fatty acids are incorporated into VLDL-TAG to a small extent, indicating at least some exogenous lipid transport. The VLDL production does not, however, increase proportionally with increased lipid load.

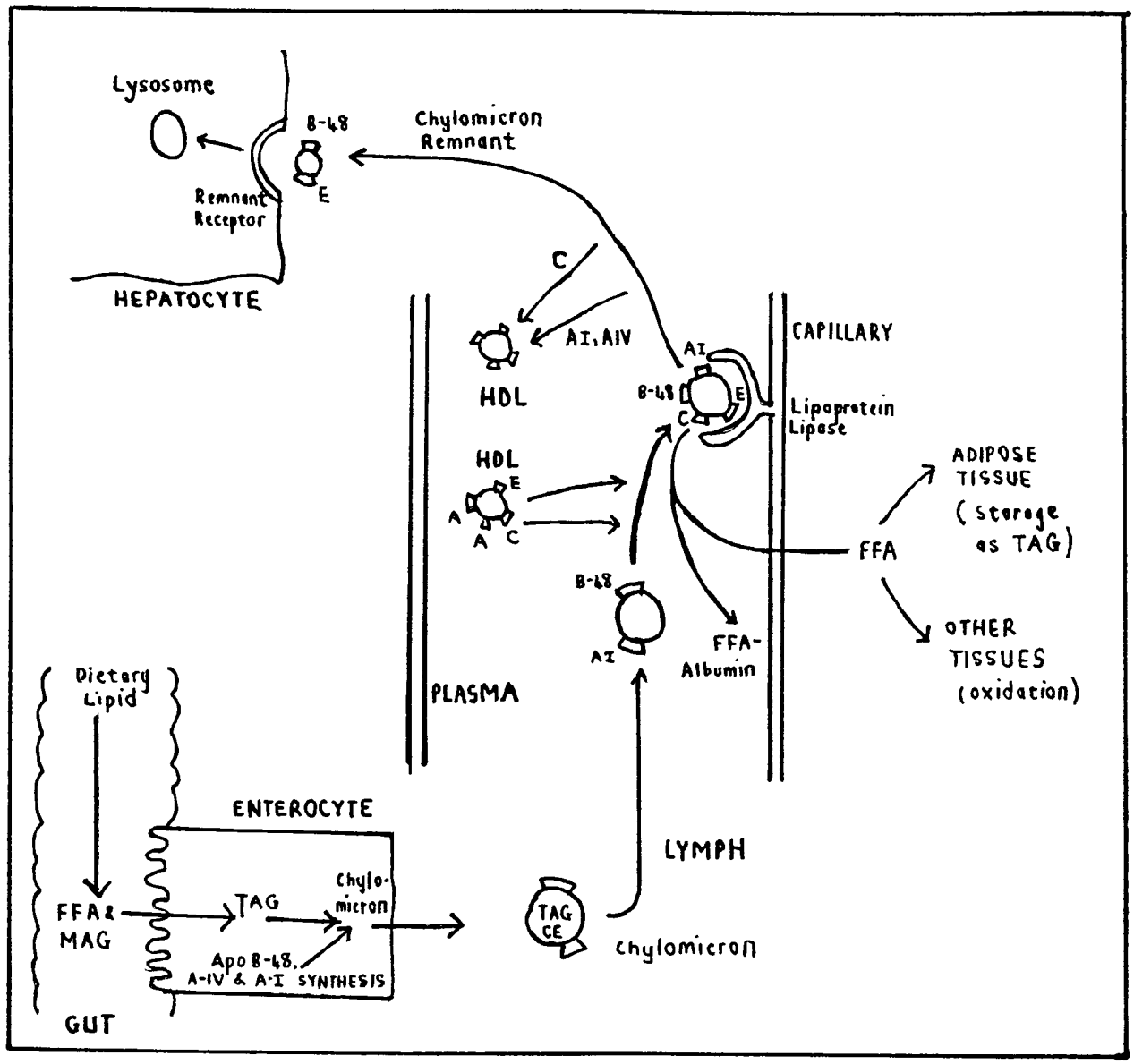


Fig. 1.11. A schematic representation of chylomicron formation and catabolism. FFA = free fatty acid, MAG = mono-acylglycerol, TAG = tri-acylglycerol, CE = cholesteryl ester, HDL = high density lipoprotein, A-I, C, B-48, A-IV = apolipoproteins. (Ref.: Kane & Havel 1989).

During fasting about half of the cholesterol and TAG present in rat mesenteric lymph is found in the VLDL fraction (Ockner *et al.* 1969).

Intestinal VLDL composition resembles that of chylomicrons rather than plasma or liver-synthesized VLDL. Being smaller particles though, the content of surface constituents is greater (phospholipid 15%), free cholesterol 2.3% and protein 4%). The cholesteryl ester content (6%) is also greater than in chylomicrons (~1% of total mass). The apolipoprotein content is similar to that of chylomicrons although Apo A-IV (20-30%) and Apo B content are greater, while the apo C content is lower (Green *et al.* 1979). Intestinal VLDL have thus often been regarded simply as "small chylomicrons" and their subsequent metabolic fate is also believed to be similar. However, studies by Tso *et al.*. (1984b & 1987) using the detergent Pluronic L-81 have shown selective inhibition of chylomicron synthesis by this chemical with the production of VLDL from the intestine left intact, thus suggesting independent pathways or mechanisms of production. Indeed, a study of the lipoproteins associated with the Golgi apparatus of rat intestinal epithelial cells by Mahley *et al.*. in 1971 showed subcellular compartmentalization of VLDL- and chylomicron-sized particles in secretory vesicles with only limited mixing of the two.

During fasting in normal rats the intestine was calculated to contribute about 11% to the total plasma VLDL and this rose to 14-17% in (non-lipid) fed rats (Risser *et al.* 1978). However, in diabetic animals the amount of intestinal VLDL was twice that observed in normals suggesting an important role for the intestine in the pathogenesis of the abnormal lipid metabolism of diabetes mellitus (Popper *et al.* 1985).

1.4 (iii) Low Density Lipoproteins (LDL)

While LDL (ρ: 1.006 - 1.063 g/ml) has been isolated from rat mesenteric lymph, the quantities are small and the particles are believed to originate from the catabolism of hepatic VLDL in the plasma rather than from *de novo* intestinal synthesis or chylomicron

catabolism (which results in chylomicron remnants). The mesenteric LDL therefore derives from plasma ultra-filtration and the increased LDL observed in the postprandial state probably represents increased bowel perfusion. However, some researchers have presented evidence to support an intestinal origin for a lipoprotein particle (ρ: 1.006 - 1.030 g/ml) found in the mesenteric lymph of rats fed a cholesterol-olive oil diet (Riley *et al.* 1980). While the lipid composition of this particle was similar to that of plasma LDL, apolipoprotein A-I was the major protein (compared with apo E in the case of plasma LDL), thus suggesting an intestinal source.

Monolayer cultures of the human intestinal cell line, CaCO-2, secreted particles in the VLDL ($\rho < 1.006$), LDL (1.006 - 1.063) and HDL (1.063 - 1.21) density ranges into the basolateral medium (Traber *et al.* 1987). Apolipoproteins B-100 (57%) and E (30%) comprised the major [^{35}S] methionine-labelled (i.e. newly synthesized) proteins of the LDL fraction. Half of all the newly synthesized Apo B-100 secreted by this cell line was found in the LDL density range with most of the rest recovered in particles less than density 1.006 g/ml.

When CaCO-2 cells were incubated with [^{14}C] oleic acid more than half the radio-labelled lipid recovered was isolated from the LDL fraction with only 42% found in the $\rho < 1.006$ fraction. Thus particles of LDL density while thought not to be significant secretory products of the normal rat intestine are significant lipoproteins of the CaCO-2 cell line (Traber *et al.* 1987).

1.4 (iv) <u>High Density Lipoproteins (HDL)</u>

Using techniques of lipoprotein flotation-ultracentrifugation and electron microscopy of negatively stained (phosphotungstate) HDL lipoproteins, three distinct types of HDL particles (density 1.063 - 1.21 g/ml) have been described from rat mesenteric lymph:

(1) A normal spherical HDL particle, similar to that found in plasma, with a diameter of 10-12 nm. Following fat feeding these particles have been found to increase in number as a result of increased (vascular) perfusion of the bowel and filtration into lymph (Glickman & Magun 1986a).

(2) A discoidal HDL particle constituting 50% of lymphatic HDL in the fasted state and 30% in the fat-fed state. These particles measure 19 by 5.5 nm in diameter and resemble nascent HDL found in liver perfusion systems (Hamilton *et al.* 1976). In lymph they are not reduced by biliary diversion (as are chylomicrons and VLDL) suggesting that they are not derived from chylomicron or VLDL breakdown. Unlike hepatic-derived HDL which contains mainly apo E, this discoidal lymph particle has apolipoprotein A-1 as its major protein constituent. It is also rich in phospholipid.

(3) Small spherical HDL particles, 7.8 nm in diameter, are found especially in the 1.13 - 1.18 g/ml density range. The apolipoprotein constituents are mainly A-I (66%) and A-IV.

Intestinal HDL is thought to have a higher TAG content than plasma HDL (11% versus 2.8%) (Green *et al.* 1979). Under fasting conditions 85% of the apo A-1 in rat mesenteric lymph was found in the $\rho > 1.006$ g/ml fraction while in fat-fed states this dropped to 50% suggesting a redistribution of apo A-1 from HDL to lighter TAG-rich lipoproteins in the fat-fed state (Glickman & Green 1977).

Firm evidence for intestinal synthesis and secretion of HDL was provided by Magun *et al.*. in 1985 who isolated nascent small spherical HDL from the Golgi organelles within rat enterocytes. No discoidal forms were obtained, however, although discoidal HDL has been isolated from the basolateral media of CaCO-2 cells (where both the small spherical and discoidal forms have been found) (Hughes *et al.* 1987).

Windmueller and Wu (1981) have identified newly labelled HDL apolipoproteins in the venous effluent of the isolated perfused intestine, suggesting direct *in vivo* secretion of lipoproteins into the portal vein system. In the absence of TAG absorption a greater proportion of intestinally synthesized apoproteins were secreted directly into the portal sytem. The quantitative, physiological significance of this route of secretion has not yet been determined although it may be important in the secretion of excess apolipoproteins not utilized in the processes of chylomicron assembly. Following secretion of the immature discoidal HDL into the plasma, it is believed to be processed into mature spherical HDL by the acquisition of unesterified cholesterol and phospholipid with subsequent esterification to cholesteryl ester mediated by the enzyme lecithin:cholesterol acyltransferase (LCAT) (which requires apo A-1 as a co-factor). Subsequent remodelling of the particle may occur through various apolipoprotein interchanges, the incorporation of surface remnant material from TAG-rich lipoproteins, and the actions of lipoprotein lipase, hepatic lipase and lipid transfer proteins. It is interesting to note that the plasma of patients with the rare recessively inherited disorder of LCAT deficiency contains HDL particles similar to the small spherical and discoidal forms described in mesenteric lymph (Forte *et al.* 1971, Soutar *et al.* 1982).

1.5 INTESTINAL LIPOPROTEIN UPTAKE

Thus far the role of the intestine in the production of plasma lipoproteins or lipoprotein constituents has been emphasized. However, another function of this organ is that of lipoprotein uptake and clearance from the circulation.

Low density lipoprotein (LDL), which results from the hydrolysis of TAG-rich, hepatocyte-derived VLDL in the plasma, is the major cholesterol-carrying lipoprotein of human plasma. It is taken up by a variety of tissues by receptor-dependent and receptor-independent processes. Using techniques of constant radio-labelled LDL infusion into the circulation of hamsters, Dietschy and co-workers have been able to quantify the relative contributions of the various organs to LDL uptake (Spady *et al.* 1983a). In the hamster most of the LDL (73%) is taken up and degraded by the liver with 90% of this uptake mediated by the LDL receptor. The intestine is the second most important organ, accounting for 10% of LDL turnover. The jejunum and ileum of the small intestine together account for 7% of LDL clearance. Unlike the liver, receptor-independent (as opposed to LDL-receptor-dependent) uptake is quantitatively more significant in the intestine, accounting for 44% of total intestinal LDL uptake. Only 10% of hepatic LDL uptake is mediated independently of the LDL receptor.

While the hepatic LDL receptor is suppressed in hamsters fed a diet of cholesterol and saturated TAG causing an elevation of plasma LDL, the intestinal uptake of LDL appears to be unresponsive to dietary manipulation (which also produces a wide variation in intestinal cholesterol synthesis) (Stange & Dietschy 1983). However, it has been estimated that 4 times more cholesterol is acquired by the intestinal epithelial cells through local *de novo* synthesis than by LDL-cholesterol uptake, so LDL uptake appears from this evidence to be quantitatively unimportant from the point of view of meeting the intestinal cells' cholesterol needs for membrane synthesis and differentiation as well as chylomicron packaging (Stange & Dietschy 1985). Stange and Dietschy, using the

technique of Weiser (see Chapter 2.3(ii)) to fractionate enterocytes from the villus-crypt axis, found that the immature, dividing crypt cells took up more LDL than those of the mature cells nearer the villus tips (Stange & Dietschy 1983) (see Fig. 1.6).

However, immuno-histochemical studies of the rat intestine using a mono-specific anti-LDL receptor antibody by Fong *et al.* in 1989 showed that the greatest density of immune staining occurred in the cells of the villus base but that significant staining was also present at least up to the level of the mid-villus of the jejunum as well as duodenum and ileum. LDL receptor density in the crypt regions was consistently less than that of the base-mid villus but was uniform throughout the intestine. These findings contradicted the earlier Dietschy evidence and led the authors to speculate on a greater role of LDL in providing cholesterol for absorbing cells than previously thought. Since these epithelial cells later migrate to the upper tip of the villus and are ultimately sloughed into the lumen, much of the cholesterol acquired by LDL uptake (and not utilized for lipoprotein assembly or cell membrane synthesis), would be excreted from the body. The intestine is, in fact, the only organ involved in the excretion of cholesterol from the body, the luminal sterols being derived from unabsorbed dietary cholesterol, bile salts (synthesized from cholesterol in the liver), and sloughed epithelial cells. The faecal sterols coprostanol and cholestanone result from the actions of intestinal bacterial enzymes on cholesterol (Montgomery *et al.* 1977).

In 1983 Suzuki *et al.*. provided evidence for specific high density lipoprotein (HDL) binding sites on isolated rat intestinal mucosal cells. Sviridov *et al.*. (1986) also demonstrated saturable, high affinity, reversible binding of HDL with isolated human small intestinal epithelial cells. Internalization and degradation of [^{125}I]HDL_3 was also observed. The intestine may thus be involved not only in the synthesis and secretion of HDL but also in its catabolism. HDL is believed to clear excess cholesterol from peripheral tissues by transporting it to the liver. By incorporation into bile salts the

cholesterol is eventually excreted into the bowel lumen. However, in the light of the HDL-intestinal cell interactions described it is possible that HDL may deliver cholesterol (and other lipoprotein components) directly to the intestinal epithelial cells for use in lipid transport or eventual excretion into the bowel lumen.

The interactions of LDL and HDL with the intestine may provide an exogenous source of apolipoproteins for chylomicron assembly and lipid transport from the cells. The evidence available suggests that this is not true of apolipoprotein B where the isoform on newly synthesized intestinal lipoproteins is B_L (apo B-48) rather than B_H (apo B-100) which is found on circulating LDL. In the case of apolipoprotein A-1 only the immature precursor form or apolipoprotein-pro A-I is present on intestinally synthesized lipoproteins whereas circulating HDL contains the mature apo A-1 (the "pro-" peptide having been cleaved) (Assmann *et al.* 1989).

1.6 THE CONTRIBUTION OF THE INTESTINE TO WHOLE-BODY LIPID DYNAMICS

The overall contribution of the intestine to whole-body lipid dynamics is summarized in Fig. 1.12 and involves both synthetic and catabolic functions in the production of lipoproteins and their clearance from the plasma. During TAG transport the intestine contributes significantly to the plasma apolipoprotein pool. In 1979 Wu and Windmueller performed isotope incorporation studies to determine the relative contributions of various organs to the plasma apolipoproteins of the rat. They found that the liver and the intestine were the only organs which synthesized and secreted plasma apolipoproteins. 19% of the total apolipoprotein pool was synthesized in the intestine, and 81% by the liver. The percentage contribution of the intestine to individual apolipoproteins was: apo A-IV, 59%; apo A-I, 56%; apo B, 16%; apo C-II, 10%; apo C-III-0, 7%; apo C-III-2(-1) < 10%; apo C-III-3, <1%, and apo E, also <1%. The rat intestine was therefore a major source of apo A-1 and apo A-IV which are also the major proteins of plasma HDL. A subsequent study by Kraft *et al.* (1989) on plasma apo A-IV isoforms in humans showed no change following liver transplantation indicating exclusive intestinal synthesis of human plasma apo A-IV.

In the plasma the surface components of TAG-rich lipoproteins (the apolipoproteins and phospholipid) are rapidly redistributed to the HDL fraction following lipolysis. The intestine is therefore intimately involved in plasma HDL metabolism. However, in addition to this indirect contribution to plasma HDL via the transfer of material, the intestine itself is a source of HDL through direct synthesis and secretion of nascent, immature HDL particles which are probably transformed in the plasma to mature HDL. The relative contribution of the intestinally synthesized HDL to the total plasma HDL pool is not known. The intestine also synthesizes VLDL which transports mainly endogenously derived lipid. According to one study intestinal VLDL contributes about

11% of the total plasma VLDL in the fat-fed rat and about 14-17% in non fat-fed animals (Risser *et al.* 1978).

Finally, the intestine is an important organ (second only to the liver) for the clearance of cholesterol-rich LDL from the plasma. There is also evidence for HDL binding and internalization by intestinal epithelial cells although the physiological significance of this process remains to be determined.

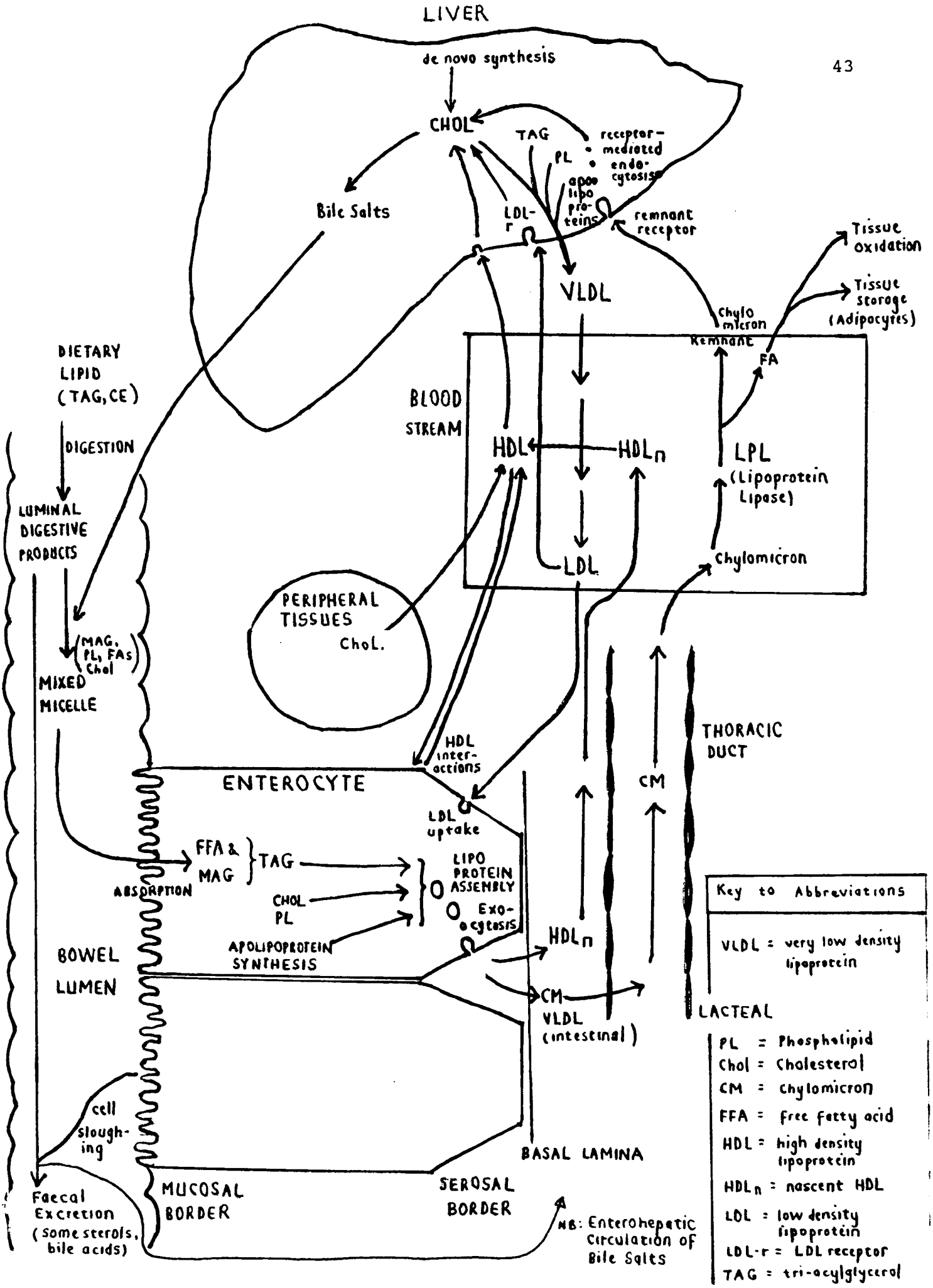


Fig. 1.12. Lipid fluxes across the intestinal epithelium and their relation to whole-body lipid dynamics.

1.7 QUESTIONS CONCERNING INTESTINAL LIPID TRANSPORT AND APOLIPOPROTEIN BIOSYNTHESIS

The complex nature of the process of lipid absorption and transport in itself raises several important questions regarding the regulation of the process in the short- and long-term, the effects of any changes that may occur on whole-body lipid dynamics, and the implications that such changes may have with respect to certain disease states e.g. atherosclerosis and diabetes mellitus.

The dietary lipid flux is periodic or episodic in nature. Most carnivorous mammals do not ingest food on a continuous basis but feed at certain times. The dietary lipid content (as a proportion of the total meal) and composition of the various lipids (saturated versus unsaturated fat, cholesterol content) may vary from meal to meal. Nevertheless, the intestinal epithelial cells have to provide the "packaging materials" for fat transport (the apolipoproteins, phospholipid and some cholesterol) whenever the lipid arrives and have to cope with varying lipid loads. This project aims in particular to examine whether the processes of apolipoprotein biosynthesis on the one hand, and lipid assembly and elaboration, on the other, are linked or co-ordinated and, if so, how this is achieved.

Apolipoprotein availability is essential for lipid transport out of the epithelial cells and active apolipoprotein synthesis also appears to be necessary for the process (see Chapter 1.8). The question is: how is this "availability" met? Is TAG absorption a specific stimulus (directly or indirectly) for active apolipoprotein synthesis; i.e., is apolipoprotein synthesis regulated by dietary lipid flux? If so, at what level is this regulation effected? By pre-translational gene activation and expression? By increased messenger RNA "translatability", or by post-translational protein activation? Are there factors other than TAG absorption which stimulate apolipoprotein synthesis and secretion? Over what time period do regulatory changes take place? The processes of lipid transport are, in fact, very rapid. Chylomicrons appear in the intercellular spaces

of the epithelial cells as little as 12 minutes after the instillation of lipid in the bowel lumen (Jersild 1966). Is there an intra-cellular pool of apolipoproteins available for lipid packaging? How large is this pool and to what extent is it able to meet the apolipoprotein "demands" of the cell? Do degradation mechanisms play a role in regulating intracellular apolipoprotein availability? Do the regulatory mechanisms differ according to various segments of the small intestine (e.g. jejunum or duodenum versus ileum)? Do they vary along the villus-crypt axis of the same bowel segment?

Other questions also arise concerning the nature and composition of the intestinally produced lipoprotein particles under varying dietary perturbations. How does the chemical composition of the various intestinal lipoprotein particles change with variations in dietary lipid content and composition? Do meals rich in saturated fats and cholesterol, for example, cause the production of lipoproteins with a different apolipoprotein composition compared with those caused by meals rich in unsaturated fats? Do these differences affect the subsequent metabolism of the particles and, given the important contribution of the intestine to overall lipid dynamics in the body, do such alterations have implications in the aetiology of atherosclerosis?

Finally, what are the effects of chronic alteration in diet on the processes of lipid absorption and apolipoprotein biosynthesis? Does chronic (as opposed to acute) dietary fat loading give rise to adaptations in the intestine e.g. enhancement in its capacity to absorb lipids and up-regulation of apolipoprotein biosynthesis? Do chronic high saturated fat and cholesterol diets result in alterations of apolipoprotein synthesis, secretion, chylomicron composition and subsequent metabolism, and do these chronic alterations play a role in atherogenesis? Adaptive changes are known to occur in the intestine e.g. the rate of lipase secretion by the pancreas is enhanced with chronic dietary lipid. In uncontrolled diabetes mellitus associated with polyphagia and increased metabolism generally, gut hypertrophy occurs. Adaptive changes are also seen in

various segments of the small bowel following surgical resection. The ability of the ileum to absorb fat, for example, may be significantly enhanced when it is chronically exposed to a higher nutrient load or following resection of part of the small bowel.

1.8 INTESTINAL APOLIPOPROTEIN BIOSYNTHESIS AND ITS REGULATION

1.8 (i) Apolipoprotein B

The molecular and cell biology of apolipoprotein B has previously been reviewed by Scott (1989 & 1990). The most important facts will be summarised here: Two major isoforms of apolipoprotein B have been found in mammalian species: Apolipoprotein B-100, a large hydrophobic glycoprotein of 4536 amino acids and a molecular weight of 512 kiloDaltons, (also known as High Molecular Weight Apo B or Apo B_H), and Apolipoprotein B-48 (48% of the size of Apo B-100 on SDS polyacrylamide gels), with 2152 amino acid residues and a molecular weight of 242 kDa (also referred to as Low MW Apo B or B_L). Apo B-48 is colinear with the amino terminal half of Apo B-100 and terminates at residue 2152 (isoleucine) of apo B-100 (Scott 1989).

Both proteins arise from a common gene. The gene is expressed primarily in the liver and the intestine (Scott 1990). There is evidence for some expression in renal tubular cells (Hodges & Scott 1992). A unique post-transcriptional editing reaction results in the de-amination of a specific cytidine base of codon 2153 (nucleotide 6666) to a uridine, thus creating a "stop" codon (UAA) from a glutamine codon (CAA) (Powell *et al.* 1987) (see Fig. 1.13). Apo B-100 is synthesized in the mammalian liver. In humans the liver synthesizes apo B-100 only, while in rodent species both apo B-48 and apo B-100 are produced by the liver (Scott 1989). The editing mechanism - a site-specific, sequence-specific cytidine de-aminase - manifests itself in the human intestine to produce B-48, although the editing mechanism itself has been found in other tissues and cell lines that do not express apolipoprotein B (Driscoll & Casanova 1990).

Two sizes of apo B-48 (i.e. edited) messenger RNA are found in the intestine of different mammalian species: a 14 kilobase (kb) mRNA, equal in length to the mRNA for apo B-100 but with the stop codon), and a 7 kB mRNA. The latter is thought to

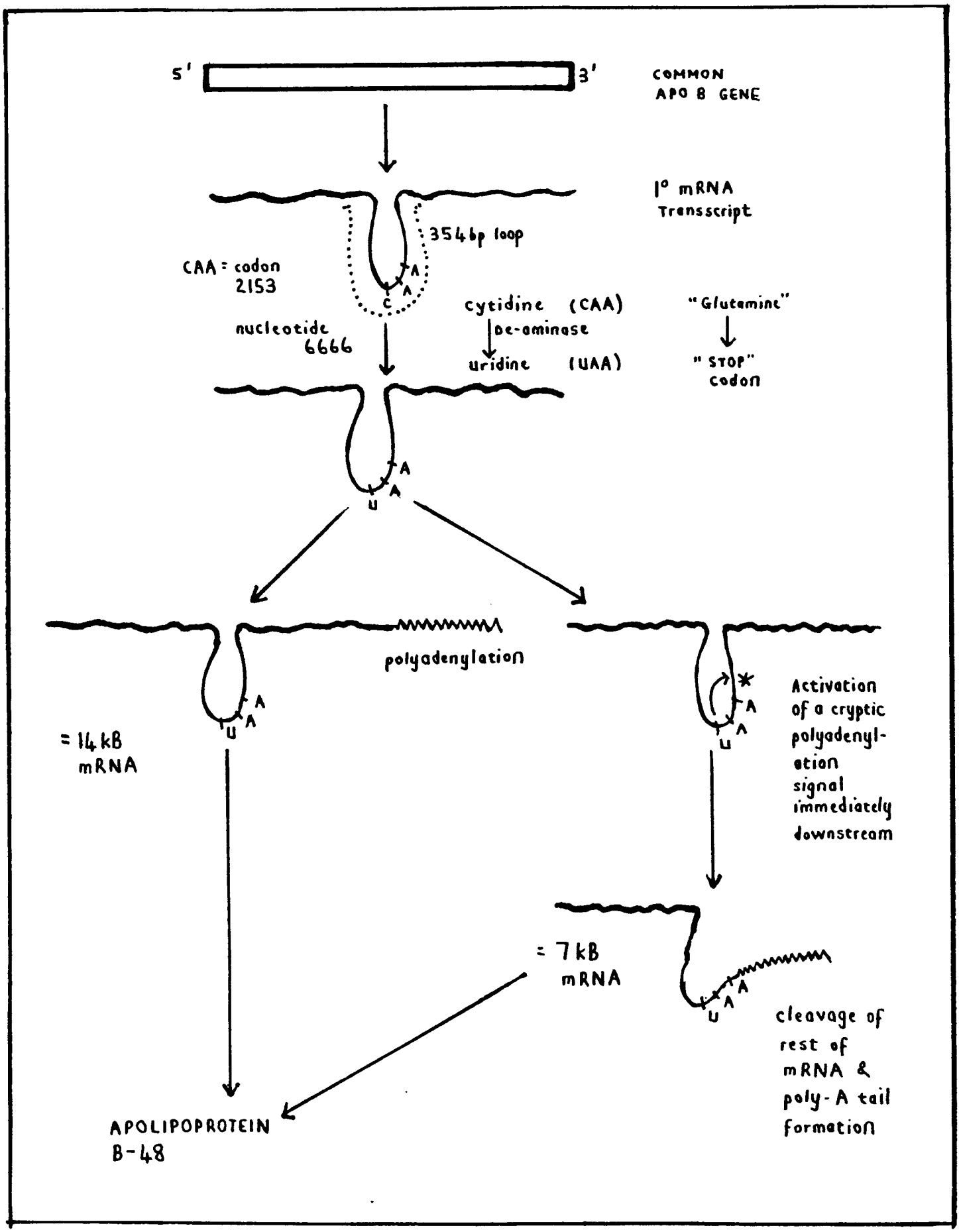


Fig. 1.13. A schematic representation of apo B mRNA editing to produce apolipoprotein B-48 in the intestine.

arise through the activation of a cryptic polyadenylation signal immediately downstream from the stop codon, resulting in cleavage of the mRNA at this site and a polyA tail formation. Both mRNAs will, of course, still give rise to apo B-48 (Fig. 1.13).

The mRNA editing activity is under developmental, hormonal and nutritional regulation. Early in development both liver and intestine express only unedited apo B mRNA (Glickman *et al.* 1986b). Around birth the intestinal mRNA becomes edited to a progressively greater extent and progresses to complete editing in adults. Editing is also induced in the rat liver but to a lesser extent and on a time-course independent of that of the intestine. Uninduced cells of the small intestinal-like cell line, CaCO-2, do not edit apo B mRNA whereas more differentiated cells do (Hodges & Scott 1992). Thyroid hormone induces apo B mRNA editing in rat liver (Davidson *et al.* 1988b & c) while fasting induces a decrease in editing activity. With carbohydrate re-feeding, which increases lipogenesis, near-complete editing of RNA results again (Baum *et al.* 1990).

Studies by Glickman *et al.*. (1978a) showed that radioactive amino acids were incorporated into apo B associated with lymph chylomicrons during TAG absorption. Windmueller and Wu (1981) estimated that 5% of the plasma apo B pool under fasting conditions originated from the intestine. This increased to 16% during fat feeding (Wu & Windmueller 1979). The active synthesis of apo B (and other intestinal apolipoproteins) is essential for chylomicron formation. This was demonstrated by studies using inhibitors of protein synthesis which resulted in TAG droplet accumulation within intestinal epithelial cells and decreased lymphatic secretion of chylomicrons (Glickman *et al.* 1972). The chylomicrons that were secreted under these conditions were larger than usual and contained reduced amounts of apo A-1, although apo's A-IV and B were still present (Glickman & Kirsch 1973).

The importance of apo B synthesis for lipid absorption and chylomicron formation is perhaps best illustrated by the autosomal recessive condition of abetalipoproteinaemia, in

which the liver and intestine are unable to form lipoproteins containing apo B and TAG droplets accumulate in these organs (Kane & Havel 1989). As a result, there is malabsorption of fat and fat-soluble vitamins; chylomicrons, VLDL and LDL are absent from the plasma, and levels of cholesterol and TAG are very low. Apo B has been shown to be absent from the enterocytes of some subjects with this condition, indicating an inability to synthesize the protein (Glickman *et al.* 1979); in others, though, increased quantities of polyadenylated apo B-100 were found, suggesting perhaps a defect in the post-translational modification of the protein. An interesting variant of this condition has been described by Malloy *et al.*. (1981): apo B-100 was deficient in the serum with severely reduced levels of plasma LDL-cholesterol, while intestinal apo B-48 synthesis and hence lipid absorption, chylomicron formation, and postprandial lipaemia were not affected. This patient therefore had a selective deficiency of hepatic apo B.

Studies of the intracellular content of apo B in the intestines of various species have produced varied results. The enterogastric instillation of corn oil in rats resulted in the apo B concentration (normally 80 μg/g cell protein) rising two- to threefold after two hours (Schonfeld *et al.* 1978). Other studies using fluorescent antibodies suggested that the increase in the cellular apo B content was more rapid, occurring as early as ten minutes after exposure of the rat intestine to lipid (Glickman *et al.* 1979). However, studies on human intestinal biopsies showed a significant decrease in the apo B content post-prandially, suggesting depletion of a pool or a rate of chylomicron-apo B secretion exceeding the rate of apo B synthesis (Rachmilewitz *et al.* 1976).

There is in fact some good evidence for a substantial intracellular apolipoprotein B pool. When protein synthesis inhibitors were administered to rats under conditions of no exogenous lipid absorption, only a 20% reduction in the apo B content of the cells was observed. Biliary diversion (which depletes the intestinal mucosa of TAG) also produced only a modest reduction in cellular apo B content. Finally, when protein synthesis

inhibitors were administered to rats actively absorbing TAG, the mucosal apo B content was reduced by about 50% but no more, suggesting a limit beyond which no further reduction in apo B could be achieved (Bisgaier & Glickman 1983).

Ultra-structural, immuno-localization studies of apo B have demonstrated its presence in the rough endoplasmic reticulum and on TAG droplets in profiles of the smooth endoplasmic reticulum, suggesting that apo B was added early on in chylomicron formation (Rubin *et al.* 1980).

Perhaps the most definitive experiments on the regulation of the synthesis of apo B-48 in the intestine have been provided by the group of Davidson, Glickman and co-workers in the 1980's. Rats were either fasted, fed fat-free chow or were administered an acute bolus of TAG. Under general anaesthetic a laparotomy was performed and radioactive amino acid instilled into a selected segment of the small intestine. After a nine-minute protein synthesis "pulse" (the labelling period was confined to 9 minutes to avoid the loss of labelled lipoprotein apolipoproteins by secretion from the cells) the tissue was removed, homogenized, and the incorporation of radioactive amino acids into apo B measured by immunoprecipitation. Results were expressed as a proportion of the total trichloroacetic acid-insoluble radioactive protein in the cell. These workers found no difference in apo B synthesis as a percentage of the total protein synthesis in both control and experimental groups of animals. Furthermore, the intracellular apolipoprotein B content (as measured by radio-immunoassay) remained virtually unaltered although it appeared to fall slightly for a short period only at the onset of the triglyceride flux. The authors concluded that apo B synthesis rates were not regulated by acute TAG flux (Davidson *et al.* 1986).

Subsequently, using similar experimental techniques, the same authors showed that chronic lipid feeding (30% saturated or unsaturated fat as opposed to 0% fat but iso-

caloric diets in controls, for six weeks) also failed to affect B-48 synthesis. However, an inverse relation was found between B-48 synthesis and mucosal cholesterol absorption i.e., the higher the mucosal cholesterol absorption, the lower the apo B-48 synthesis (as a % of total protein). Hypothyroidism (induced by the administration of propylthiouracil to rats) also resulted in decreased B-48 synthesis (Davidson *et al.* 1987).

Prolonged (unphysiological) biliary diversion, however, resulted in a decline in the apo B-48 synthesis rate and this was restored with bile salt replacement in a dose-dependent manner. Despite the changes in B-48 synthesis rates, however, apo B mRNA size and abundance as determined by RNA blot hybridization remained unaltered (Davidson *et al.* 1986, 1988a).

In 1988 Magun *et al.*. utilized the technique of nitrogen cavitation to isolate nascent intracellular lipoproteins from the enterocytes of rat small intestine (jejunal segments). They measured the distribution of apolipoproteins B-48 (and A-1) in 2 intracellular pools or compartments: (1), a non-lipoprotein-bound, membrane-associated (endoplasmic reticulum, Golgi) pool, and (2), a lipoprotein-associated pool. Both radio-immunoassays and radioactive amino acid incorporation were used in the measurements. They found that, under fasting conditions, less than 10% of the apolipoprotein was lipoprotein-associated; with fat feeding, this % increased slightly, but most of the apolipoprotein remained associated with the microsomal membranes. They concluded that only a small fraction of a large intracellular apolipoprotein pool was mobilized during lipid absorption and that the apparent increase in apolipoprotein B secretion following lipid feeding resulted from a small shift away from the membrane-associated, to the lipoprotein-bound, pool (Magun *et al.* 1988). The constant presence of this large pool meant that apolipoprotein synthesis was not the rate-limiting step in lipoprotein assembly or secretion. The model of apo B-48 synthesis that therefore emerges from these experiments is one in which a constant rate of synthesis occurs i.e. the B-48 gene is

constitutively expressed ("permanently up-regulated" by endogenous biliary lipid, perhaps) to provide a large intracellular pool of mainly membrane-associated apolipoprotein B which is more than sufficient to provide for the needs of the cell during acute (and indeed chronic) TAG flux through the intestine (Fig. 1.14).

However, questions still arise about the fate of the "unutilized" excess apo B-48 during non-fat-fed (but also during fat-fed) conditions, since only a small portion is actually utilized in lipoprotein packaging. Are there high rates of apolipoprotein B-48 degradation in the cell? On the face of it, this "strategy" of providing more than is ever needed appears rather wasteful of the cell's energy sources. Perhaps it is a mechanism to safeguard against the sudden appearance of a TAG flux, irrespective of the size of the lipid load? Secondly, can the intracellular pool of apo B ever be depleted? (30% fat/feeding for 6 weeks was not sufficient in the Glickman experiments). If such a depletion is possible, will this increase the rate of B-48 synthesis or is it already operating at "ceiling" levels? Will malabsorption occur under these conditions?

Evidence for intracellular apolipoprotein B degradation in liver was provided by Borchardt and Davis in 1987 who showed significant intracellular turnover of apolipoprotein B during pulse-chase studies in cultured rat hepatocytes. In 1991 Dixon *et al.*. examined the effect of oleate on apolipoprotein B turnover and secretion in the hepatoma cell line, Hep G2. They showed that oleate stimulated the secretion of apolipoprotein B-containing lipoproteins by inhibiting the early intra-cellular degradation of apo B. In the absence of oleate, 58% of the apo B synthesized during the labelling period was degraded within 20 min; when oleate was present, only 29% of labelled apo B was degraded in the same period. The actual synthesis of apo B was minimally affected by the addition of oleate. The authors therefore concluded that the effects of oleate on apo B secretion were post-translational and involved some kind of protection of nascent apo B from rapid intracellular degradation early in the secretory pathway.

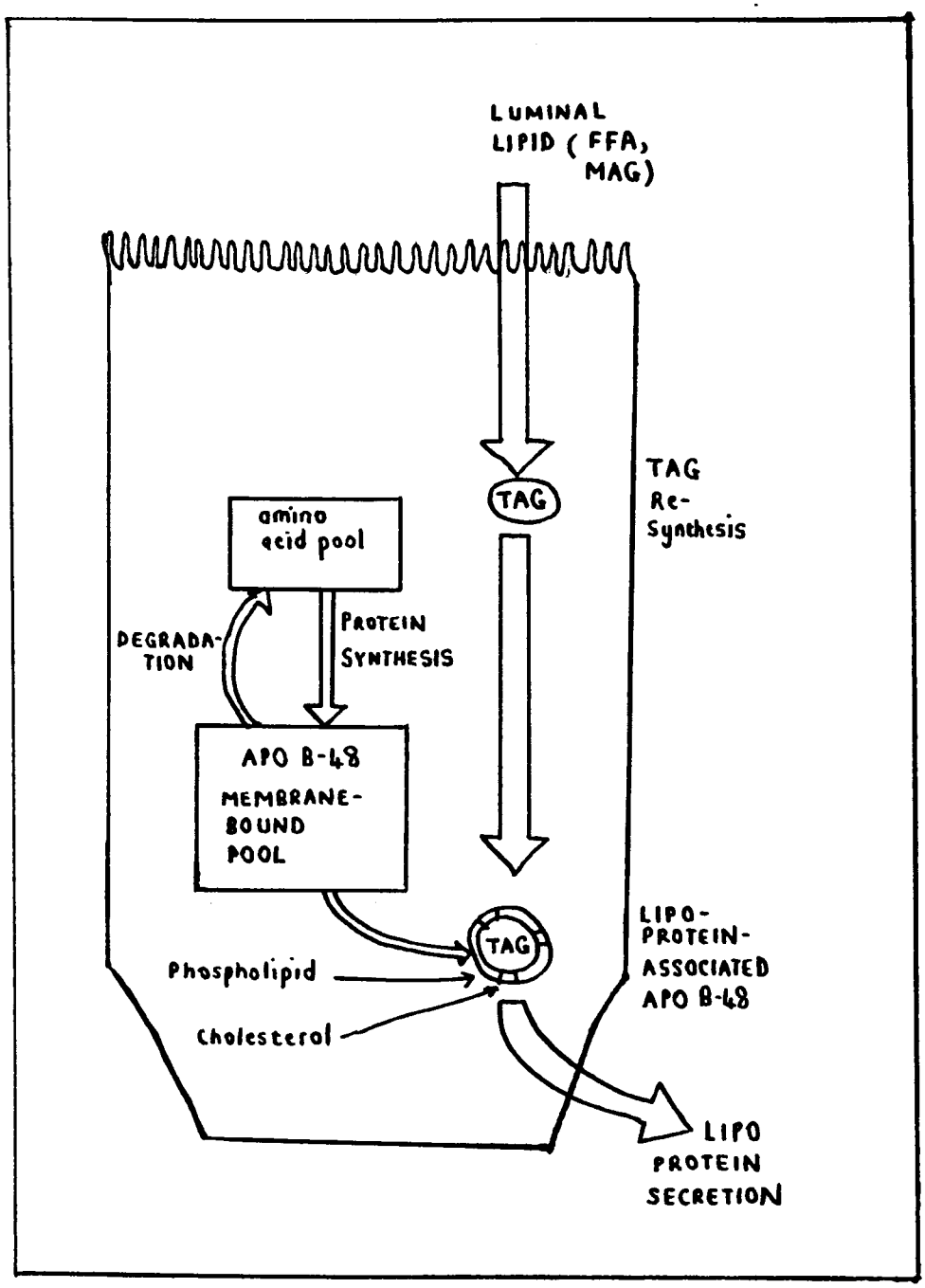


Fig. 1.14. Schematic representation of the two intracellular apolipoprotein B-48 pools. Most of the intracellular apoB is believed to reside in the membrane-bound (non lipoprotein-associated) pool.

A more recent study by White *et al.*. (1992) confirmed this finding. Using the Hep G2 model and a series of carboxyl terminally truncated apo B constructs, they concluded that oleate had no effect on the translation of apo B mRNA but promoted the secretion of apo B-containing lipoproteins by reducing the pre-secretory degradation of those forms of apo B that were able to produce buoyant (flotable) lipoproteins.

Perhaps a similar model operates in the intestine. However, no studies of intestinal apo B-48 degradation have been reported in the literature to date. A number of other studies of apolipoprotein B synthesis have been reported. In the context of the present work, one other deserves mention: A study by Renner *et al.*. in 1986 showed that saturated fat feeding resulted in a selective increase in the apo B content of chylomicrons prepared from these animals compared with unsaturated fat-feeding. They also found small differences in the clearance of saturated versus unsaturated fat-containing chylomicrons when these lipoproteins were infused into a new set of animals, and they speculated that these differences might be significant with respect to the potential atherogenicity of saturated compared with unsaturated lipids.

1.8 (ii) Apolipoprotein A-1

Apo A-1 is the major protein of plasma HDL and of TAG-rich lipoproteins from mesenteric lymph. It has a molecular weight of around 28 000 daltons in both rats and humans (Bisgaier & Glickman 1983, Green & Glickman 1981). In humans, apo A-1 is synthesized as a 267 amino acid residue pre-pro-apolipoprotein (pre-pro-apo A-1) which contains an 18 amino acid pre-peptide and a 6 amino acid pro-peptide. The pre-peptide is co-translationally cleaved inside the cell and the protein is secreted as pro-apo A-1 by the intestine into lymph and by the liver into the plasma. Plasma pro-apo A-1 undergoes intravascular post-translational cleaving to the 243 amino acid residue mature apo A-1 (Assmann *et al.* 1989). In normal human plasma most of the apo A-1 is in the mature isoform.

Apo A-1 exhibits considerable α-helical structure. It is an activator of the enzyme lecithin: cholesterol acyltransferase (LCAT) which esterifies cholesterol on HDL using a fatty acyl residue of lecithin (phosphatidyl choline). Wu and Windmueller's study (1979) indicated that 56% of the total rat plasma apo A-1 was derived from intestinal synthesis. Estimates from chyluric patients have suggested a similar proportional contribution in humans (Green *et al.* 1979).

Studies conducted in rats have demonstrated that approximately 130 to 140 mg per hour of A-1 is transported in mesenteric lymph under conditions of saline or glucose feeding (Imaizumi *et al.* 1978). During TAG absorption this value increases two-fold and is associated with a shift in the distribution of apo A-1 amongst the lipoprotein classes: a greater proportion of lymph A-1 becomes associated with the TAG-rich $\rho < 1.006$ g/ml fraction and a reduction in HDL-associated A-1 occurs (Glickman & Green 1977).

In studies of humans with chyluria apo A-1 was found to comprise 15% of chylomicron protein. However, this is thought to represent an underestimate because of the rapid transfer of apo A-1 that is known to occur from chylomicrons to HDL (Green *et al.* 1979).

Using immuno-fluorescence techniques, apo A-1 has been demonstrated in both rat and human enterocytes (Glickman *et al.* 1978a). Incorporation of radioactively labelled amino acid into apo A-1 by the small intestine or isolated enterocytes has also been demonstrated in both species (Rachmilewitz *et al.* 1978).

In rats the intracellular apo A-1 content has been measured at 80 to 150 nanograms per mg enterocyte protein (Schonfeld *et al.* 1978) and a similar figure of 200 ng/mg protein has been obtained for human duodenal A-1 content in the post-absorptive state (Green *et al.* 1982). This apo A-1 has been shown to originate from the intestine and is not

derived from the circulation through filtration. A study by Imaizumi *et al.* (1978) showed that only 3-5% of the apo A-1 on lymph chylomicrons could have originated from the plasma.

Although fat-feeding has been shown to increase the secretion of apo A-1 into lymph and early immunofluorescence studies in both rats and humans have suggested an increase in the intracellular content of apo A-1 with lipid feeding, studies of the apo A-1 content using radio-immunoassays have not shown dramatic changes (Glickman *et al.* 1978b). The mucosal apo A-1 content appears small relative to the lymphatic output. It has been estimated that the total intestinal mucosal content of apo A-1 in the rat is only 2 μg, yet lymphatic secretion during fat feeding is 20 to 50 times that amount. Thus a high rate of mucosal synthesis of A-1 might be expected (Bisgaier & Glickman 1983).

A study by Gordon *et al.* (1982a) which first demonstrated that the primary translational product of rat intestinal apo A-1 mRNA was a pre-protein, also showed no increase in enterocyte A-1 mRNA following the intra-gastric feeding of corn oil to rats. These results were supported by Blaufuss *et al.* who showed no increase in pre-pro-apo A-1 mRNA levels in rat intestinal mucosa following acute fat feeding although mRNA for another apolipoprotein, pre-apo C-III, doubled. Other workers have shown significant synthesis and secretion of apo A-1 in the absence of TAG absorption (e.g. in glucose-infused, or bile-diverted, animals) (Windmueller & Wu 1981).

The group of Davidson and Glickman has provided the most comprehensive study of the effects of dietary lipid on apo A-1 synthesis. Using the same techniques described in 1.8(i) for apo B, they found that acute fat feeding resulted in no significant change in apo A-1 synthesis or in the intracellular content of apo A-1 in jejunal enterocytes, compared with fasting, bile-diverted, or fat-free feeding. In contrast to the results obtained with jejunal enterocytes, apo A-1 synthesis and content were significantly

depressed in ileal enterocytes following 48 hours of external bile diversion. This effect was reversed by the infusion of the bile salt, taurocholate, into the bowel (Davidson *et al.* 1985). Sustained changes of transmural TAG flux (30% TAG diet for six weeks) also had no effect on A-1 synthesis (Davidson 1987). Finally, as in the case of apo B-48, they found that most of the intracellular apo A-1 was present in a membrane-associated, non-lipoprotein-bound pool, and that fat-feeding resulted in a small redistribution to a lipoprotein-bound pool (Magun *et al.* 1988).

The model therefore of apo A-1 regulation in the intestine derived from these experiments is the same as the one proposed for apo B-48: apo A-1 synthesis is not regulated by dietary lipid flux; the "constitutive" synthesis that occurs provides a large intracellular pool which is only slightly utilized for lipoprotein "packaging" during times of TAG flux.

Whether transmural TAG flux has any effect on the post-translational modification of A-1 (from pre-pro-A-1 to pro-A-1 and eventually to mature A-1) is not yet known.

1.8(iii) Apolipoprotein A-IV

Apolipoprotein A-IV is the third major apolipoprotein of chylomicrons that originates in the intestine. Human apo A-IV is a glycoprotein with a high proportion of α-helical structures, which result from the presence of multiple amino acid repeating sequences (Weinberg & Spector 1985, Elshourbagy *et al.* 1986). These amino acid repeats are amphipathic in nature and the integrity of apo A-IV's secondary structure is essential for its lipid-binding capacity (Elshourbagy *et al.* 1986, Boguski *et al.* 1984). Human apo A-IV comprises 391 amino acid residues and this includes a 20 amino acid signal peptide which is cleaved co-translationally to yield a final protein of 371 amino acids (Gordon *et al.* 1982a & 1984). The protein has a calculated MW of 42500 Da (Boguski *et al.* 1984)

although apo A-IV derived from rat plasma HDL has a MW of 46000 on SDS polyacrylamide gels (Swaney *et al.* 1974).

In the rat 60% of the apo A-IV is produced by the intestine (Wu & Windmueller 1979) and evidence based on the isoform pattern of apo A-IV from human liver transplant patients before and after liver transplantation showed no change in the isoform pattern (despite differences between the donor and the recipient before transplantation) indicating exclusive intestinal synthesis (Kraft *et al.* 1989).

The metabolic fate of apo A-IV appears to differ between humans and rats. In rats, the A-IV is believed to be transferred to the HDL fraction of plasma during chylomicron catabolism (Tall *et al.* 1979); in humans the A-IV that leaves the chylomicrons is recovered mainly in the density > 1.21 g/ml fraction of the plasma. It has been suggested that as much as 80% of human apo A-IV circulates as a free apolipoprotein (Ghiselli *et al.* 1986).

In abetalipoproteinaemia, where no chylomicrons are secreted, plasma apo A-IV levels are only about half those of normals, suggesting A-IV secretion unassociated with TAG-rich lipoproteins, perhaps in association with nascent HDL or as a free apolipoprotein (Green *et al.* 1980).

Unlike apo B-48 and apo A-1, there is greater consensus in the literature concerning the changes associated with apo A-IV during fat-feeding. In humans, the fasting intestinal mucosal A-IV content is 12 μg/mg protein; this rises to 46 μg/mg after lipid feeding (Green *et al.* 1982). A study by Apfelbaum *et al.* in 1987 showed that acute and chronic dietary TAG feeding increased apo A-IV synthesis in rat enterocytes, compared with controls. In the case of the acute TAG bolus administration, the increase was maximal at 4-6 hours post-feeding and occurred in both jejunum and ileum. Apo A-IV mRNA

abundance measured by an *in vitro* translation assay showed a significant increase at 4-6 hrs (a 50% increase in the jejunum and a 350% increase in the ileum). These findings confirmed the results of an earlier study by Gordon *et al.* (1982b). Chronic saturated or polyunsaturated fat-feeding (for six weeks) showed a similar increase in jejunal A-IV synthesis but no change in ileal synthesis (Apfelbaum *et al.* 1987). In chyluric humans, apo A-IV secretion and intestinal content were markedly stimulated by fat-feeding (Green *et al.* 1980 & 1982). In normal human subjects, the plasma level of apo A-IV is also increased with fat-feeding (Green *et al.* 1980). Studies in mice have also confirmed a lipid-regulated increase in apolipoprotein A-IV expression in the liver (Williams *et al.* 1989).

A study by Hayashi *et al.* (1990) using the non-ionic hydrophobic surfactant, Pluronic L-81, which inhibits chylomicron formation but does not affect lipid uptake or resynthesis inside the cell, provided some insight into the mechanism by which the TAG-related increase in apo A-IV synthesis is brought about. They showed that this increase did not occur when lipid was infused into the bowel lumen of rats when Pluronic L-81 was also infused; however, when the Pluronic L-81 inhibition was removed, A-IV synthesis was markedly stimulated and A-IV lymphatic secretion significantly increased. The authors concluded that the stimulus for apo A-IV synthesis by lipid was not mediated by the mere uptake of lipid into the enterocyte or by the TAG content inside the cell; rather, the events of chylomicron packaging and secretion (inhibited by Pluronic L-81) provided the stimulus for the increased A-IV synthesis. (The chylomicrons formed early on would presumably have been packaged using a preformed intracellular pool of A-IV, as in the case of apo B-48 and apo A-I).

The physiological role of apo A-IV in the body is unclear. Recent studies by Fujimoto *et al.* (1993) have suggested that apo A-IV acts in the central nervous system as a satiety factor, suppressing food intake after a lipid meal.

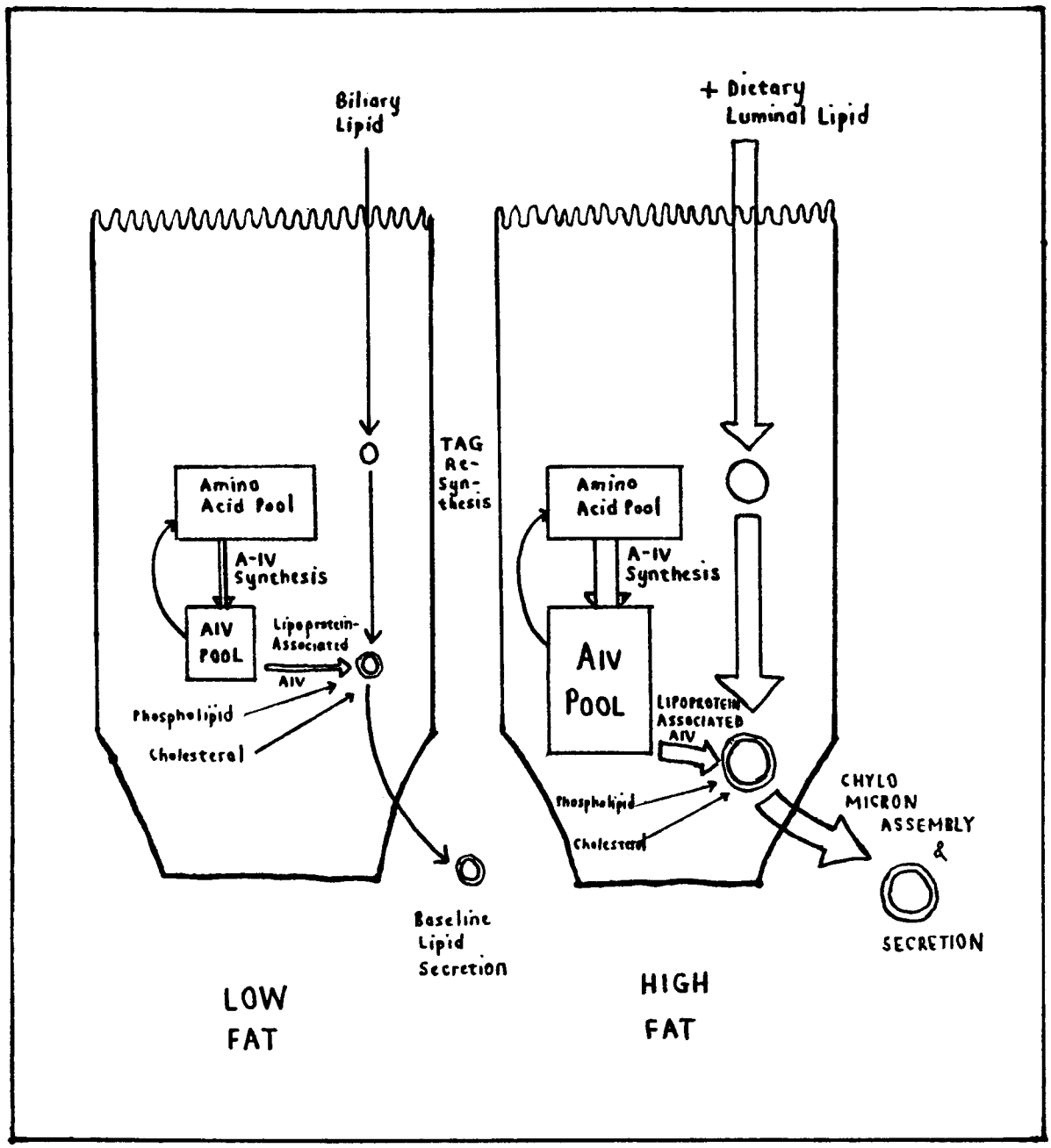


Fig. 1.15. A proposed model for apolipoprotein A-IV fluxes in the intestine during periods of low and high lipid flux. With increased dietary lipid flux, apolipoprotein A-IV synthesis increases; the size of the intracellular A-IV pool increases and more apo A-IV becomes lipoprotein-associated. The stimulus for the increased A-IV synthesis is probably derived from the greater rate of chylomicron formation occurring at the onset of the increased lipid flux. (See text for details).

1.8(iv) Conclusion

No unifying mechanism appears to exist whereby all intestinal apolipoproteins are regulated. Only apo A-IV synthesis is stimulated by dietary TAG; apo B-48 and apo A-1 are essentially unaltered by acute or chronic dietary lipid flux. It is interesting that apo A-1 and apo A-IV, which possess considerable sequence homology, are processed differently and appear to be controlled by different mechanisms. Studies of apo A-1 synthesis have also suggested fundamental differences between the regulation in the jejunum and the ileum. Differences in the saturation of dietary TAG did not give rise to differences in apolipoprotein regulation for a given apolipoprotein.

1.9 PROJECT AIMS

This project aimed to address the questions (discussed in Section 1.7) of the effects of dietary lipid flux on the synthesis, secretion and degradation of the apolipoproteins B-48, A-IV and A-1 in a novel system for the study of the intestinal epithelium, namely freshly isolated epithelial cell (enterocyte) sheets incubated *in vitro*. The cells were prepared from the small bowel of the Syrian Golden Hamster, Mesocricetus aureatus, using techniques described in Chapter 2. Following optimization and characterization of the system, the specific apolipoproteins were identified (Chapter 3) and measurements were performed of apolipoprotein synthesis, secretion and degradation under various dietary perturbations (Chapter 4).

Specific questions that were addressed were: (i) Is TAG flux a specific stimulus for apolipoprotein biosynthesis? (ii) Does the composition of the TAG (saturated versus unsaturated fat) affect the rates of apolipoprotein synthesis and secretion? (iii) If acute dietary TAG does not stimulate or "upregulate" apolipoprotein biosynthesis, does more sustained feeding (designed to deplete the intracellular apolipoprotein pool by prolonging the TAG flux and thus increasing the utilization of apolipoproteins for lipoprotein packaging) do so? (iv) Are apolipoproteins degraded within the epithelial cells? If so, to what extent? Are there differences in the degradation rates for low fat-fed as opposed to high fat-fed animals? (v) How does low fat feeding compare with an acute bolus administration of TAG, overnight high fat feeding (saturated or unsaturated) and chronic (6 week high fat feeding) with respect to apolipoprotein synthesis, secretion and degradation?

Preliminary studies of intracellular pre- and post-translational modification of apolipoproteins were also performed using tunicamycin, an inhibitor of N-linked glycosylation.

CHAPTER TWO

AN ANIMAL MODEL AND A SYSTEM FOR THE STUDY OF INTESTINAL PROTEIN BIOSYNTHESIS AND SECRETION: FRESHLY ISOLATED SHEETS OF HAMSTER-DERIVED, INTESTINAL EPITHELIAL CELLS (ENTEROCYTES) INCUBATED *IN VITRO*

2.1 THE SYRIAN GOLDEN HAMSTER: A SMALL-ANIMAL MODEL OF HUMAN LIPOPROTEIN METABOLISM

Adult Syrian Golden hamsters (*Mesocricetus auratus*) were chosen as a suitable small-animal laboratory model for the study of human lipoprotein metabolism. The experimental evidence behind the choice has been provided largely by the work of Dietschy and co-workers. Unlike most other small experimental animals, the metabolism of cholesterol in these hamsters closely resembles that occurring in humans. The rates of total body and liver cholesterol synthesis are relatively low in both these species and equal to about one tenth the rates found in rats (Spady & Dietschy 1983b). Much of the plasma cholesterol in hamsters is carried in low density lipoprotein (LDL) particles. Receptor-dependent and receptor-independent mechanisms for the degradation of lipoproteins are important in both humans and hamsters for the turnover of plasma LDL. In the hamster, most of the LDL (73%) is taken up and degraded by the liver and 90% of the LDL uptake is mediated by the LDL-receptor. The jejunum and ileum together account for 7% of LDL-clearance. Unlike the liver, receptor-independent uptake (as opposed to LDL-receptor-dependent uptake) is quantitatively more significant in the intestine, accounting for 44% of the total intestinal LDL uptake. Only 10% of hepatic LDL uptake is mediated independently of the LDL receptor. Increased dietary cholesterol and saturated tri-acylglycerol intakes elevate the plasma LDL-cholesterol concentrations in hamsters by suppression of hepatic LDL receptor activity and by increasing the production rate of LDL-cholesterol (Woollet *et al.* 1989).

Hamsters develop atherosclerosis in response to an atherogenic, cholesterol and saturated fat diet (Nistor *et al.* 1987). The metabolism of bile acids and the composition of hamster bile resembles that found in humans more closely than that in the rat, and hamsters have also been used to study cholesterol gallstone formation (Matoba *et al.* 1988, Pearlman *et al.* 1979).

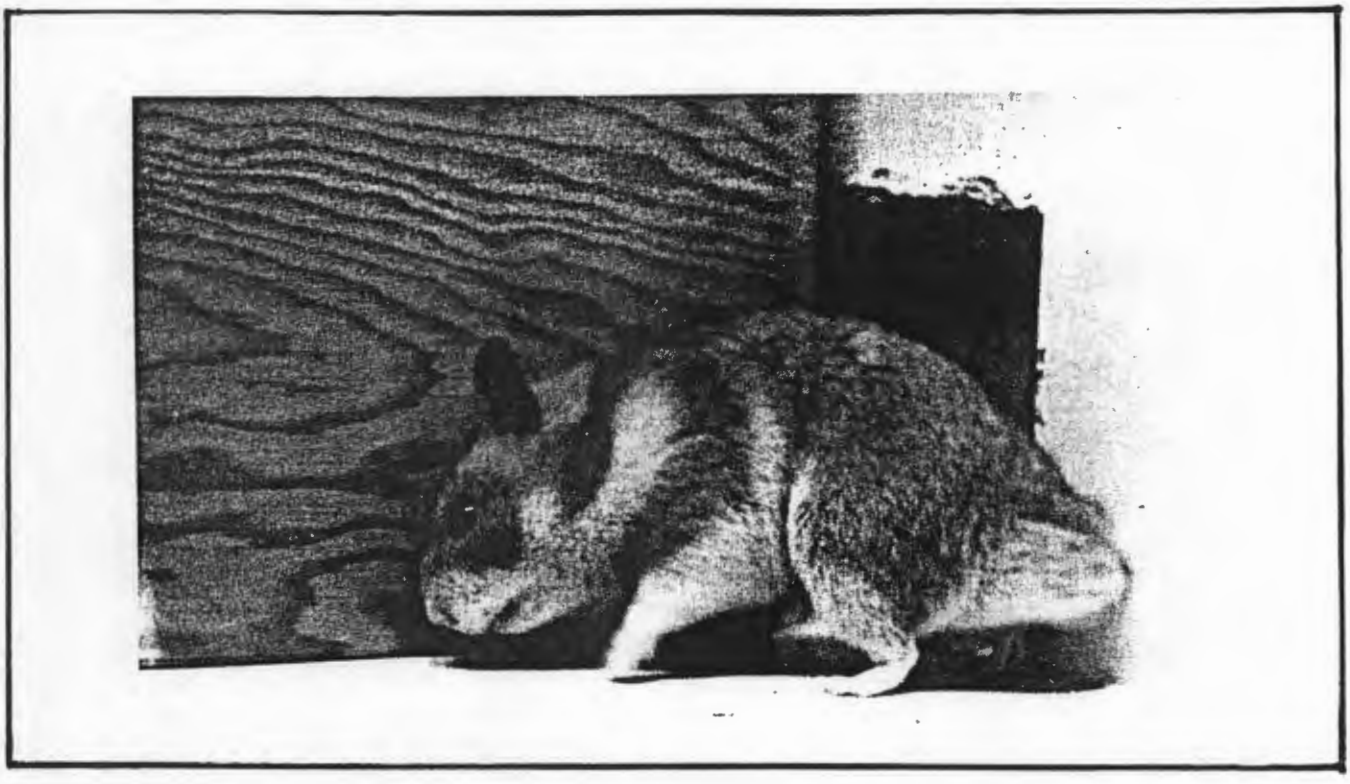


Fig. 2.1. The Syrian Golden Hamster, *Mesocricetus auratus*.

The activity of cholesteryl ester transfer protein (CETP), which transports cholesteryl ester between plasma lipoproteins, has been measured in various mammalian species and an inverse relation appears to exist between the plasma levels of CETP and the relative resistance of different species to atherosclerosis e.g. dogs, rats and sheep have a low CETP activity and a high resistance to atherosclerosis while rabbits and humans have high CETP activity and are prone to develop atherosclerosis on high cholesterol diets. Measurements of the CETP activity in hamsters suggest that they too belong to the latter group (Stein *et al.* 1990). The Streptozotocin-treated Syrian Golden Hamster has also been used as an animal model for Diabetes Mellitus (Phares 1980, Rosenberg *et al.* 1988).

The digestive tract of the hamster (Fig. 2.2) has certain peculiarities (Borer 1985). Hamsters have large internal cheek pouches which they use to store and transport food. In addition, hamsters have a compartmentalized stomach consisting of a forestomach and a glandular stomach. The forestomach is similar to the rumen found in herbivores, and ruminant-type micro-organisms isolated from the hamster forestomach are capable of fermenting cellulose. Volatile fatty acids are produced and absorbed in the forestomach. Following ingestion, food first enters the forestomach and is thought to remain there for 10 to 60 minutes. before passing into the glandular stomach. The caecum of the hamster is also a prominent structure and contains a microflora that promotes cellulose fermentation. Thus the forestomach and the caecum together allow the hamster to digest plant foodstuffs. The overall structural feature of the hamster gastro-intestinal tract characterizes the animal as a natural herbivore. Hamters feed every two hours. Experimentally induced longer inter-meal intervals are associated with weight loss and a reduction in the plasma insulin concentration (Borer 1985).

In the 1960's the hamster small intestine was often used to study the mechanisms of sugar and amino acid transport (Bihler & Crane 1962, Kinter *et al.* 1965, Alvarado

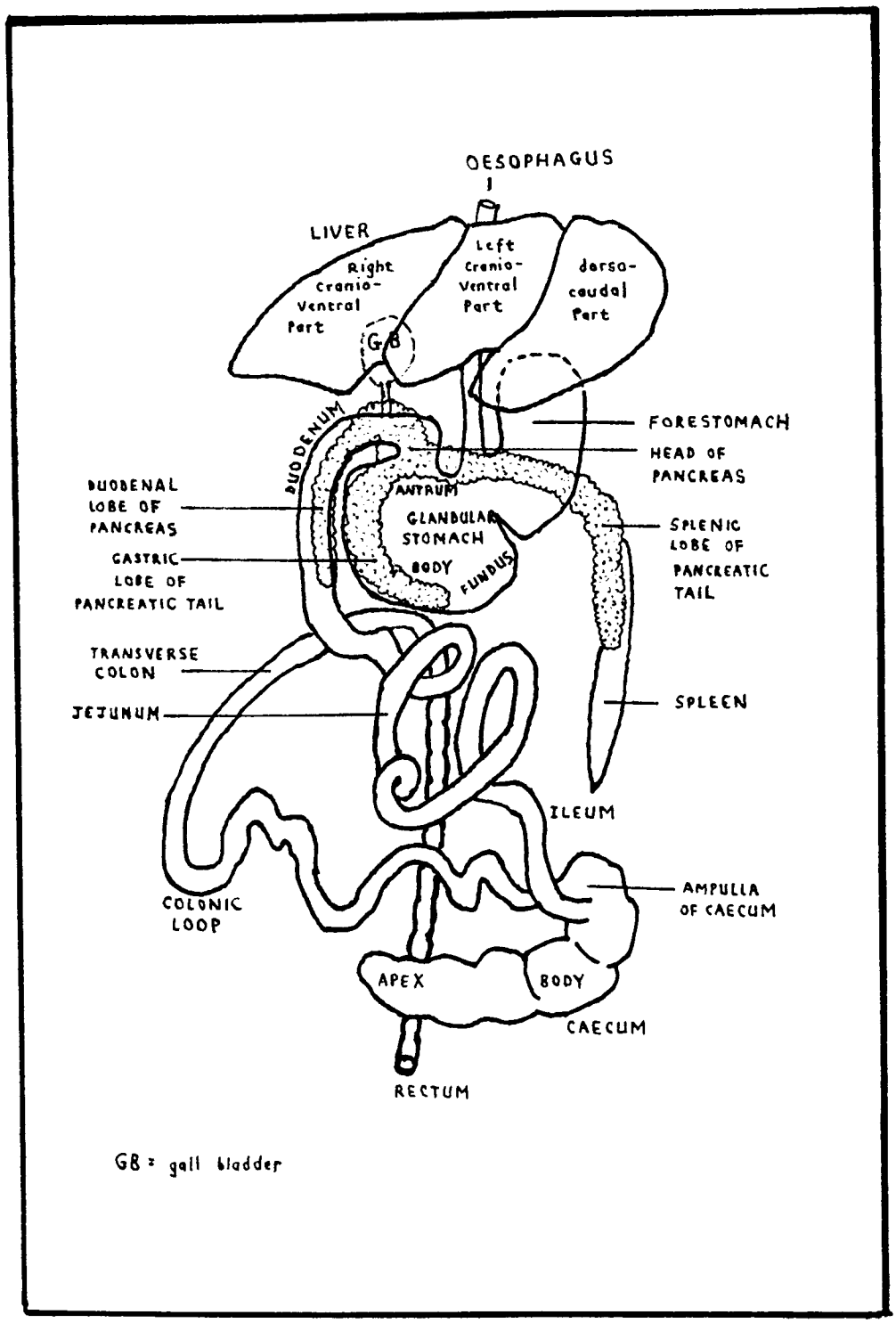


Fig. 2.2. The hamster gastro-intestinal tract. (Ref.: Borer, 1989).

1968). However it has seldom been used to study lipid absorption and transport. A morphometric electron microscopic analysis of the membranes and organelles of hamster small intestinal enterocytes was carried out by Buschmann and Manke in 1981. Following the instillation of corn oil into the stomachs of male golden hamsters, a comparison was made between the enterocytes from the proximal jejunum of lipid-fed versus fasted hamsters. Following lipid instillation the enterocytes become engorged with lipid; the mean heights of the columnar epithelial cells increased by 20% and the enterocyte volume increased by 30%. This volume increase was accounted for largely by an increase in the endoplasmic reticulum and Golgi luminal volume with lipid. There was also a significant appearance of lipid in the cytoplasmic matrix, however, and the total nuclear volume also increased. The intercellular spaces at the basolateral margins of the enterocytes distended with chylomicrons.

A few other studies related to hamster lipid absorption have been reported in the literature. The importance of calcium ions in the secretion of absorbed lipid was illustrated by a study involving the use of everted hamster jejunal sacs following *in vitro* incubation in a bile salt solution containing radio-labelled oleic acid. Calcium ions were presumed in this study to promote the exocytotic release of chylomicron-containing vesicles from the baso-lateral surfaces of the enterocytes (Strauss & Jacob 1981). In 1969 an *in vitro* study of the effect of dietary fat content on the site of fat absorption in hamster small intestines showed that while most fat absorption took place from the proximal jejunum, increasing the lipid load enhanced lipid absorption from the distal ileum and had no effect on the proximal jejunum (Hoving & Valkema 1969). Under physiologic conditions, however, the ileum is probably exposed to very little luminal lipid.

2.2 METHODS FOR STUDYING INTESTINAL ABSORPTION, APOLIPOPROTEIN SYNTHESIS AND LIPOPROTEIN PRODUCTION

Various *in vivo* and *in vitro* techniques have been used in the study of intestinal absorption and lipoprotein production. The rat has proven to be the most popular small laboratory animal in this regard.

2.2(A) *In Vitro* Techniques

(i) The Lymph-fistula Rat Model

This operatively complex system is one of the commonest methods used. It has the advantage of providing information not only with regard to digestion, absorption and intracellular metabolism of absorbed lipid, but also about rates of lipid transport into lymph. The method was first introduced by Bollman *et al.* in 1949 and was modified by Shepherd and Simmonds in 1959 to include a steady-state infusion of lipid by an intraduodenal cannula. The lipid may be infused for up to 6 hrs; the rates of flow and composition of lymph lipid at steady states may then be compared with the pre-infusion rates. Either the intestinal mesenteric lymphatic duct or the thoracic duct may be cannulated. The surgery is performed on an anaesthetized animal and recovery from the anaesthetic is usually rapid. Thoracic duct cannulation requires considerable experience and there is a major drawback in that the duct contents also contain lymph from the liver and, therefore, lipoproteins synthesized and secreted by this organ. The recovery of intestinal lymph is usually complete in the thoracic duct cannulation. In the mesenteric approach, intestinal lymph recovery may be incomplete as accessory ducts are also present that bypass the mesenteric duct at the usual cannulation site. Another disadvantage of both methods is that the lymph normally also contains some lipoproteins filtered from the plasma. Nevertheless, the system has been successfully used to study lipoprotein production following the infusion of micellar lipid preparations (often radiolabelled) into the intestine (Tso & Simmonds 1984a).

In experiments performed by Glickman *et al.* the bile ducts of some animals were cannulated and exteriorized to provide external bile diversion and thus the experimental control conditions for no lipid absorption from the lumen. The synthesis of apolipoproteins in the intestine has also been studied by Glickman *et al.* using an *in vivo* pulse-labelling technique with a subsequent immuno-precipitation of the proteins of interest from lysed enterocyte preparations. Following prior dietary perturbations either through ad libitum feeding, the continuous infusion technique, or single dose instillation via a duodenal catheter, the rats were anaesthetized, a loop of proximal small intestine was created by ligaturing it around two ends, and a large quantity of radio-labelled amino acid (^{3}H-leucine) was then instilled into the loop. The intestine was replaced intra-abdominally, the animals were kept warm, under general anaesthetic, for nine minutes, then sacrificed. The bowel loop was removed, flushed clean of unabsorbed labelled amino acid and enterocytes prepared from the bowel using the method of Weiser (1973) (see later). It is from such experiments in rats that much of the data on the regulation of apolipoprotein biosynthesis by dietary tri-acylglycerol has been derived (see, for example, Davidson & Glickman 1985, Davidson *et al.* 1987; Apfelbaum *et al.* 1987). The surgical techniques involved in these experiments require expertise and are extremely difficult to perform in the hamster, an animal about one-third the size of the rat. Considerations of expense precluded the use of this technique in my experiments as large quantities of radio-labelled precursor amino acids are required to sufficiently label the proteins of interest.

(ii) The Cori Procedure for Lipid Absorption Studies

This technique was originally designed to study the absorption of sugars from the gastro-intestinal tract but has since been modified to study lipid absorption. It involves administering a labelled lipid emulsion or solution by lavage followed, after a period of time, by killing of the animal, isolation of different segments or organs of the gastro-intestinal tract by ligating them off, and then removing the various segments.

Unabsorbed luminal lipid is removed by washing and the effluents kept for analysis. The various organs as a whole or the scraped mucosa is then analyzed for lipid. By measuring the amount of infused lipid left in the organ, the amount of lipid absorbed, catabolised by the intestinal cell, and transported to the lymph, may be calculated by subtraction. The lipid transported as lipoproteins in lymph and in the blood cannot be quantified directly, however (Cori 1925).

2.2 B *In Vitro* Techniques

These have been successfully used in studying uptake and the intracellular events of lipid absorption but have, in the past, proven less helpful in studying the assembly and secretion of lipoproteins by the intestine. A major problem with *in vitro* techniques has been to validate the biochemical and physiological integrity of the systems used . Seven in vitro techniques will be reviewed:

(i) Everted sacs; (ii) Ring Segment Incubations; (iii) the isolated perfused bowel loop; (iv) isolated intestinal epithelial cells; (v) subcellular organelles; (vi) morphologic studies, and (vii) cell culture.

(i) Everted Sacs

This technique was first described by Wilson and Wiseman in 1954. Eversion of an isolated intestinal sac segment exposes the mucosal surface to a large volume of "external" medium for cell support (O_2, nutrients) as well as for absorption (Fig. 2.3). The absorbed and secreted products of absorption then accumulate in the inner serosal fluid and may be analyzed. However, the properties of the absorptive cell membrane *in vitro* may not be the same as *in vivo* and reductions in the absorption of certain nutrients have been noted in the *in vitro* system when compared with the *in vivo* situation. As in all *in vitro* techniques, the mucosa is isolated from its normal blood and nerve supply as well as the normal lymphatic drainage. This isolation may be critical when studying the intracellular events of lipid absorption viz. TAG re-synthesis, apolipoprotein biosynthesis

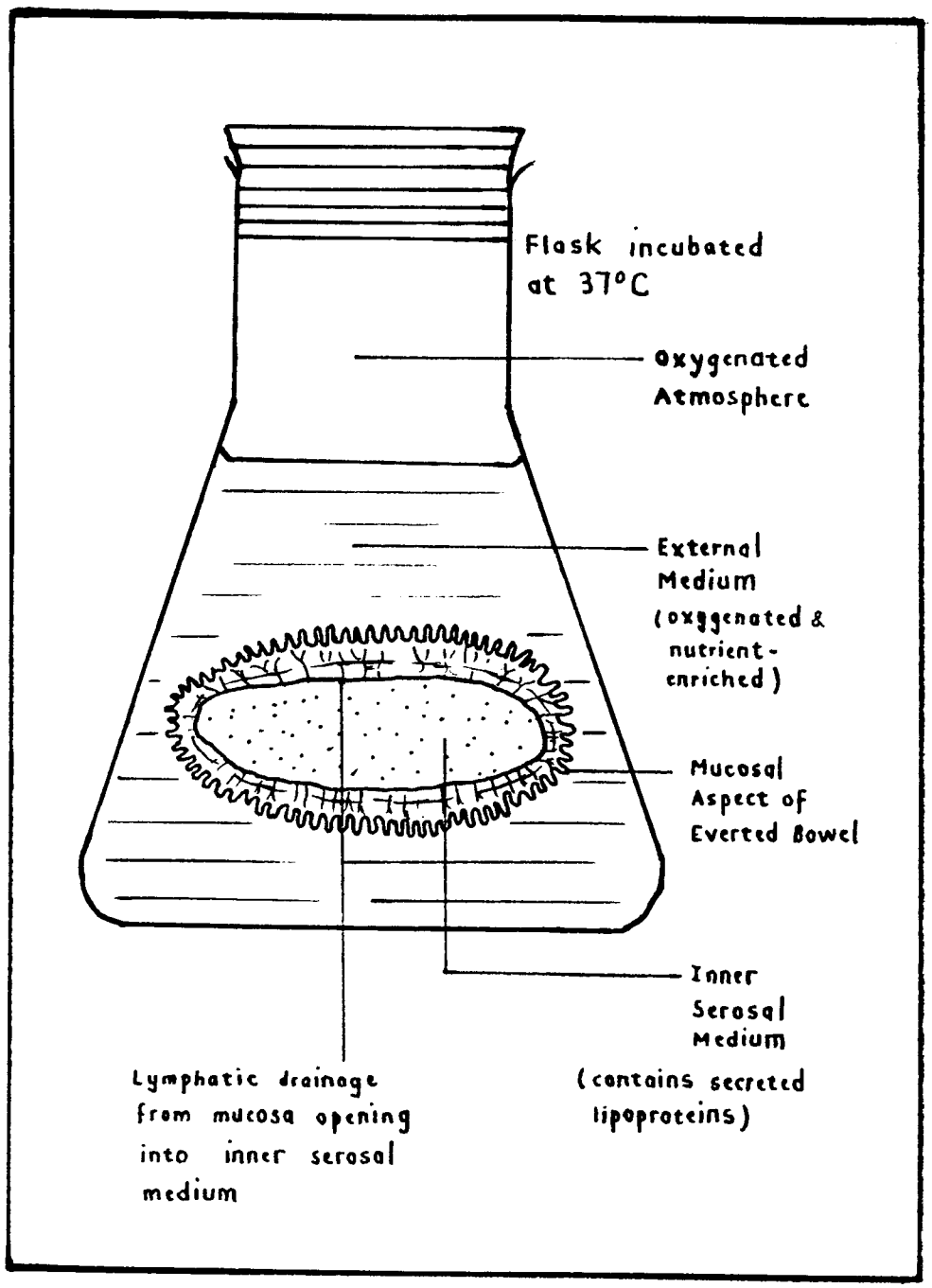


Fig. 2.3. A schematic representation of the everted sac technique.

and lipoprotein assembly and secretion. Deficiencies of nutrients, substrates, and lack of humoral mechanisms of control may seriously hinder the normal intracellular processes. There is in fact experimental evidence to show that the re-esterification of absorbed free fatty acids, for example, is less efficient in vitro than *in vivo* (Tso & Simmonds 1984a). Another deficiency of this method as a model of lipid absorption is the lack of significant transport of chylomicrons from the intestinal absorptive cells to the tissue fluid and serosal medium. The nascent lipoproteins are unable to be caught or trapped immediately following their secretion from the enterocytes. A study by Strauss & Jacob (1981) on the everted hamster jejunum showed, however, that by including Ca^{++} 2.5 mM and Mg 1.2 mM in both the mucosal and serosal media, the impairment of transport processes could be overcome (in part, at least).

(ii) Ring segment incubations

Thin transverse slices of intestinal tissue accumulate absorbed amino acids and sugars from the surrounding medium. These slices have been incubated for 4 hrs or longer *in vitro* with no apparent disintegration or autolysis of the epithelial cell layer (Crane & Mandelstam 1960). No studies of lipid absorption have been reported with this technique, although apolipoprotein biosynthesis using radio-labelled amino acids has been studied in ring sements incubated *in vitro*.

(iii) Isolated, perfused bowel loop

This method has been extensively used by Wu and Windmueller (1979) to study apolipoprotein secretion by the rat intestine. The procedure is complex and involves the preparation of an isolated intestinal segment with cannulation of the attached mesenteric lymph duct, superior mesenteric artery and superior mesenteric vein. The test meal is infused via a duodenal infusion tube; the vascular perfusate used is whole rat blood with added heparin and antibiotics. A 95% O_2:5% CO_2 mixture was used to continuously oxygenate the perfused blood. The addition of certain hormones to the vascular

perfusate was found to prolong the viability of the isolated small intestine for up to 5 hrs (as evaluated by microscopy and measurements of O_2 uptake, etc). When free fatty acids were added to the duodenal infusion, chylomicrons were found in the lymph collected. Once again the hormonal influences normally present in the living animal that may regulate lipoprotein assembly and secretion are absent. An advantage of the system, though, is that newly secreted lipoproteins may be obtained prior to their catabolism in the general circulation. By controlling the constituents of the duodenal infusion and the vascular perfusate, it is at least theoretically possible to study, individually, factors that may regulate chylomicron formation and transport.

(iv) Isolated Intestinal Epithelial Cells

Kimmich (1975) has written a comprehensive historical account of the development of methods for isolating and studying intestinal epithelial cells *in vitro*. A review of the most relevant facts will be presented here.

Systems for studying isolated intestinal epithelial cells were initially developed to elucidate the mechanisms of sugar and amino acid transport across the epithelial cell and the role of monovalent cation gradients in the process. Early methods of separating the mucosal epithelium from the underlying mucosa depended on scraping techniques (using the edge of a glass microscope slide). This yielded epithelial cell sheets, fragmented villi, clumps of cells, individual cells as well as organelles from damaged cells. However, these cell preparations showed a rapid loss of glycolytic activity over a 20 to 40 minute incubation period in a supportive medium *in vitro*.

Subsequently, mechanical devices were constructed to peel the cells off in large sheets, including a vibrating motor to detach cells from an everted segment of intestinal tissue (Sjostrand 1968). Viability was usually assessed by measuring the glycolytic capacity of the cells e.g. rates of lactate production, but the cells were usually short-lived.

Subsequently, chemical and enzymatic methods, usually in combination with mechanical methods e.g. digital squeezing or stirring and shaking of the bowel, were employed to remove the cells from the intact tissue. Trypsin was used but poor viability (related to the duration of trypsin treatment of the intact bowel) was reported (Harrer *et al.* 1964). Lysozyme treatment resulted in a combination of villi fragments, connective tissue and muscle (Huang 1965). The chelating agent EDTA in a calcium and magnesium-free Krebs-Ringer phosphate was first used in 1967 when everted sacs were incubated for one hour at 4°C, then subjected to vigorous shaking to detach the epithelial cells (Sognen 1967).

Mucus has often proved troublesome as cells tend to aggregate in its presence. The addition of hyaluronidase (1 mg/ml final concentration) was found by some to limit the extent of clumping (Kimmich 1975). In general, however, the presence of enzymes or chelators tended to reduce the metabolic capacity of the cells which deteriorated rapidly after preparation. The addition of bovine serum albumin (BSA) in the preparative media was found to limit cell breakdown. Kimmich *et al.* have used intact intestinal tissue incubated in the presence of Krebs-Ringer phosphate with hyaluronidase (1 mg/ml) and BSA (1 mg/ml). Clumping of cells remained a problem, though, and cell dispersion was achieved by drawing up the cell suspensions in a plastic pipette. Cell viability has been estimated using the Trypan Blue Dye Exclusion technique but this is often unsatisfactory when large suspensions or clumps of cells are present. The glycolytic activity measured by lactic acid production and $^{14}CO_2$ production from ^{14}C-glucose has generally been used as a metabolic marker of cell viability, and glycolytic rates usually expressed as millimoles of lactate produced per milligram of cell protein per hour have often been used to compare different cell preparation techniques (Kimmich 1975). The success rates of the different methods often varied according to the animal species from which the bowel was obtained. Thus a method that may have yielded poorly viable cells from a rat intestine might produce a richly viable preparation from a hamster intestine.

A major theoretical disadvantage of the use of isolated intestinal cells in the study of lipid absorption and secretion is the exposure of the basolateral surface of the cells (normally "hidden" *in vivo*) to the constituents of the luminal fluids, especially the bile salts. Apart from the potential toxicity to the cells, the usefulness of isolated cells may be limited when attempting to elucidate the luminal uptake stage of absorption. In 1972, however, Yousef and Kuksis reported *in vitro* release of chylomicrons from isolated cells that had been "pre-loaded" *in vivo* by dietary-derived lipid. Another potential disadvantage of the use of isolated cells or cell sheets is the loss of the normal endocrine and paracrine influences that would be expected to operate *in vivo* as well as the loss of the effect of cell-to-cell contacts. Finally, any isolation procedure, however mild, may be expected to result in a change in the structural integrity of the cells.

In this project, I have chosen to study intestinal epithelial cell sheets isolated from the hamster small intestine using a modification of a technique first described by Towler *et al.* in 1978.

The cells are "pre-loaded" with dietary lipid by dietary modifications performed on the intact animal. Potential advantages of the isolated epithelial preparations are that large quantities of pooled cell suspensions may be easily obtained from a single animal and these may be aliquotted with reproducibility of results. Various time point incubations and *in vitro* experimental manipulations are possible. (The animal is, of course, killed at a single time point folloiwng the *in vivo* dietary modification). Isolated cell preparations generally require smaller quantities of radio-labelled precursor substances than do intact tissues or organs and are therefore relatively inexpensive. Finally, the primary, intact cell secretion products (lipoprotein particles and apolipoproteins) may be studied prior to the modifications they normally undergo soon after leaving the cells.

(v) Subcellular organelles

The preparation of nascent intracellular lipoproteins from subcellular organelles has been extensively used in studying liver lipoproteins. The application of these techniques to the intestine, however, appears to have been limited by two problems: the presence of mucus in the small intestine, which interferes with subcellular fractionation, and the tiny quantities of material that are eventually obtained following isolation of the relevant subcellular organelle fraction. For instance, in order to perform chemical analyses on rat Golgi apparatus contents, at least 10 to 12 rats have had to be used in order to gain sufficient material (Mahley *et al.* 1971). (This would be equivalent to using 30 to 36 hamsters when one considers the relative sizes of the animals).

Before isolating the relevant subcellular fractions, the intestinal epithelial cells themselves have to be isolated from the whole bowel (see Sect 2.2(iv)). Cell disruption techniques including Dounce homogenization and nitrogen cavitation (the French Pressure cell and the Parr "bomb") have been used (e.g. Magun *et al.* 1988). The subcellular organelle of particular relevance to the nascent lipoproteins is the Golgi apparatus and this has usually been isolated by differential ultra-centrifugation techniques often involving the use of sucrose gradients. The final vesicle fraction obtained is then resuspended and broken to release the lipoproteins. Breakage techniques using sonication or chemical methods for dissolving the vesicle or organelle membranes (e.g. sodium carbonate or dilute bile salt solutions) have been employed (Fujiki *et al.* 1982, Howell & Palade 1982). Some of these methods, however, may affect the normal integrity and structure of the nascent lipoproteins. The lipoproteins are then harvested by flotation-centrifugation. Nascent high density lipoproteins, very low density lipoproteins and chylomicrons have been isolated using these techniques. Attempts have also been made to differentiate between immature and mature intracellular vesicles and to compare the lipoprotein composition in the 2 subpopulations (Mahley *et al.* 1971, Magun *et al.* 1985 & 1988).

(vi) Morphological studies

Morphological studies of fat absorption using electron microscopy have yielded important information regarding lipoprotein assembly and transport by small intestinal epithelial cells (See Chapt 1.3). Transmission electron microscopy and negative staining of lymph lipoproteins with phosphotungstate has also helped in characterizing the shapes and sizes of the intestinally derived lipoproteins (Forte & Nordhausen 1986).

(vii) Cell culture

Attempts to maintain isolated intestinal epithelial cells in primary culture have been unsuccessful. However, since 1987, the human colonic carcinoma cell line, CaCO-2, has been extensively used as a cell culture model for the *in vitro* study of human intestinal lipoprotein metabolism (Hughes *et al.* 1987). This cell line, when grown in culture, develops many features characteristic of fully differentiated small intestinal mucosal epithelium, including the expression of brush border hydrolases and structural characteristics typical of the small intestinal enterocyte; for example, the CaCO-2 cells possess brush border microvilli on the apical surface and tight junctions between cells. The cells form domes (resembling villi shapes) when grown on an impermeable support. When grown as monolayers on porous filters (Fig. 2.4), the cells secrete radio-labelled triacylglycerol-containing chylomicrons and other lipoproteins into the basolateral medium in response to the addition of ^{14}C-labelled oleic acid bound to albumin to the apical medium (Traber 1987). CaCO-2 cells labelled with ^{35}S-methionine produced apolipoprotein B-100, B-48, A-IV, A-I and C-III in the basolateral medium (Hughes *et al.* 1987 & 1988; Traber *et al.* 1987).

CaCO-2 cell lines have been used in numerous studies of apolipoprotein synthesis and secretion as well as of cholesterol metabolism. The importance of calcium ions in the secretion of apolipoprotein B has also been demonstrated (Hughes *et al.* 1988). CaCO-2 cells secreted cholesteryl ester transfer protein (lipid transfer protein-1 or LTP-1) into

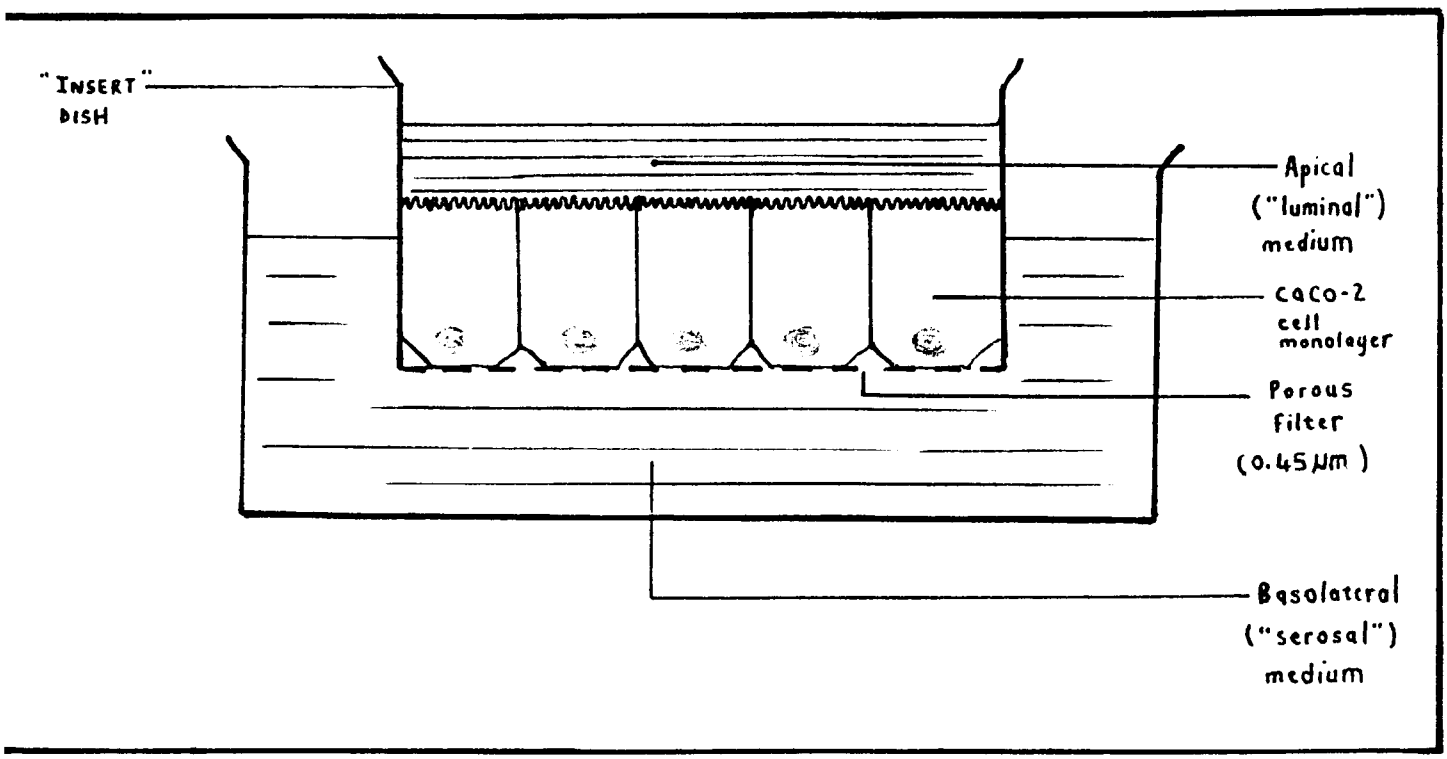


Fig. 2.4. A schematic representation of CaCO-2 cells grown as a monolayer on a porous filter.

the basolateral medium of the cells in response to fatty acid in the apical medium. The degree of cholesteryl ester transfer activity recovered was dependent on the concentration of fatty acid added (Faust & Albers 1988).

Despite the above findings the use of this colonic carcinoma cell line as a model for normal small intestinal lipid absorption and lipoprotein secretion has been seriously questioned. The CaCO-2 cells appear to produce significant amounts of apolipoprotein B-100 in contrast to most studies of normal mammalian small intestine which have revealed the production of the smaller molecular weight Apo B-48 only. Glickman *et al.* (1986b) have previously reported that the isoform of apolipoprotein B synthesized by the intestine depended on the developmental stage of the tissue. At 11 weeks gestational age the fetal intestine produced more Apo B100 than B-48, at 16 weeks the quantities of B-48 and B-100 were approximately equal, while the adult small intestine produced B-48 only (Glickman *et al.* 1986b). The CaCO-2 cells, therefore, may resemble fetal intestinal cells more closely than fully mature (adult) enterocytes.

While the major lipoproteins isolated from the intracellular organelles and lymph of normal intestine have been chylomicrons, some very low density lipoproteins (VLDL) and high density lipoproteins, the CaCO-2 cell line appears to secrete significant quantities of low density lipoproteins (LDL) with small amounts of VLDL and HDL but no particles characteristic of chylomicrons (Traber et al. 1987). Studies of cholesteryl ester transfer protein (CETP) mRNA in various organs of the hamster have revealed only very small quantities in the small intestine compared with other organs such as adipose tissue and muscle, raising further questions about the physiological significance of the CaCO-2 findings (Jiang *et al.* 1991). More recently it has also been shown that fatty acid esterification to tri-acylglycerol in CaCO-2 cells occurs primarily via the glycerol 3-phosphate pathway in contrast to normal intestinal absorptive cells where the mono-acylglycerol pathway is dominant (Trotter & Storch, 1993).

2.3 METHODS OF CELL PREPARATION, INCUBATION AND ANALYSIS

2.3(i) The preparation of freshly isolated small intestinal epithelial cell (enterocyte) sheets: Towler method

Male Syrian Golden Hamsters weighing between 160 and 220 g were housed four to a cage and were exposed to a 12 hour light-dark cycle beginning at 3.00 pm. They were maintained on a standard laboratory rodent chow which contained 4% fat (mainly polyunsaturated) by weight, and were allowed free access to water. The Guidelines on Animal Experimentation as set out by the South African Medical Research Council were strictly adhered to. On the morning of the experiment the animals were fasted for two hours prior to sacrifice. They were then anaesthetized using diethyl ether inhalation, weighed and exsanguinated by cardiocentesis. The blood was kept for subsequent analysis. The bowel was exposed by laparotomy and the caecum identified (see Fig. 2.2). The entire small bowel was then removed from the caecal to the duodenal end by gently freeing it from the mesentery.

A modified method of Towler *et al.* (1978) was used to obtain intestinal epithelial cell sheets. The small bowel was rinsed with chilled, oxygenated phosphate-buffered saline (PBS; 0.15 M NaCl, 0.0014 M NaPi, pH 7.25) introduced at one end of the bowel usin a 5 ml syringe and plunger, to remove food debris from the lumen. The proximal jejunal segment was identified (approximately one-fifth the length of the small intestine 3 cm distal to the insertion of the hepato-pancreatic duct) and excised from the rest of the small bowel. The selected segment was rinsed with PBS, weighed and kept moist in a Petri dish containing chilled PBS while the two free ends were carefully attached to the tips of two 5 cc syringe barrels using nylon surgical tying material. Five mls of well-oxygenated isolation medium (NaCl 160 mM, KCl 3 mM, Na_2HPO_4 11.2 mM and KH_2PO_4 16 mM containing glucose 10 mM, glutamine 2 mM, lactate 2.5 mM, penicillin 100 units per ml, streptomycin 100 μg/ml) was then introduced into the lumen

through one of the syringe barrels, placed in a 200 cc Erlenmeyer flask containing pre-warmed PBS (T = 37°C), and incubated for 15 minutes (Fig. 2.5).

The bowel and attached syringes were then removed, the isolation medium was discarded, and the loop rinsed with 5 ml quantities of chilled PBS until the effluent was clear. The loop was then filled with 5 mls of well-oxygenated suspension medium (NaCl 137 mM, KCl 2.2 mM, Na_2HPO_4 1.4 mM, glucose 10 mM, glutamine 2 mM, lactate 2.5 mM; penicillin 100 units/ml, streptomycin 100 μg/ml, HEPES, pH 7.4, 20 mM), carefully placed on a chilled surface, and gently patted along its length for 30 seconds before being turned onto the other side and the patting repeated (see Fig. 2.6). The initial suspension obtained was decanted and discarded. The tapping procedure was repeated five times with sequential 5 ml quantities of suspension medium and increasing the vigour of patting with each fresh 5 ml of medium used. The cell suspensions thus obtained were decanted through the syringe barrels and pooled into a centrifuge tube on ice, then spun at low speed (1800 rpm, JA20 rotor, Beckman Model J-21C centrifuge machine) for 2 minutes. The suspension medium was decanted carefully so as not to disturb the cell pellet, the cell pellet was gently resuspended in suspension (wash) medium and the centrifugation repeated. Finally, the cell pellet was gently resuspended in 10 ml of Incubation Medium (Krebs-Ringer bicarbonate with phenol red as pH indicator; fuels and antibiotics: NaCl 118.46 mM, KCl 4.74 mM, KH_2PO_4 1.18 mM, $MgSO_4$ 1.18 mM, $NaHCO_3$ 24.88 mM, CaCl 2.54 mM, glucose 10 mM, glutamine 2 mM, lactate Na 2.5 mM, Penicillin 100 units/ml and Streptomycin 100 μg/ml), final pH 7.4.

2.3 (ii) Enterocyte Preparation: Weiser Method

Following the removal, rinsing and selection of jejunal segment obtained as described above, it was everted over a glass rod so that the mucosal surface was exposed to the outside (Fig. 2.7). The bowel was then tied at one end, the glass rod removed and the

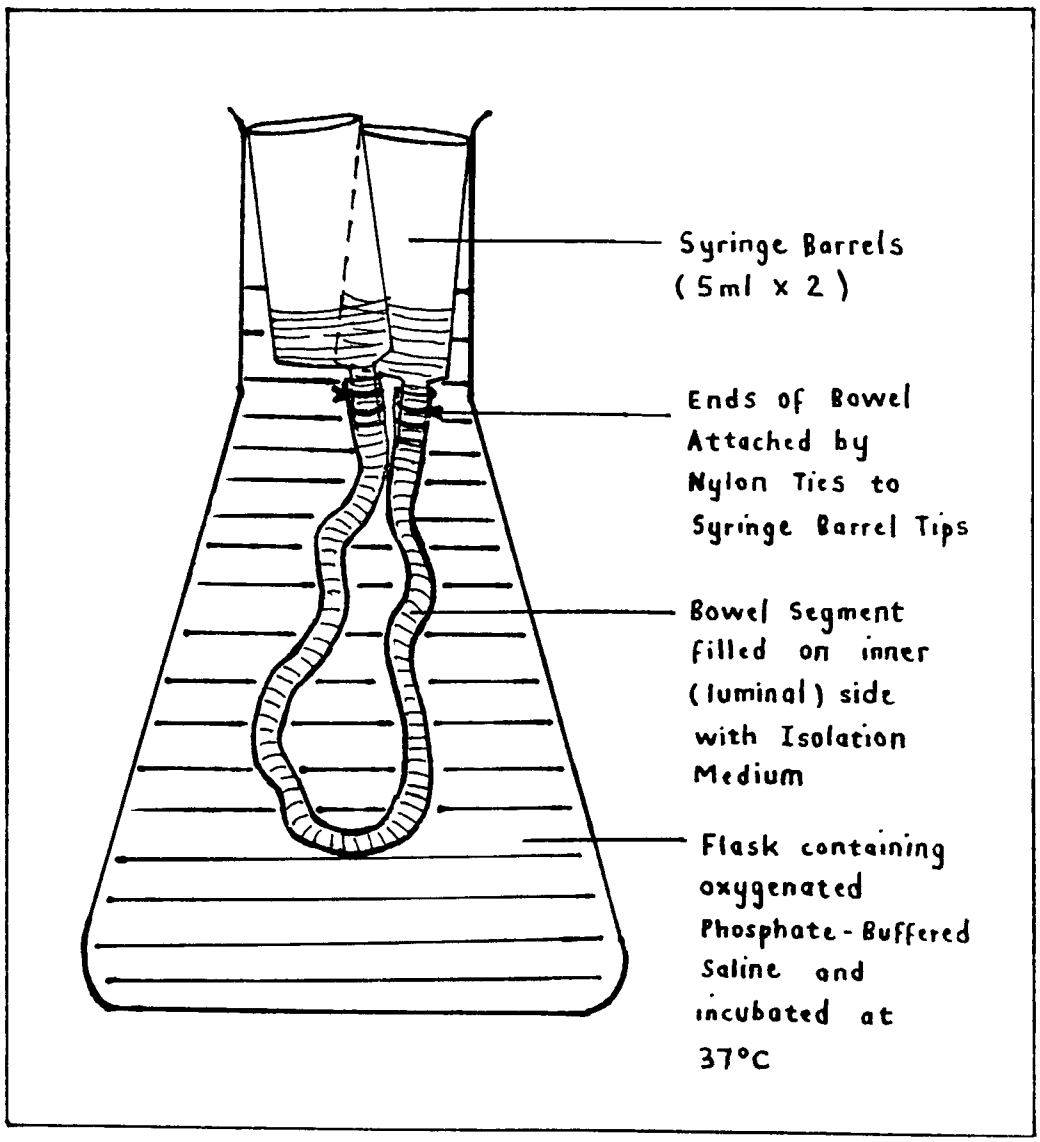


Fig. 2.5. The Towler Method: Pre-incubation of the washed bowel segment prior to harvesting of cells (see text 2.3(i) for details).

. 2.6. The Towler Method: "Patting" procedure for loosening epithelial cell sheets from the bowel wall (see text 2.3(i) for details).

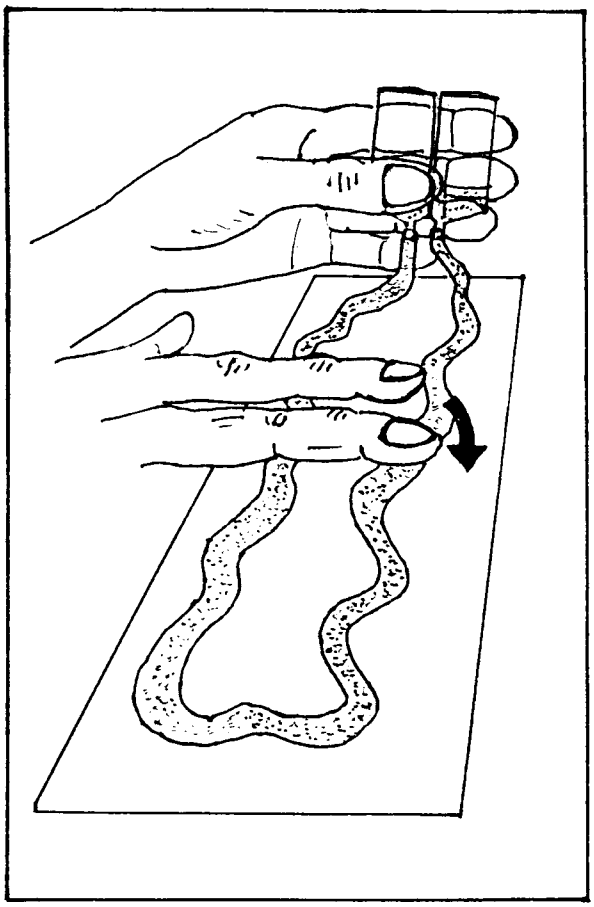
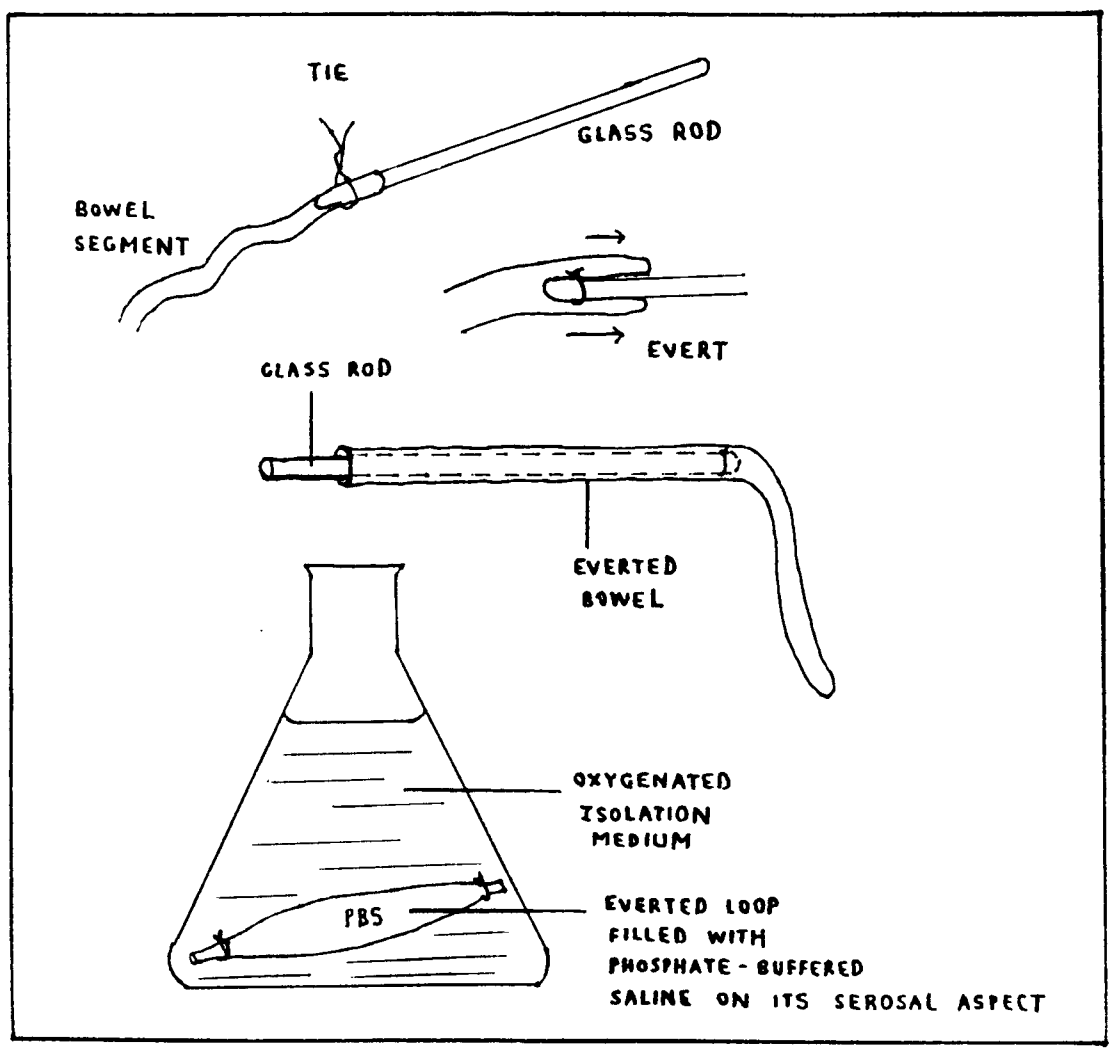


Fig. 2.7. The Weiser Method of preparing epithelial cells (see text 2.3(ii) for details).



bowel filled with 2.5 ml of PBS introduced by a syringe. The other end was then tied and the everted bowel sac containing PBS was incubated in 10 ml well-oxygenated isolation medium at 37°C for fifteen minutes. The isolation medium was then discarded and replaced with 7.5 ml of suspension medium (as above) but also containing 1.5 mM Ethylenediamine Tetra-acetate (EDTA), pH 7.4. The EDTA-containing suspension medium as well as the air in the incubation flask was gassed with 100% O_2 and incubated again at 37°C in an orbital shaking water bath for 15 minutes. The suspension medium (containing freed epithelial cells) was decanted into a centrifuge tube and replaced with fresh suspension medium-EDTA, re-gassed, and incubated for 15 minutes. The procedure was repeated a number of times (Weiser *et al.* 1973 & 1978). The cell suspensions thus collected were pooled in a centrifuge tube on ice and further washing of the cell suspension was carried out as in the modified Towler method already described.

2.3 (iii) Cell Viability Estimation

Following resuspension of the cell pellets in incubation medium and just prior to their incubation, cell viability was checked using the Trypan Blue Dye Exclusion Test (Adams 1990). The reagent used was 0.4% Trypan Blue Solution in PBS, pH 7.2. 0.1 ml dye solution was added for every 1 ml cell suspension to yield a final dye concentration of 0.04%. The two were mixed gently and examined under a light microscope at room temperature within 1 minute of being mixed. The proportion of viable cells (i.e. cells that excluded the dye, as opposed to those that took up the stain) was estimated and expressed as a percentage of the total number of cells in the high power field.

2.3 (iv) Protein Synthesis Experiments and Radioactive Protein Quantification

Precise amounts of freshly prepared enterocyte suspensions in incubation medium were dispensed into 2.5 ml Erlenmeyer incubation flasks containing radio-labelled precursor

amino acid (either ^{3}H-leucine or ^{35}S-methionine, 0.5 μCi for every 100 μl of cell suspension). Other constituents were added to the incubation medium as determined by the experiment protocol. The incubation flasks were sealed with rubber bungs and the atmosphere inside the flasks gassed with a 95% O_2 : 5% CO_2 gas mixture, until the phenol red indicator in the medium changed to pH 7.4 colour. The flasks were incubated in an orbital shaker (75 rotations per minute) at a temperature of 37°C. At set time intervals the flasks were removed and placed on ice. Protein precipitation of the entire sample (cells and medium) was carried out by the addition of ice-cold trichloro-acetic acid (TCA), 10% final concentration, containing 5 mM leucine or methionine as cold carrier. The TCA-treated samples were kept on ice for thirty minutes and subsequently filtered using a Millipore filtration apparatus: a 2.5 cm diameter glass fibre filter disc (GF/C very fine glass microfibre paper) (1.2 μm pore size) was placed onto a sintered glass disc fitted into a rubber stopper which, in turn, fitted the neck of a 500 ml side-arm filtration flask. A cylindrical glass funnel was held in place on the filter disc and sintered glass, using a clamping device. Negative pressure was applied through the side-arm of the filtration flask. The TCA-treated sample was mixed well, decanted into the funnel, and filtered. The filter discs were washed five times with 5% TCA containing 5 mM cold amino acid carrier and a sixth wash with 96% ethanol was carried out. The filter discs were dried and the radioactivity counted using 10 ml fluor (Beckman "ReadySolve" liquid scintillation cocktail for aqueous samples) in a liquid scintillation counter with a quench standard and a programme for tritium (^{3}H) or ^{14}C (for ^{35}S-methionine labelled samples).

When separate medium and cell radio-labelled protein analysis was required, the cell suspensions at the end of the incubation were transferred to 2 ml Eppendorf tubes and spun at 2000 rpms for 2 minutes in a refrigerated Beckman TJ60 centrifuge machine. The medium was aspirated and the cell pellet resuspended in homogenization buffer (NaCl 150 mM, Na_2HPO_4 1.4 mM, cold amino acid carrier 4 mM, 1% Triton X-100,

0.15% sodium dodecyl sulphate (SDS), benzamidine 1 mM and EDTA 1 mM, pH 7.4). The samples were then sonicated, on ice, for 5 seconds using a sonicator with a microtip probe (Ultrasonic Processor W-385, 40% duty cycle, setting "2" energy output). Aprotinin (10 i.u./ml and phenylmethylsulphonyl fluoride (PMSF) 1 mM, final concentration, were added to the sonicate and the sample was spun at 10 000 g for 10 mins in a Beckman^{TM}11 microfuge machine. The supernatant thus obtained was used for further analysis. Protease inhibitors and preservatives were also added to the medium samples: PMSF 1 mM, aprotinin 10 i.u./ml, sodium azide 100 mg/l and EDTA 3.2 mM, pH 7.4 (final concentrations). The media were also spun at 10 000 g for 10 mins to centrifuge down any cellular debris, and the supernatant from this spin kept for analysis.

The filter-paper disc method of Mans and Novelli (1961) was used to quantify the radioactive amino acids incorporated into protein. Aliquots of the cell sonicates or media were spotted onto Whatman GFC filter discs, dried and then sequentially submerged using a filter disc carrier and dipping device, into the following solutions: (1) 10% trichloro-acetic acid (TCA) and 5 mM cold amino acid carrier, for 30 mins at 4°C; (2) 5% TCA and cold carrier, for 15 mins at room temperature; (3) Ether:ethanol mixture (1:1 v/v) for 5 mins at room temperature; and (4) Ether, for 5 minutes at room temperature. The dried filter discs were then counted in a liquid scintillation counter.

2.3 (v) Protein Estimations

The results of protein synthesis experiments were expressed as dpm of radio-labelled amino acid incorporated into TCA-precipitable material per microgram cell protein. The cell protein was determined using the Markwell modification of the method of Lowry. This modification improves the solubility of proteins from lipid environments (e.g. lipoproteins and membranes in the reaction mix and involves the addition of sodium

dodecyl sulphate to Lowry Markwell Solution A and the replacement of Na-K tartrate with disodium tartrate (Lowry *et al.* 1951, Markwell *et al.* 1978).

2.3 (vi) Enzyme Assays of Cell Stability

The activities of lactate dehydrogenase (LDH), a cytosolic enzyme, and alkaline phosphatase, an integral brush border enzyme, were assayed in the cell lysates and media to determine cell stability and viability during a time course incubation. The degree of cell disruption achieved by the method of sonication in homogenization buffer as compared with other methods of cell disruption e.g. Dounce Homogenization, was also evaluated by measuring the release of LDH. The LDH assay was based on the reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{LDH}]{} \text{Lactate} + NAD^+$$

At 340 nm the two forms, NAD^+ (oxidized) and NADH (reduced) have large differences in their respective light absorption. The reaction mix consisted of Tris HCL, pH 7.4, 100 mM; Na pyruvate 5 mM, and NADH 0.2 mM. The absorbance at 340 nm (A_{340}) of 1 ml of reaction mix in a cuvette was measured in a spectrophotometer at room temperature, a small aliquot of cell lysate or medium was then added, mixed quickly, and the rate of disappearance of NADH was determined by sequential A_{340} readings at 30 second intervals for up to 3 minutes. The enzyme activity was expressed as mAU/min and divided by 6.2 to give the number of nanomols of pyruvate reduced per minute (Reeves & Fimognari 1963). In cell-medium comparisons the respective LDH activities were expressed as percentages of total LDH activity for a given time point.

The assay of alkaline phosphate activity was based on the reaction: p-Nitrophenol phosphate → p-Nitrophenol + H_3PO_4. The product is yellow in colour with an A_{max} of 18.5 at 405 nm for a 1 cm light path. The reaction mix consisted of: NaCl 160 mM,

$MgSO_4$ 1 mM, $ZnCl_2$ 0.5 mM, glycine, pH 9.5, 49 mM, and p-Nitrophenyl phosphate (dicyclohexylammonium salt), 5 mM. The A_{405} of the reaction mix was measured in a spectrophotometer at room temperature. An aliquot of the cell lysate or medium sample was mixed with the reaction mix and incubated at room temperature for 15 to 30 minutes, until a yellow colour appeared. The A_{405} was again measured and the elapsed incubation time noted. Alkaline phosphatase activity was expressed as:

A_{405} in mAU/incubation time (minutes) i.e. A_{405} in mAU/min
(Bessey *et al.* 1946, Chan & Atkins 1983).

Once again the respective cellular and medium activities were determined for each time point incubation and expressed as percentages of the calculated total alkaline phosphatase activity (cells + medium).

2.4 RESULTS: CHARACTERIZATION OF THE SYSTEM AND OPTIMIZATION OF CONDITIONS

The average hamster small intestine (from first part of duodenum to ileocaecal junction) measured about 42 cm in length. The proximal 17 cm segment (distal to the opening of the hepato-pancreatic duct in the duodenum) was used for the cell preparations (see Fig. 2.2). The original method described by Towler *et al.* (1978) included the use of calcium sequestration (50 mM trisodium citrate in the isolation medium) to release the epithelial cells from the bowel wall. In the light of experimental evidence suggesting the critical role of Ca^{++} ions in the secretion of nascent lipoproteins and the potential loss of viability induced by calcium sequestrants (e.g. Strauss & Jacob 1981), the citrate was omitted in our experiments. Nevertheless, cell yields expressed as milligrams of cell protein obtained per centimetre of proximal small bowel were similar for preparations with and without citrate. When examined histologically during the "tapping" procedure, the epithelial cells were seen to be removed *én masse* from the bowel wall with the freed cells coming off in large sheets that resembled the shapes of the villi from which they had been removed (Figs. 2.8 - 2.10). Most of the cells (>80%) were present in large sheets or clusters, a few smaller groups of epithelial cells were seen, and only a few individual cells were present. As far as could be assessed by light microscopy, virtually all the cells in the preparation had the morphological appearance of columnar epithelial cells. It is, however, conceivable that some stromal material (and thus non-epithelial cells) may have been obtained and were buried within the large villi-shaped clusters. It is also possible that some of the villus shapes seen may have represented severed villi tips containing stromal material. However, no cells other than columnar epithelial cells could be identified.

In the case of the Weiser method in which calcium sequestration techniques were employed (citrate in the isolation medium and EDTA in the suspension medium) the cells

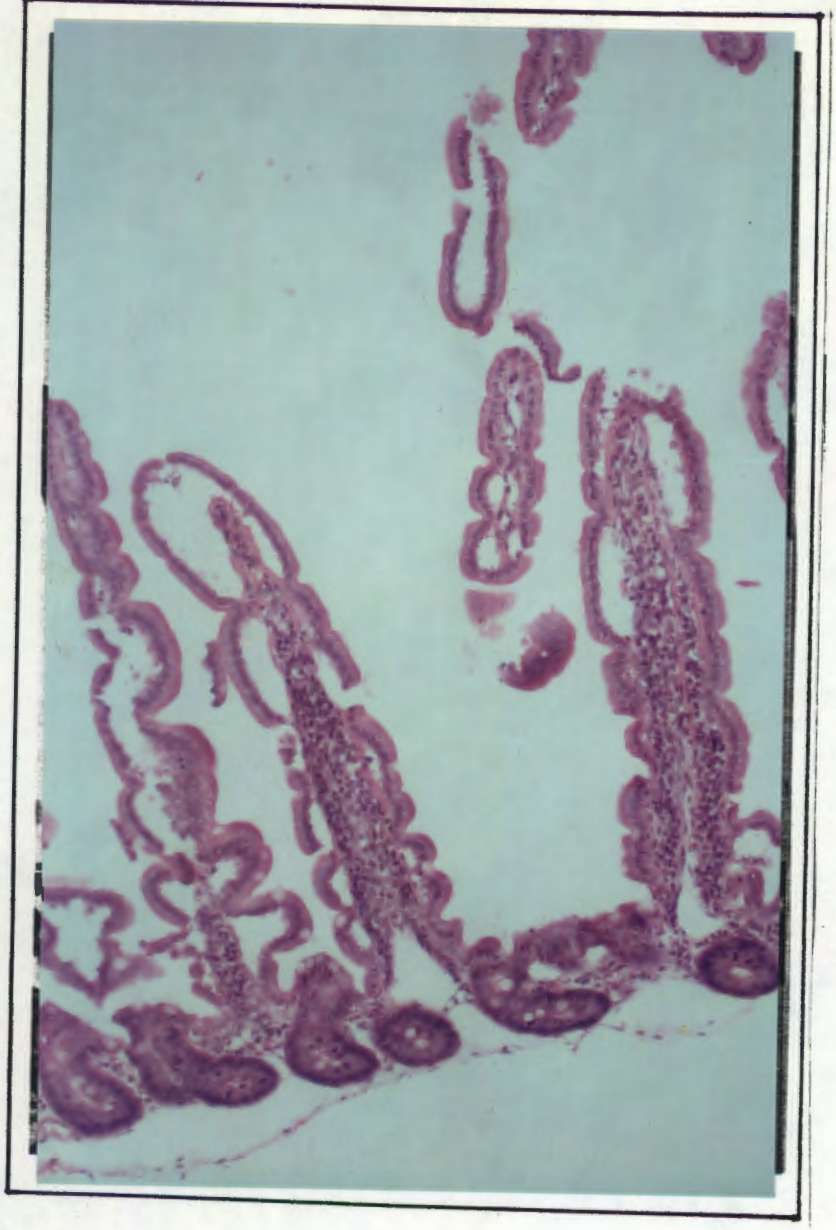


Fig. 2.8. A longitudinal section through the villi of jejunal mucosa showing detachment of the epithelial cells from the stroma after the patting procedure. Stain: Haematoxylin and eosin.

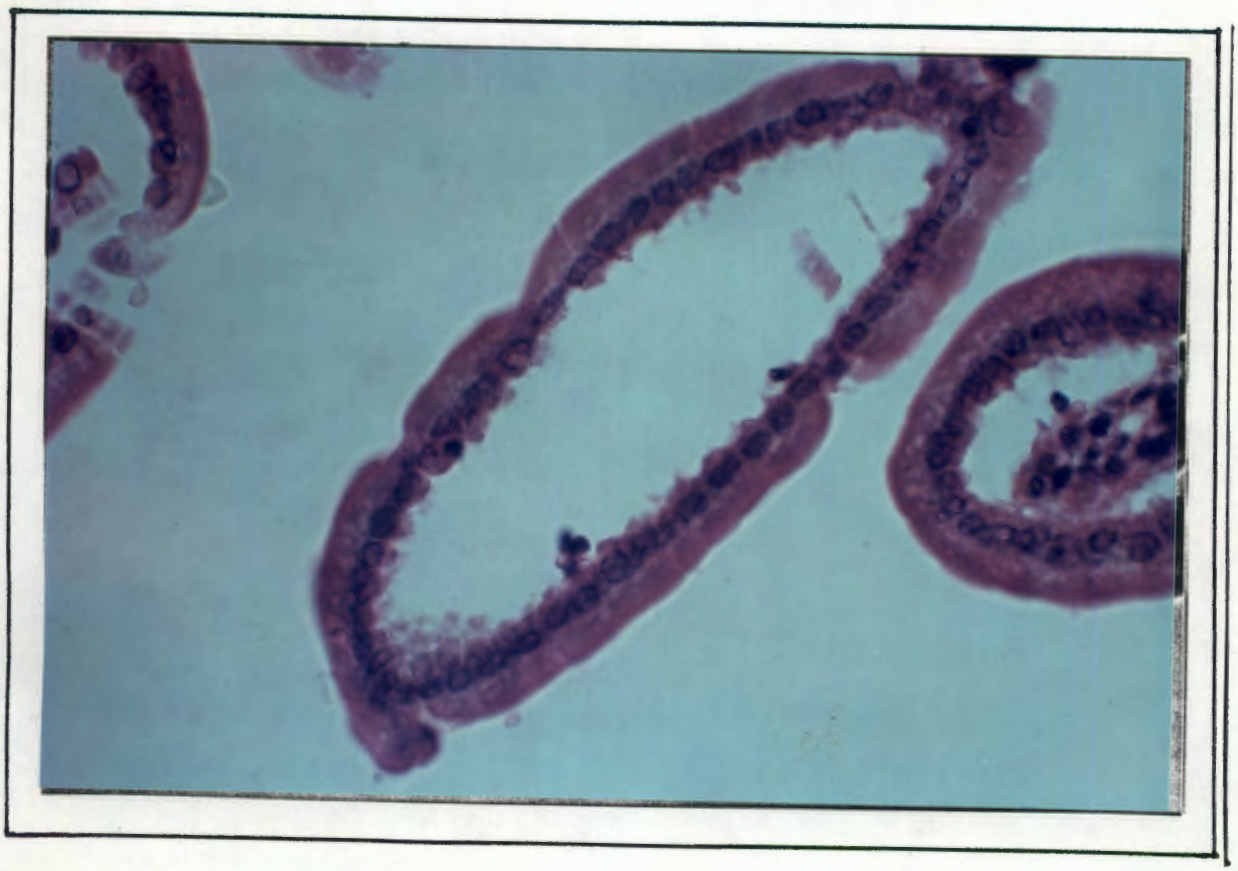


Fig. 2.9. The freed epithelial cells viewed in cross-section. Stain: Haematoxylin and eosin.

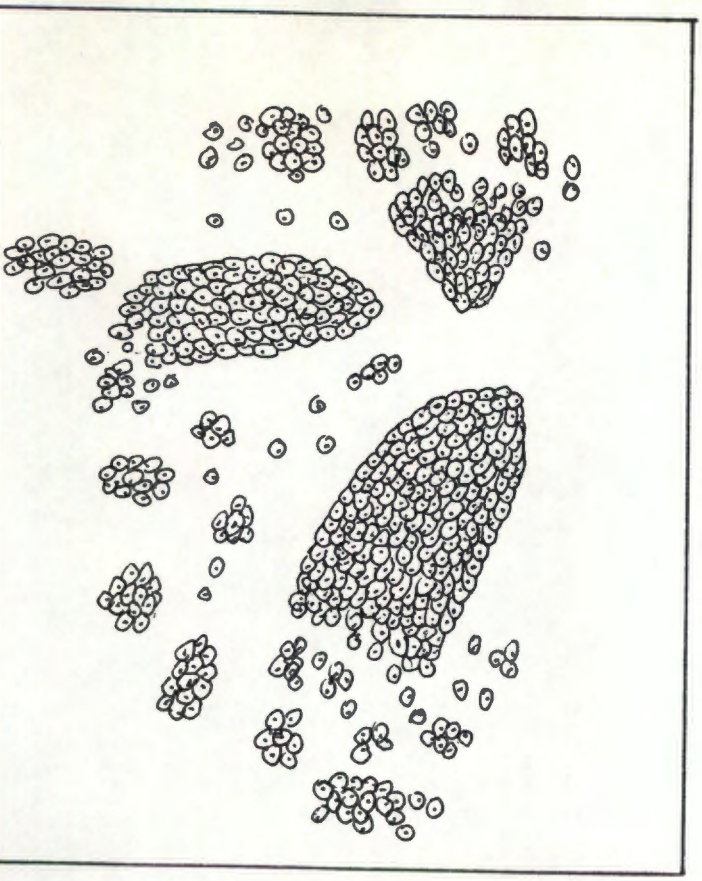


Fig. 2.10. Appearance of the freed epithelial cell sheets viewed under light microscopy following the patting procedure. The sheets resemble the shapes of the villi from which they have been detached *én masse*.

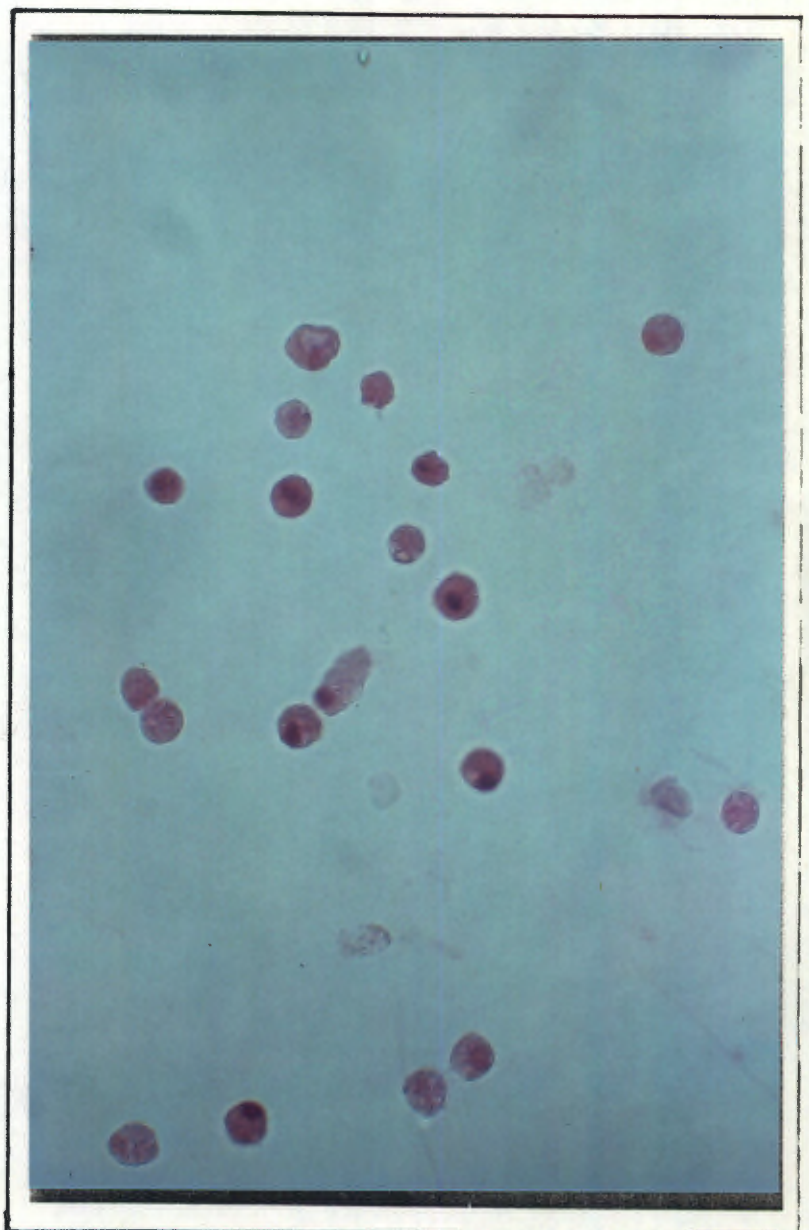


Fig. 2.11. Epithelial cells released from the bowel wall using the Weiser Method. Individual cells are seen and they have lost their characteristic columnar shape.

were released either singly or in small groups. All the cells appeared again to be squamous columnar epithelial cells although when present individually they tended to lose their columnar shape and to become rounded (Fig. 2.11). Cell viability as judged by the Trypan Blue Dye Exclusion technique, was estimated at greater than 95% at the start of the incubation experiments for both the Weiser and Towler techniques. However, it should be pointed out that in the case of the Towler method the Trypan Blue technique represented at best, only a rough estimate, as most of the cells were present in large clusters and individual cells could not be accurately counted.

Protein Synthesis Experiments

Sheets of hamster jejunal enterocytes prepared by the Towler method and incubated in Krebs-Ringer HCO_3 containing fuels (glucose, glutamine and lactate), antibiotics and ^{3}H-leucine, incorporated radio-labelled amino acid linearly into protein for up to six hours *in vitro*; cells prepared by the Weiser method were less active and less sustained (Fig. 2.12). In all subsequent experiments, the Towler method was used. Ice-cold 10% TCA was used for protein precipitation. This method will in theory precipitate both protein and amino-acyl tRNA but I found that the use of hot TCA (95°C) yielded TCA-precipitable radioactivity only 5% less than when cold TCA was used. In future experiments, therefore, cold TCA was used for protein precipitation.

The protein synthesis observed was eukaryotic (enterocytic) in origin and did not derive from bacterial contamination or overgrowth, as demonstrated by the effects of adding cycloheximide, an inhibitor of eukaryotic protein synthesis at the concentrations used, and chloramphenicol, an inhibitor of bacterial protein synthesis, respectively (Fig. 2.13). In all preparative and incubation media penicillin 100 units per ml, and streptomycin 100 μg/ml, were routinely added.

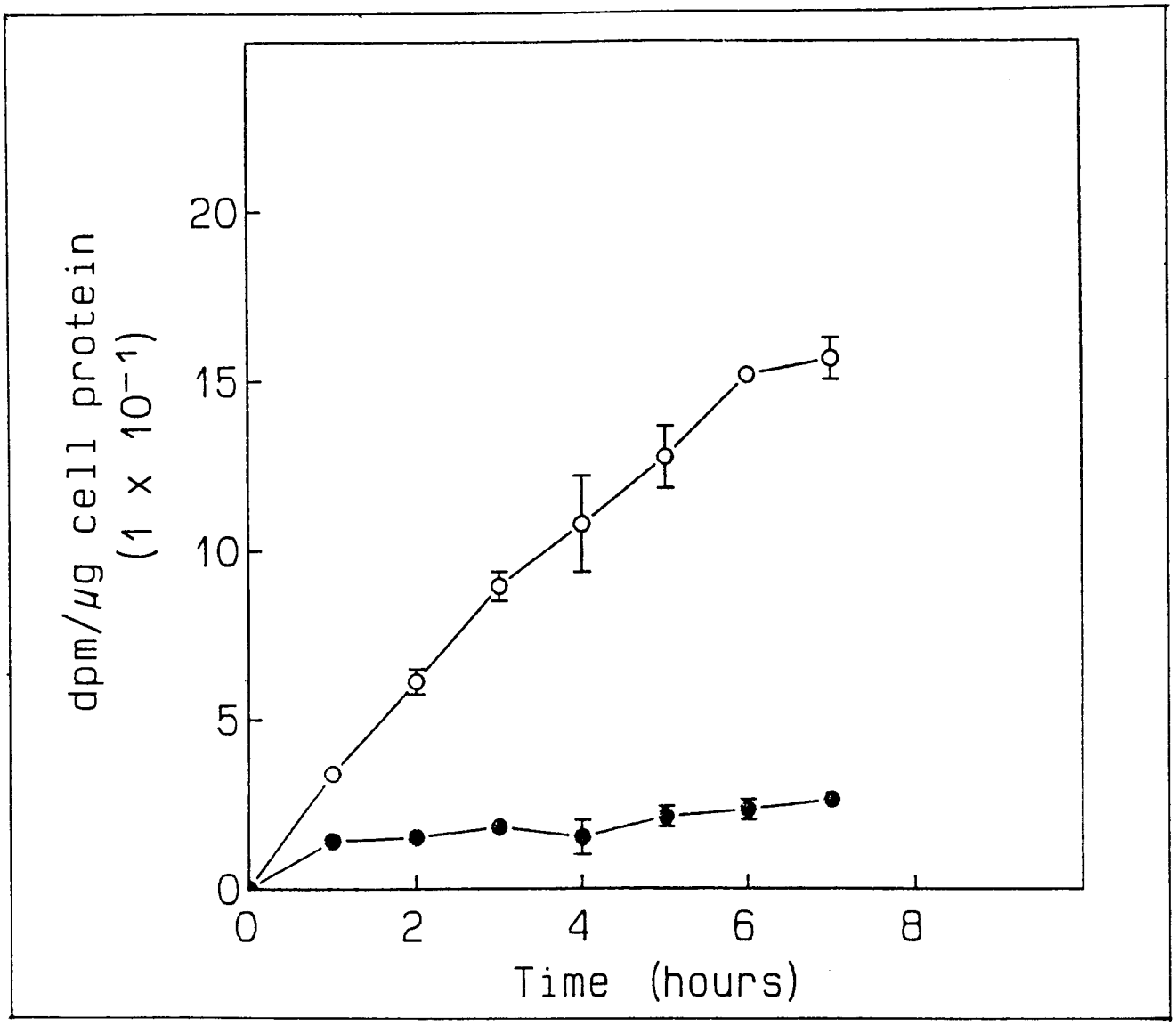


Fig. 2.12. Time course incorporation of ^{3}H-leucine into TCA-precipitable material following an incubation of jejunal enterocyte suspensions from a chow-fed hamster with 0.5 μCi of ^{3}H-leucine.

0 = Towler Method of cell preparation

● = Weiser-Dietschy Method

(= X ± SD) (mean of triplicate incubation samples)

The experiment was performed thrice using jejunal enterocytes from a chow-fed hamster on each occasion. A representative result is shown here.

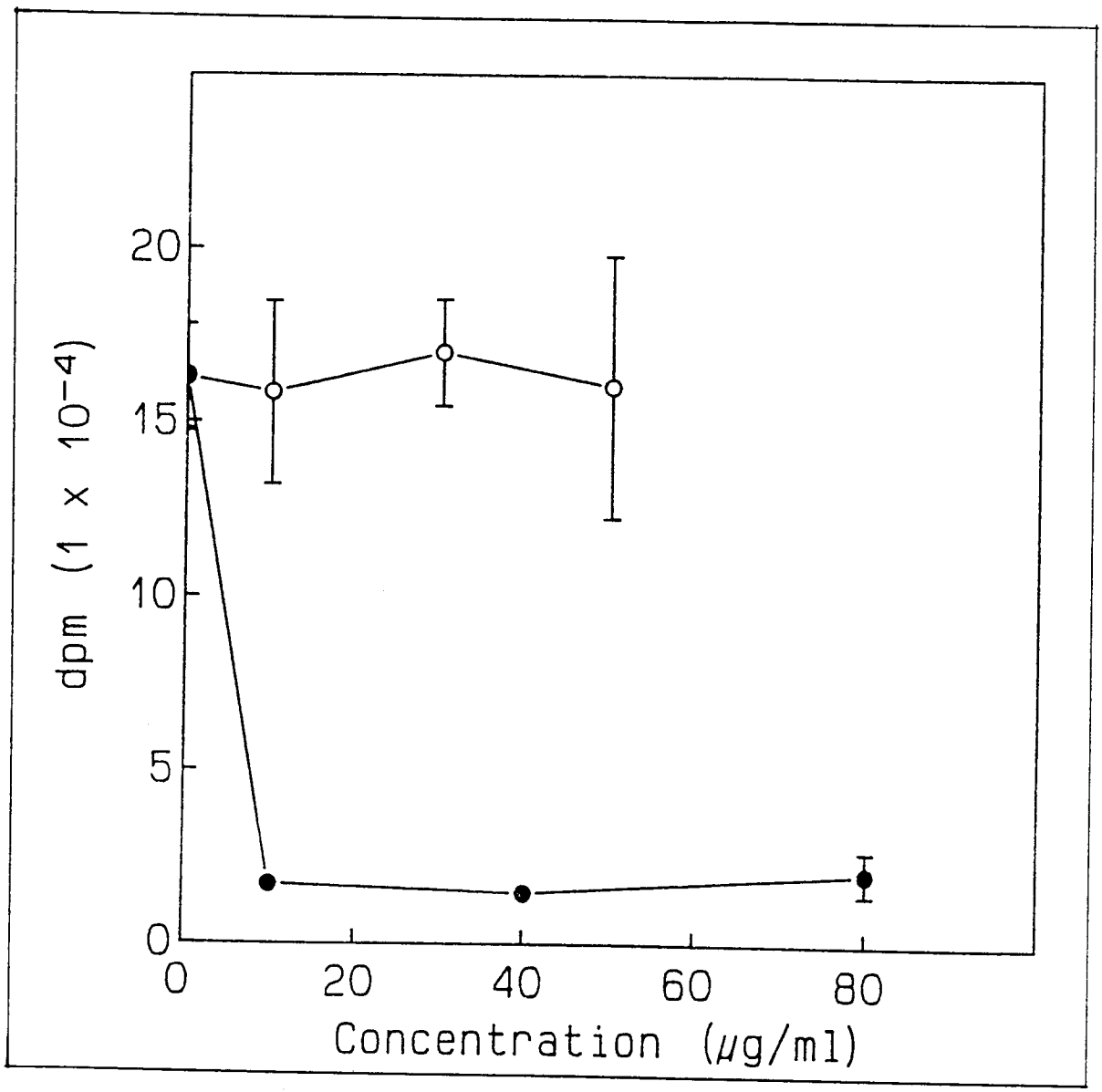


Fig. 2.13. The effects of various concentrations of cycloheximide (●) and chloramphenicol (0) on the incorporation of ^{3}H-leucine into TCA-precipitable protein following a 3 hr incubation of epithelial cell sheets from hamster jejunum.
(Experiment performed twice & incubation continued for 5 hrs, although only the 3 hour time point is shown here.)

Optimization of medium conditions was sought by varying some of the constituents of the Krebs-Ringer bicarbonate (KRB) medium. The standard KRB with 20 mM HCO_3 and 2.54 mM Ca^{++} was found to be the optimal incubation medium (Fig. 2.14). More alkalotic conditions were well-tolerated by the cells while acidosis proved detrimental (as measured by the incorporation of ^{3}H-leucine into protein after a 3 hour incubation). The beneficial effect of adding albumin to the incubation medium as reported by Kimmich (1975) was not shown in this study. The addition of 5 mM Na azide, an inhibitor of oxidative phosphorylation, resulted in a drastic reduction in protein synthesis (and probable cell death) (Fig. 2.14).

The importance of the addition of exogenous fuels to the isolated epithelial cell sheets incubated *in vitro* was demonstrated (Fig. 2.15). Glucose 10 mM, glutamine 2 mM and lactate Na 2.5 mM were routinely added to all preparative media. The importance of these exogenous fuels appeared to increase with increasing duration of *in vitro* incubation time.

Glutamine has been shown to be an important intestinal fuel especially during starvation and overnight fasting in mammals. It contributes 35% of the CO_2 production in the small intestine of the post-absorptive rat (Windmueller & Spaeth 1980) and is converted to glutamate which, following transamination, is eventually converted to malate and pyruvate. The pyruvate may then be oxidised via the tricarboxylic acid cycle.

The extent of radio-labelled leucine incorporated into protein appeared to depend on the concentration of glutamine added (Fig. 2.16A & B). At 2.5 mM glutamine (final concentration in the incubation medium) cell suspensions synthesized protein more efficiently than those which lacked glutamine. (Both sets of cell suspensions, derived from a single chow-fed hamster, were incubated in the presence of 10 mM glucose and 2.5 mM lactate and glutamine was added to both preparative media).

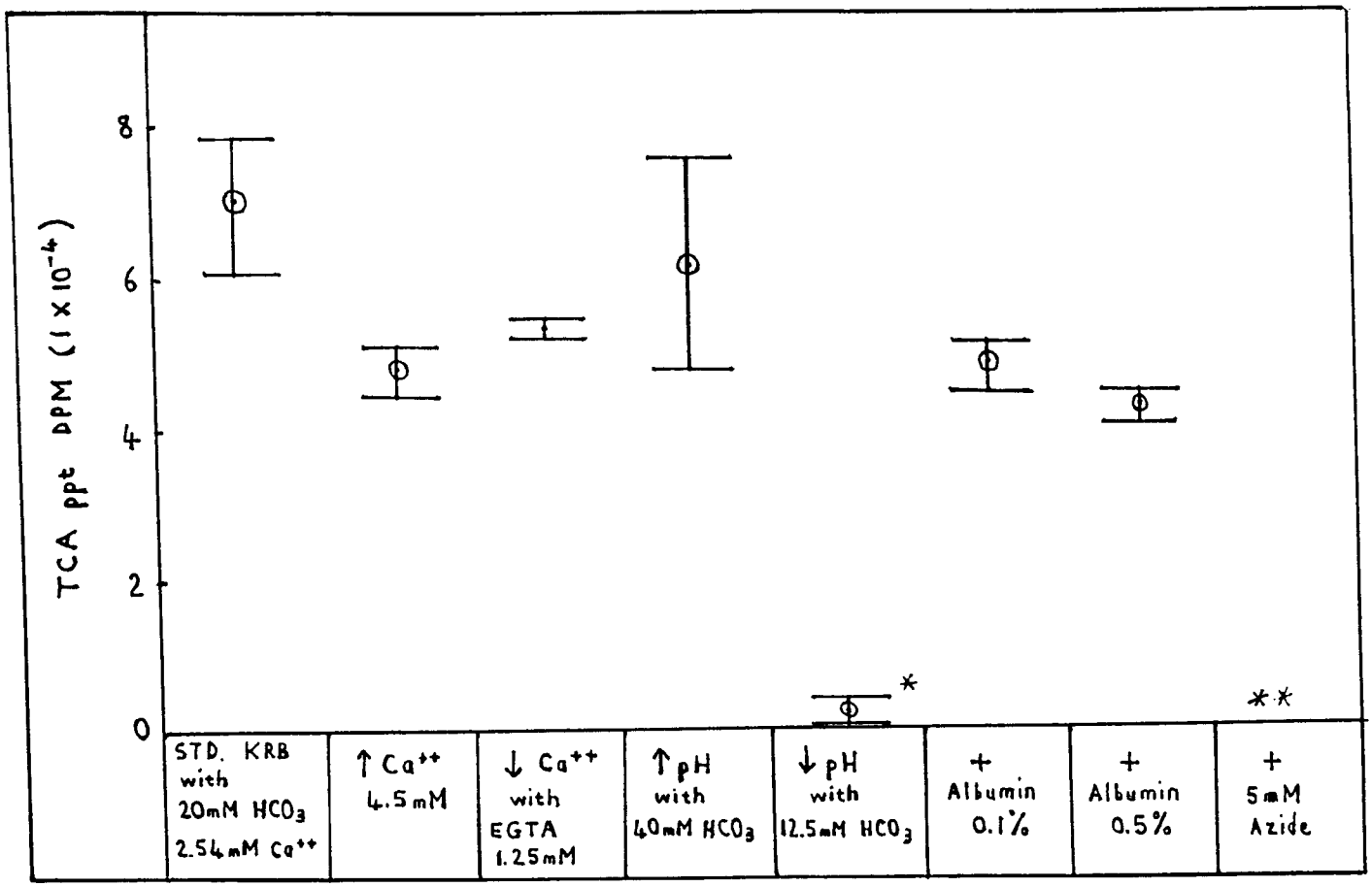


Fig. 2.14. The effects of various modifications of the standard Krebs-Ringer-bicarbonate incubation medium on the incorporation of ^{3}H-leucine into TCA-precipitable material by hamster jejunal enterocyte sheets incubated for 3 hrs. All the incubation systems were derived from the jejunum of a chow-fed hamster.

* Significantly different from std. KRB system.	Std System: 7.9 ± 1.2 ($\bar{x}$ ± SD) ↓pH: 1.1 ± 1.8 ($\bar{x}$ ± SD) p = 0.01 (< 0.05)
** + Azide	0.4 ± 0.4 significantly different from std. KRB system p < 0.05 (0.01)

TRIPLICATE INCUBATION SYSTEMS/ MODIFICATION

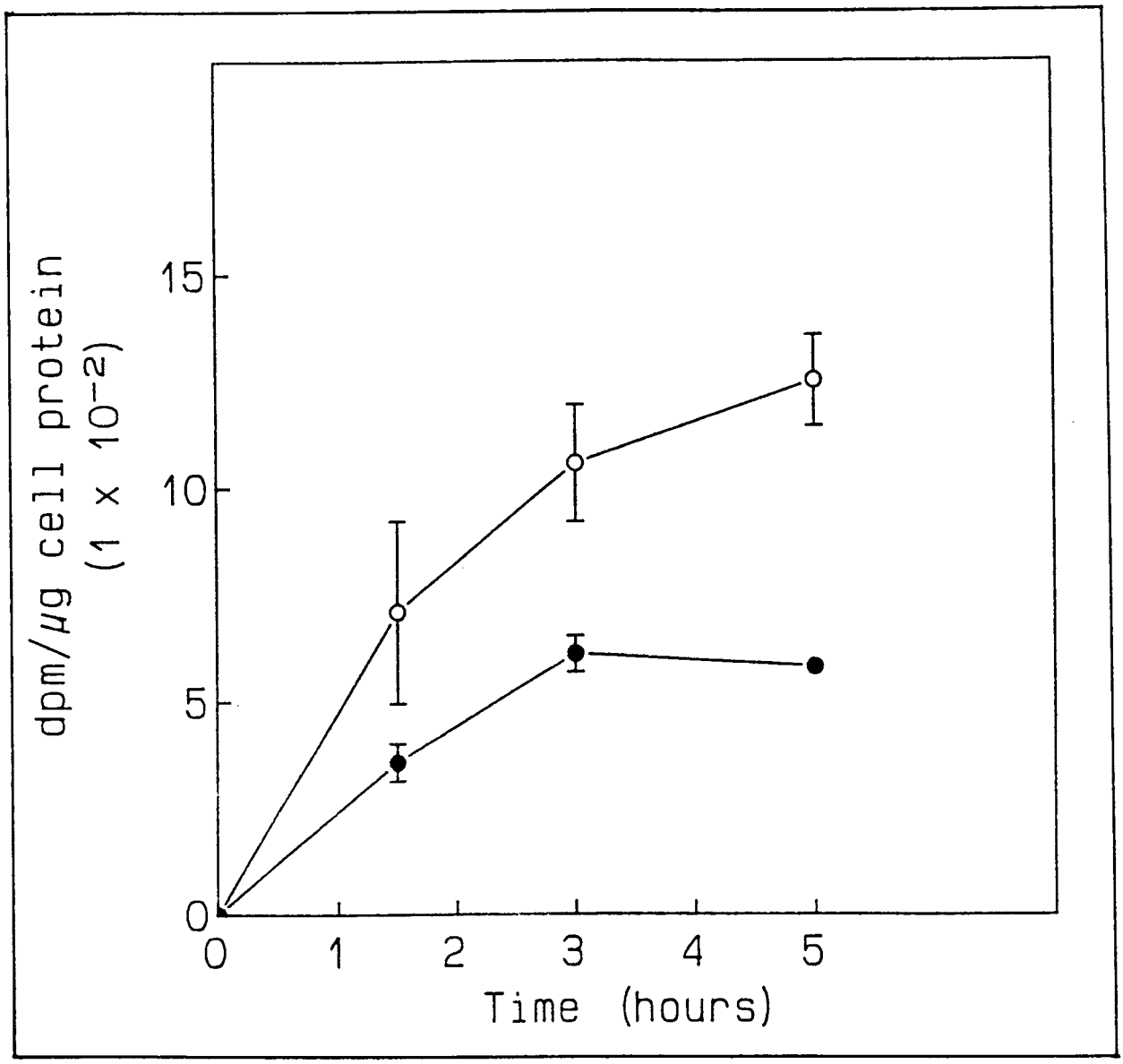


Fig. 2.15. Time course incorporation of ^{3}H-leucine into TCA-precipitable radioactivity by enterocyte sheets incubated in the presence (0) or absence (●) of exogenously added fuels in the incubation medium. The fuels were added to the preparative media (Suspension & Isolation medium) of both sets of incubations. The cell suspension was derived from a single hamster small intestine and the 2 cell suspensions split only after washing them in the suspension medium.

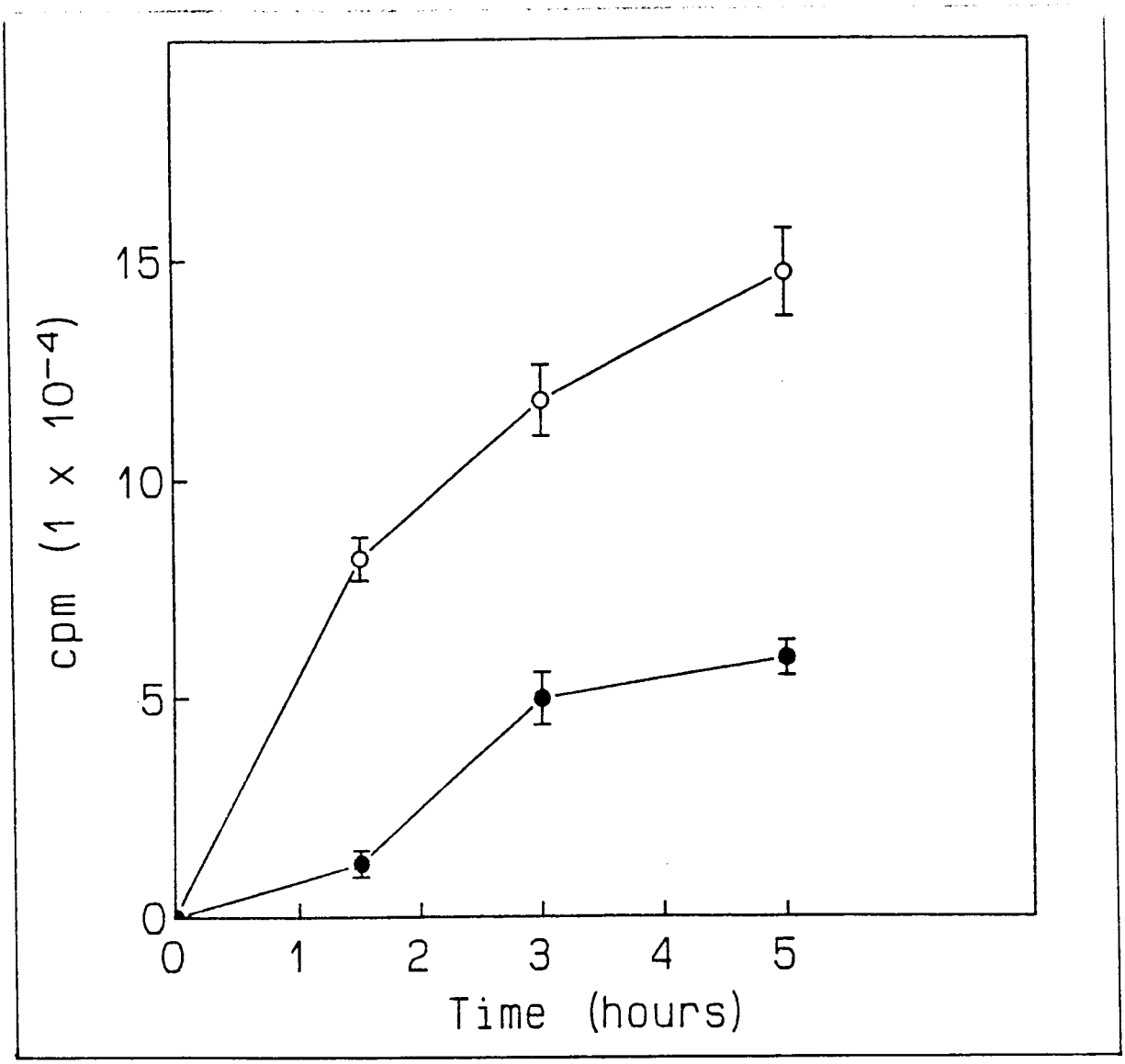
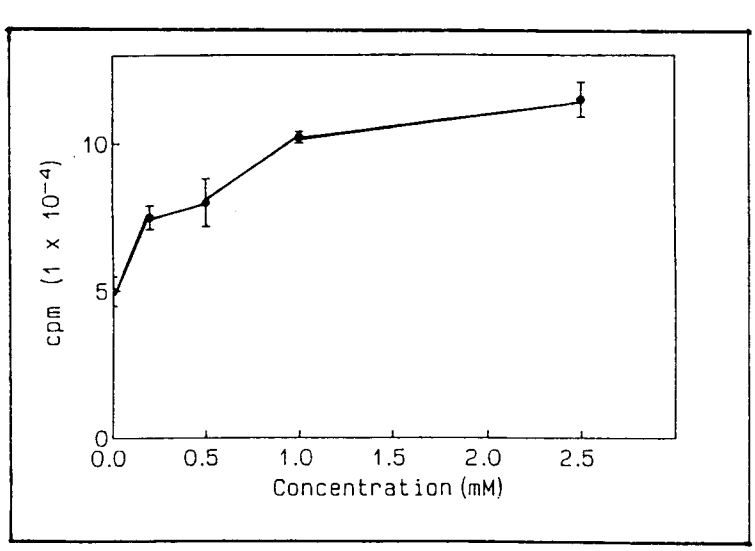


Fig. 2.16. (A) The effect of incubating jejunal enterocyte sheets in the presence (0) or absence (●) of glutamine (2.5 mM final concentration) in the incubation medium, on ^{3}H-leucine incorporation into TCA-precipitable radioactivity.

(B) The effect of increasing concentrations of glutamine on ^{3}H-leucine incorporation into TCA-precipitable material by jejunal enterocyte sheets incubated in Krebs-Ringer -HCO_3 medium.



Cell stability and viability during incubations

Only a minority of cells died or were damaged during a 5 hour incubation (Fig. 2.17A & B). Lactate dehydrogenase is a cytosolic enzyme released by disrupted or dead cells while alkaline phosphatase is an integral brush border enzyme that may be released by both dead or damaged cells.

While ^{3}H-leucine was used in all optimization experiments, ^{35}S-methionine was considered a more useful precursor amino acid when studying specific proteins of interest, as it had a much higher specific activity (1000 Ci/mmol as opposed to ^{3}H-leucine, 5 Ci/mmol) and a higher energy of radioactive decay so that fluorographic analysis of specific proteins could be made. Potential disadvantages of ^{35}S-methionine, however, are its short half life (87.2 days compared with ^{3}H, 12.26 years) and its relative chemical and especially radio-chemical instability. ^{35}S-methionine was purchased from ICN (Trans ^{35}S-label) and a typical batch contained about 70% ^{35}S-methionine and 20% ^{35}S-cysteine with other ^{35}S compounds such as methionine sulphoxide and ^{35}S-cysteic acid constituting the other 10% (manufacturer's information). It was stored, aliquotted, at -70°C and gassed with nitrogen to prevent oxidation. Fig. 2.18 demonstrated the non-linear incorporation of ^{35}S-methionine into radioactive protein compared with ^{3}H-leucine. Both cell suspensions were derived from the same small bowel preparation. That the problem of non-linear ^{35}S-methionine incorporation did not lie with the cells themselves, was demonstrated by the experiment of Fig. 2.19 (A & B) (see accompanying legend) .

The most likely explanation for the result was that with increasing incubation time, more ^{35}S-methionine became chemically and radiolytically converted (e.g. to ^{35}S-methionine sulphoxide), with consequent loss of specific ^{35}S-methionine activity. The addition of fresh ^{35}S-methionine at regular intervals replenished the supply and maintained the specific activity. For shorter incubation times, however, ^{35}S-methionine incorporation

remained more or less linear (Fig. 2.20) and this proved useful later in pulse-labelling and pulse-chase experiments (See Chapters 3 & 4).

Significant quantities of radio-labelled protein were released into the incubation medium with time. The nature of this release (secretion versus lysis) and the identity of the released proteins will be addressed in Chapter 3.

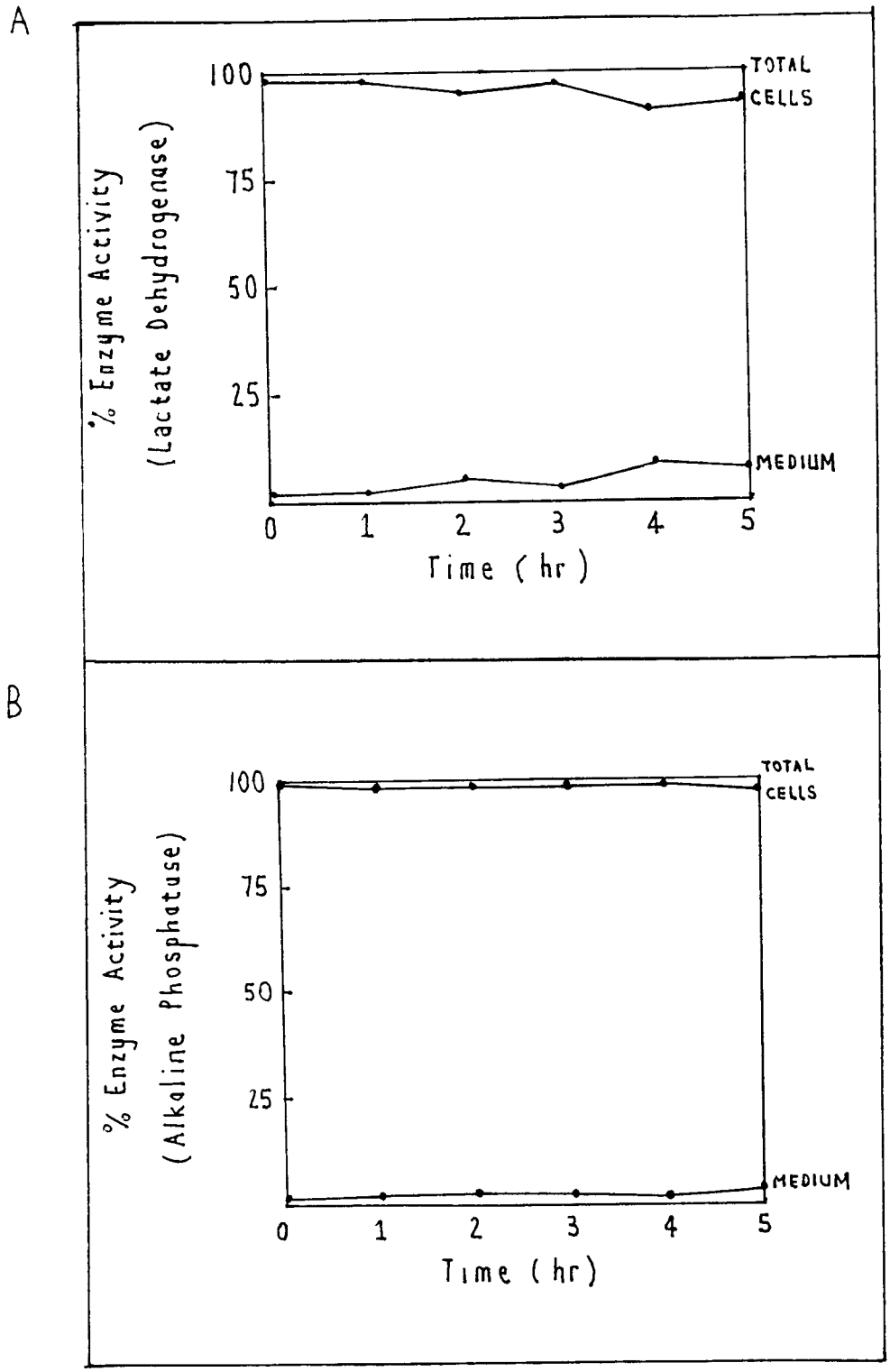


Fig. 2.17. Measurements of the activities of (A) Lactate Dehydrogenase, and (B) Alkaline Phosphatase in cell lysates and media samples from a 5 hr time course incubation of jejunal enterocyte sheets. The activities of the enzymes in the cells and media are expressed as a percentage of the total enzyme activity at the respective time points.

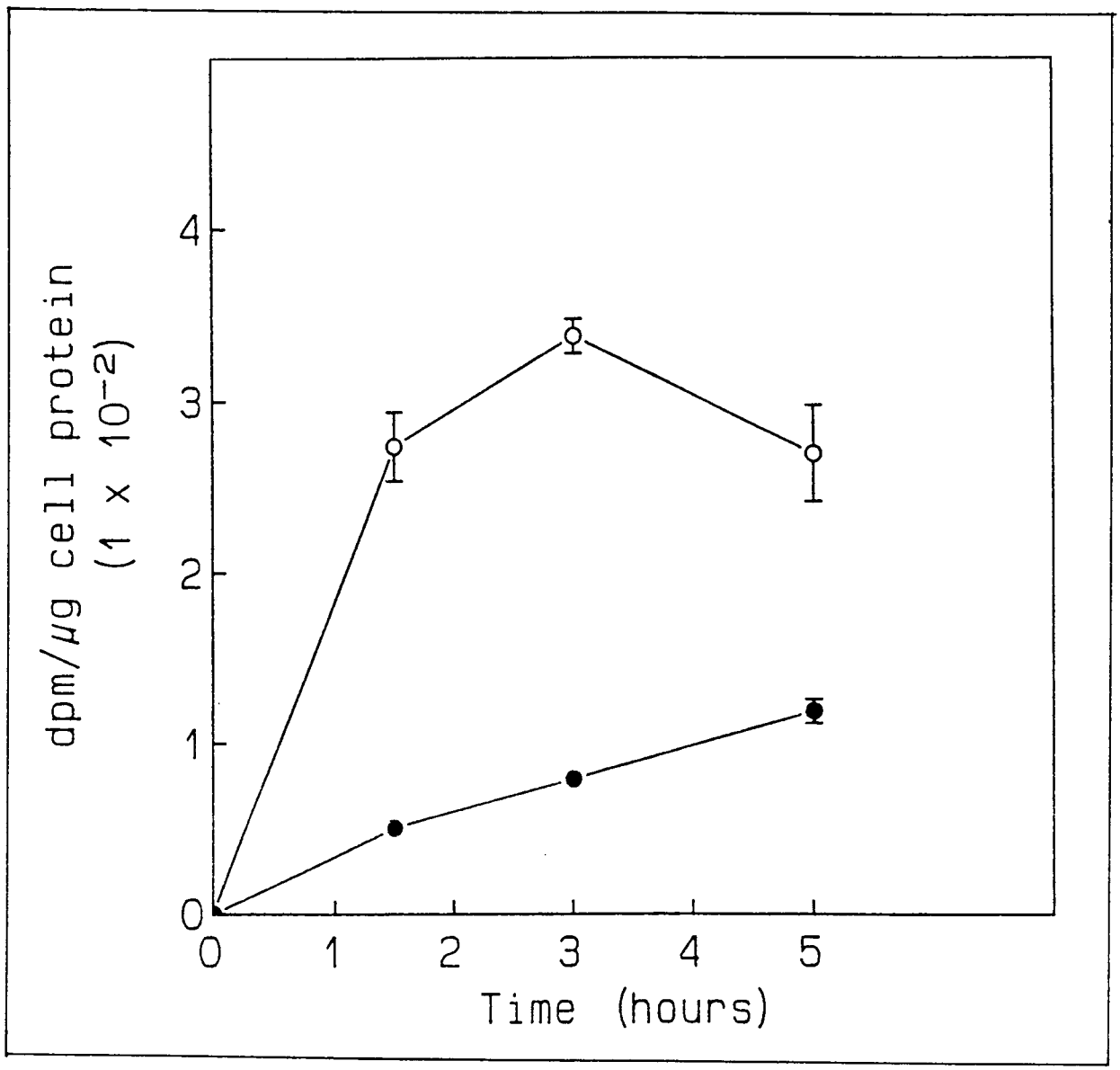


Fig. 2.18 Time course incorporation of ^{3}H-leucine (●) and ^{35}S-methionine (0) into TCA-precipitable radioactive material when enterocyte suspensions from a hamster jejunum were incubated with one or other of the 2 labelled precursor amino acids.
The experiment presented here was representative of 3 similar experiments, all of which showed a drop in the 35S-methionine incorporation after 3 hours.

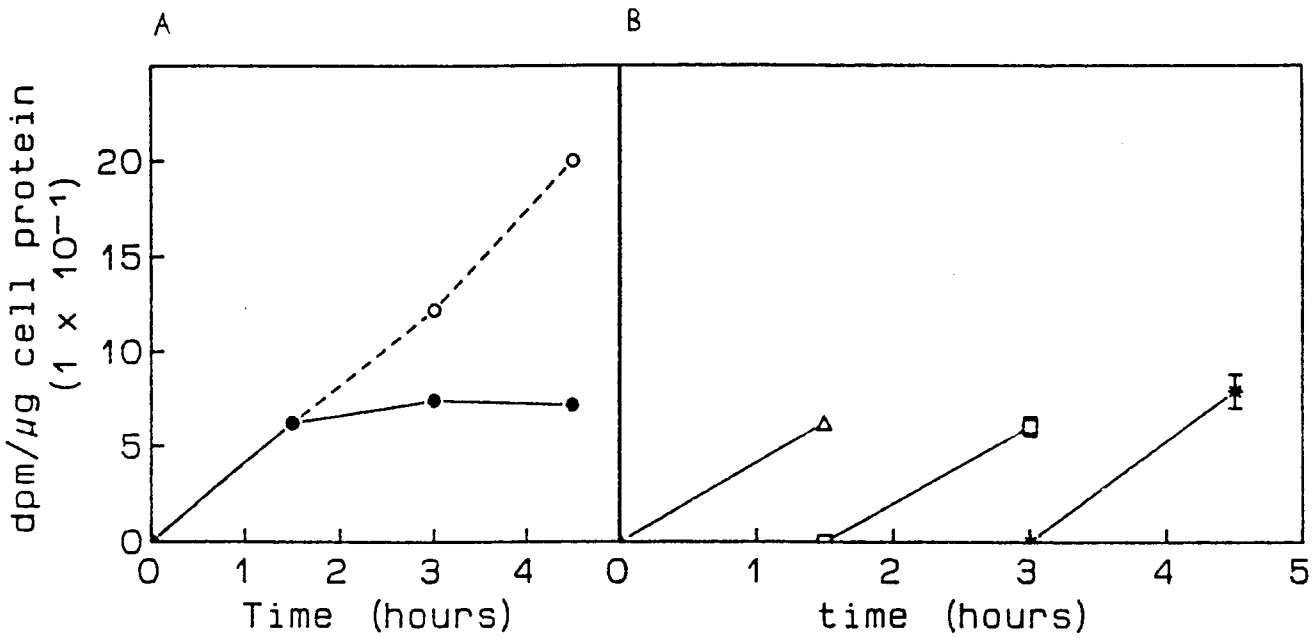


Fig. 2.19. (A) Time course incorporation of ^{35}S-methionine into protein when all the radioactive methionine was added at T = 0' (⟶ line).

(B) Incorporation of ^{35}S-methionine into protein for 1½ hours when fresh ^{35}S-methionine was added (1) at T =0';△(2) at T = 1½ hrs (i.e. following a 1½ hr incubation of the cell sheets with no radio-label),□and (3), at 3 hrs (i.e. following a 3 hr incubation of the cells with no label).*

When fresh ^{35}S-methionine was added regularly during the incubation, the system was restored to linearity (o----o, A).

The experiment was performed twice (2 animals, 2 sets of enterocyte preparations) and on each occasion the addition of fresh ^{35}S-methionine restored the system to linearity.

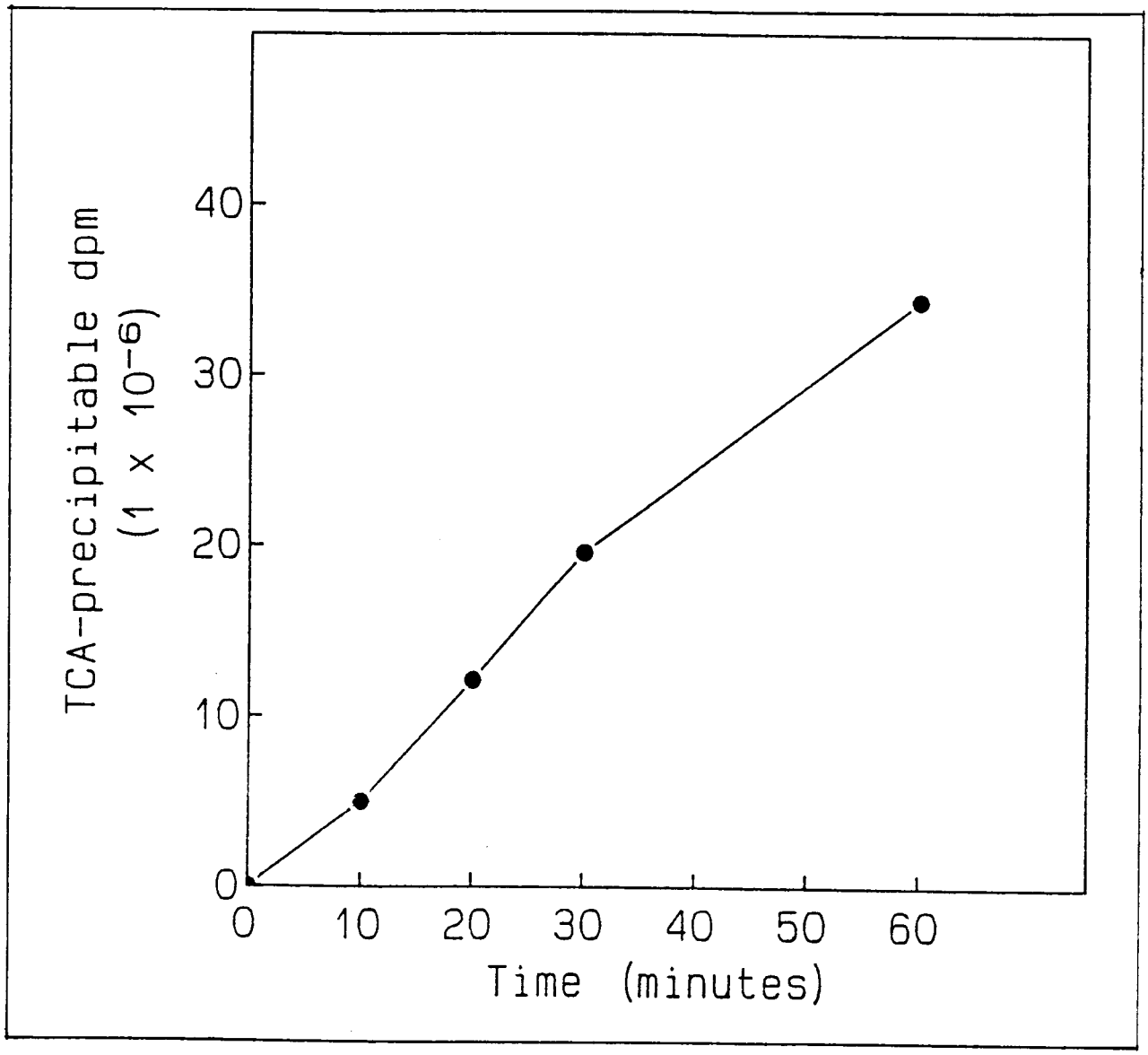


Fig. 2.20. Time course incorporation of ^{35}S-methionine into TCA-precipitable cellular material during a sixty minute incubation period.

2.5 CONCLUSION

Using the Towler method, sheets of intestinal epithelial cells were isolated from the jejunum of adult male Syrian Golden hamsters, a useful small-animal model of human lipoprotein metabolism. These cells were found to linearly incorporate radio-labelled precursor amino acids into protein for up to 5 hours when incubated *in vitro*. Optimal incubation conditions were established and tests of cell stability and viability indicated that only a minority of cells died or were damaged during the incubation period. This system therefore appeared to be potentially useful as a method for studying the synthesis and secretion of specific proteins of interest.

CHAPTER THREE

IDENTIFICATION AND QUANTIFICATION OF BIOSYNTHETICALLY LABELLED PROTEINS OF INTEREST IN LIPID METABOLISM

3.1 THE PREPARATION OF A RABBIT-DERIVED, ANTI-HAMSTER APOLIPOPROTEIN ANTISERUM

The technique of immuno-precipitation was chosen as the most suitable method in the enterocyte biosynthesis system for identifying and quantifying the specific proteins of interest. A rabbit-derived (polyclonal), anti-hamster apolipoprotein antiserum was prepared by immunizing rabbits with lipoprotein-associated (ρ < 1.21 g/ml) hamster apolipoproteins.

3.1 (i) Antigen preparation

(This method was first used in our laboratory by Dr A.D. Marais, and I wish to acknowledge his kind assistance in teaching me the techniques).

A group of 20 hamsters, previously maintained on a standard laboratory chow, were administered sunflower oil by pipette (0.5 ml per animal) on the morning of the experiment. 3 hours later the animals were anaesthetized with di-ethyl ether and exsanguinated by cardiocentesis. The pooled blood was spun at 2000 g for 15 minutes in a Beckman J-21C centrifuge using a JA20 rotor and the lipaemic plasma supernatant was aspirated. (The aim of the initial lipid administration was to increase the quantities of post-prandial apolipoproteins in the plasma in order to maximize the potential antigenic material for immunization, although we had no proof that this was indeed the case. It did, however, make aspiration of floated lipoproteins easier as they could be readily identified as a creamy layer on the top). The protease inhibitor, phenylmethylsulphonyl fluoride (PMSF) (1 mM final concentration) was added to the plasma as a preservative and the lipoproteins (ρ < 1.21 g/ml) were prepared from the plasma by flotation-ultracentrifugation as follows: The plasma was adjusted to a density of 1.25 g/ml using dried Potassium Bromide (KBr) powder according to the formula:

$$\text{g/100 ml KBr} = \frac{100(\rho \text{ desired} - \rho \text{ of present solution})}{1.010}$$

$$\overline{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}$$

$$1 - (0.312 \times \rho \text{ desired})$$

ρ = density (g/ml)

ρ desired = 1.25 g/ml

ρ of present solution = ρ plasma = 1.010 g/ml

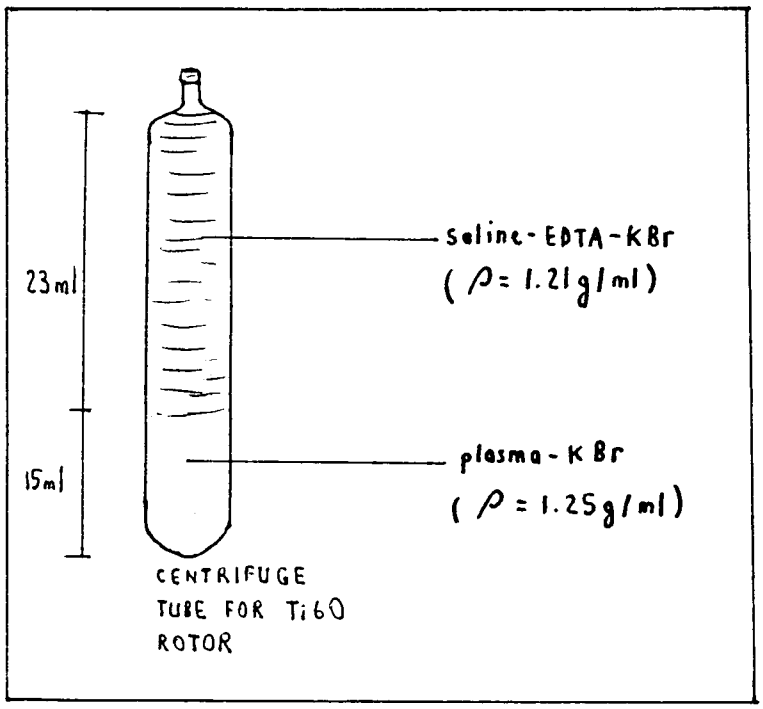
The plasma was underlayered into a solution of saline-EDTA-KBr (ρ = 1.21 g/ml) in a centrifuge tube for the Beckman Type 60 Ti rotor (see Fig. 3.1). This was followed by a 48 hour spin, at 60 000 rpm, using a Ti60 rotor (K factor = 63) in a Beckman L5-65 ultracentrifuge, in order to float all lipoproteins of density < 1.21 g/ml (i.e. chylomicrons, VLDL, LDL and HDL) (Segrest & Albers 1986).

At the end of the centrifugation the floated material which formed a creamy layer on the surface was aspirated using a pipette and dialysed over 3 days in dialysis tubing (Spectropor Membrane Tubing, MW cutoff 12-14000) against saline-EDTA (ρ = 1.006) to remove the KBr. The external dialysis medium was changed daily.

A protein estimation of the retentate was performed using the Markwell modification of the Lowry method and a sample of the dialysed material was separated on a 5-20% SDS-gradient polyacrylamide gel and a 4% polyacrylamide stacking gel (Hoefer "Sturdier" Gel Apparatus, Hoefer Scientific Instruments, San Francisco) based on the original method of Laemmli. Prior to loading, the samples were denatured at 95°C for 2 minutes using a gel-solubilizing mix containing 2.5% SDS, 10% glycerol, 5% 2-mercaptoethanol and 65 mM Tris HCl, pH 8.0. The proteins were electrophoresed initially at 10 mA for 45 minutes and thereafter at 35 mA until completion of the run (approximately 5 hrs), using a Pharmacia Electrophoresis Power supply (EPS 500/400).

The gel proteins were stained with a 0.125% Coomassie stain solution, destained, and dried onto blotting paper using a gel drying apparatus set at 60°C (Dual Temperature Slab Gel Dryer Model SE 1150, Hoefer Scientific Instruments, San Francisco) and

Fig. 3.1. Preparation of gradients for lipoprotein flotation (ρ < 1.21 g/ml) from hamster serum by ultra-centrifugation.



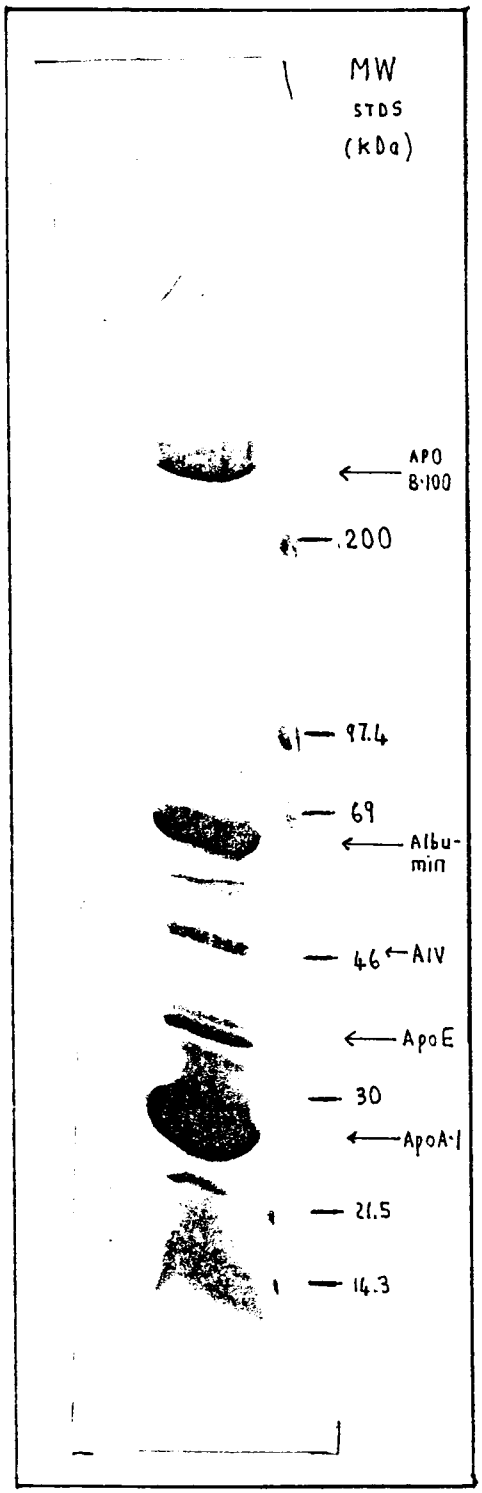


Fig. 3.2. A photocopy of Coomassie-stained proteins separated on a 5-20% SDS polyacrylamide gradient gel of floated material (ρ < 1.21 g/ml) obtained from postprandial, lipaemic hamster serum. The positions of MW standards (kDa) are shown, as are the probable identities of the prominent protein bands on the gel.

attached to a vacuum pump with a suction pressure of 0 torr. Coomassie-stained protein bands corresponding to the expected positions, respectively, of apolipoprotein B-100, albumin, apolipoproteins E, A-IV, A-I and C's, were present (Fig. 3.2). Densitometric scanning of the gel lane revealed that the putative apo B-100 band constituted about 10% of the total protein in the antigen mixture.

3.1 (ii) Immunization and Antiserum Collection

It was decided to immunize each rabbit with at least 100 μg of apo B in the antigen mixture. This corresponded to a volume of 375 μl of the original dialysed antigen material (not the SDS-denatured material used in the gel analysis) which was mixed with an equivalent volume of Freund's Incomplete Adjuvant (FICA) and injected subcutaneously at multiple sites (4 to 5) over the back of each animal. A control rabbit was immunized with lipoprotein-depleted serum (LPDS). Control (pre-immune) rabbit serum was also obtained. Four weeks later a second innoculation of the same antigen mixture was repeated and subsequent boosts were performed at four to six weekly intervals with bleeding of the animals 2 weeks after the boosts (Garvey *et al.* 1977).

The rabbits were venesected using a paediatric scalp vein set attached to a syringe and inserted into the major ear vein of the animals. The blood was allowed to clot for 30 to 60 minutes at 37°C; the clot was separated from the sides of the collecting vessel ("ringing") using a straightened paper clip and the clot allowed to contract by maintaining the sample at 4°C overnight. The serum was then decanted from the clot on the following morning and heated at 56°C for 30 minutes to inactivate complement. Thiomersal (0.08 mg/ml) and sodium azide (0.02%) (final concentrations respectively) were added to the antisera which were aliquotted into small volumes and stored at -70°C. Samples were thawed only once prior to use. Subsequent tests of the antisera using biosynthetically labelled enterocyte lysates (see later) revealed that the rabbit designated "Rabbit 5" produced the best antibody responses and this animal's antiserum will henceforth be referred to as "R5 antiserum".

3.2 OTHER SOURCES OF ANTISERA AND ANTIBODY PREPARATIONS

(i) "OSAN", a polyclonal, rabbit-derived, anti-human apolipoprotein B antiserum, was purchased from Behring Diagnostica.

(ii) A polyclonal, rabbit-derived, anti-rat apolipoprotein A-IV antiserum was a gift from the laboratory of Dr R.M. Glickman, Gastro-intestinal Unit, College of Physicians and Surgeons, Columbia University, New York.

(iii) A polyclonal, rabbit-derived, anti-rat LDL antiserum was donated by Dr R. Hay from the laboratory of Dr G.S. Getz, University of Chicago.

(iv) Anti-Acylation Stimulating Protein (ASP) IgG, purified using Protein A from rabbit polyclonal antiserum, was provided by Dr K. Cianflone, McGill Unit for the Prevention of Cardiovascular Disease, Montreal, Canada.

(v) A polyclonal rabbit-derived, anti-rat apolipoprotein A-1 antiserum was obtained from the laboratory of Dr G.S. Getz, University of Chicago.

(vi) Goat-derived, anti-human Lipid Transfer Protein-1 (LTP-1), was given by Dr Janet Adolphson, Northwestern Lipid Research Laboratory, Seattle, Washington.

(vii) Antisera to "hepatic" Fatty Acid Binding Protein (hFABP) and "intestinal" Fatty Acid Binding Protein (gFABP) were obtained from Dr N.M. Bass, Dept. of Medicine and Liver Center, University of California School of Medicine, San Francisco.

3.3 IMMUNO-PRECIPITATION TECHNIQUES

3.3 (i) Cell lysate and media preparations

The preparation of cell lysates and media samples following an in vitro incubation of enterocyte suspensions with radio-labelled [^{35}S]-methionine, has been described in Chapter 2.3. The final (radioactive) cell lysate in a 1% Triton-X100 and saline-EDTA homogenization buffer solution (pH 7.4) containing antiproteases, was used for immuno-precipitation studies. Protease inhibitors were also added to the media samples which were spun at 10 000 g for ten minutes to remove any cellular debris.

3.3 (ii) Protein A

Initial immuno-precipitation experiments utilized Protein A as the precipitating agent. Formalin-treated, bacterial cell wall-derived Protein A (Pansorbin, Calbiochem) has a high adsorption capacity for the Fc portion of many mammalian immunoglobulins including rabbits, humans, and guinea pigs (Langone 1982). The original method utilizing Protein A to immuno-precipitate antigen-antibody complexes was first described by Kessler in 1975 but a modified technique devised by Davidson and Glickman in 1985 was used in my experiments.

Heat-killed, formalin-treated Staphylococcus aureus cells (Pansorbin, Calbiochem) were washed well using a "NETTAM" buffer (0.15 M NaCl, 5 mM Na_2 EDTA; 1% Triton X100; 0.1% bovine serum albumin; 2 mM methionine; 0.02% Na azide, 65 mM Tris HCl, pH 7.40) and used within 24 hours of washing. Equivalent volumes of "NETTAM" and Protein A were mixed and spun for one minute in an Eppendorf microfuge at 10 000 g. The supernatant was discarded, the Protein A pellet was resuspended in the same volume of NETTAM, mixed, and spun again. After a second wash, the final pellet was resuspended in the same volume of NETTAM to maintain a Protein A concentration of 10% (w/v).

In the "pre-clearing" step, washed Pansorbin was added to a measured quantity of the cell lysate. (200 μl of 10% Protein A was used for every 100 μg antibody intended for use in the immuno-precipitation. Cell lysate volumes of 200 μl were generally used). The mixture was incubated at room temperature for 15 minutes and then at 4°C for 30 minutes. Following a 10 000 g spin for 10 mins in a refrigerated microfuge (Beckman ^{TM}11), the supernatant obtained (i.e. cell lysate pre-cleared with Protein A) was added to 200 μl of NETTAM and excess antiserum (25-50 μl) was added. (A control sample with non-immune serum was also included). The mixture was incubated at 4°C for 18 hrs. On the following day 200 μl Pansorbin were added and the mixture re-incubated for 45 minutes at 4°C. The cell pellet was then collected by centrifugation (10 000 g x 10 mins, 4°C), resuspended in 100 μl of NETTAM buffer and layered over a cushion of 600 μl of 1 M sucrose-NETTAM (3.423 g sucrose dissolved in a final volume of 10 ml NETTAM). This was then centrifuged (10 000 g x 10 mins), the supernatant was aspirated and the pellet resuspended in 100 μl of NETTAM buffer and once again layered over a sucrose-NETTAM cushion. A total of three resuspension-wash-centrifugations were performed.

Following the final wash the Protein A pellet (containing bound antigen-antibody complexes) was resuspended in 100 μl of a 2.5% sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 65 mM Tris HCl pH 8.0 gel sample application buffer solution, heated at 95°C for two minutes, then pelleted at 10 000 g for 10 mins. The supernatant fraction thus obtained was further analysed using SDS polyacrylamide gel electrophoresis and fluorography (see 3.3 (v)).

3.3 (iii) Protein G

In some immuno-precipitation experiments goat antiserum was used. Polyclonal goat IgG is not bound by Protein A but Protein G, a cell surface protein derived from

Streptococcus Lancefield Group G, exhibits strong binding (Bjorck & Kronvall 1984; Akerstrom *et al.* 1985). Protein G (G-Sorbin, Zymed, No. 10-1351) was used as a substitute for Protein A in immuno-precipitation experiments using goat antisera. One ml of a 10% suspension of G-sorbin binds 2 mg of rabbit immunoglobulin.

3.3 (iv) Protein A-Sepharose

A second technique of immuno-precipitation using Protein A linked to sepharose CL-4B beads (Protein A-Sepharose, Sigma, Catalogue No. P-3391) was also employed and later became the standard method for all experiments in which quantitative measurements of apolipoprotein synthesis, secretion and degradation were made.

Measured quantities of cell lysates obtained following in vitro incubation of freshly isolated enterocytes from hamster jejunum with [^{35}S]-methionine were reacted with small volumes of specific antisera. Non-immune serum control reactions were also performed. 50 μl of cell lysate suspension were mixed with 150 μl of "NETTAM" (see 3.3 (ii)) containing 0.1% bovine serum albumin (BSA) and then reacted with 50 μl of antiserum. The cell lysate-antiserum reactions were carried out in 1.5 ml sealed Eppendorf microfuge tubes which were constantly rotated overnight (for around 12 hrs) at 4°C to allow for continuous mixing.

On the following morning Protein A-Sepharose was weighed out for the day's immuno-precipitations. 8 mg of Protein A-sepharose was used for each immuno-precipitation (IP). (Thus, for 4 IPs, 32 mg of Protein A-Sepharose was weighed out).
N.B.: The amount of Protein A-Sepharose that would be required for each IP was initially estimated from the following information:

(i) the average IgG serum concentration in mammals is 12.5 mg per ml (Range: 8-18 mg/ml). Thus, in 50 μl antiserum there would be around 0.625 mg = 625 μg IgG;

(ii) the binding capacity of swollen Protein A-Sepharose (as supplied by the manufacturers) was that 1 ml of swollen gel bound 20 mg of human IgG; i.e., 20 μg IgG would require 1 μl swollen gel.

Hence 625 μg IgG would require 31 μl swollen gel which is approximately equal to 8 mg of Protein A-Sepharose. The binding capacity was, however, only known in terms of human IgG although it might have been expected to be similar for rabbit IgG which was used in most of my immuno-precipitations. Experiments, however, using 12 and 16 mg Protein A-Sepharose instead of the standard 8 mg revealed no further increase in the amount of radio-labelled immuno-precipitated protein product on the subsequent fluorogram. In any case, the completeness of the IP was checked in all cases by attempting a re-immuno-precipitation of the relevant protein in the initial cell lysate supernatant obtained from the first IP (see Chapt. 3.4).

The weighed out Protein A-Sepharose was then swollen using 0.6 ml of the NETTAM IP Wash solution containing 0.1% BSA. The Protein A-Sepharose swelled to 4 times its original weight in volume (i.e. 8 mg swelled to 32 μl). The swollen Protein A-Sepharose was washed 3 times using NETTAM - 0.1% BSA IP Wash solution by brief centrifugation in a microfuge (10 000 g x 1 min) and careful removal of the supernatant by pipette aspiration so as not to disturb the Protein A-Sepharose pellet. The pellet was then resuspended in 0.6 ml of NETTAM - 0.1% BSA, and re-spun. The washed Protein A-Sepharose pellet was finally resuspended in a volume of NETTAM-BSA equivalent to that to which it originally swelled. 55 μl of the swollen Protein A-Sepharose (equivalent to 8 mg) was added to each lysate-antiserum mixture which had been incubating overnight; the contents were mixed by repeated flushing of the pipette tip in the solution, and the tubes were mixed by rotation at 4°C for 1½ hours. Thereafter the samples were spun in an Eppendorf microfuge at 10 000 g for 2 minutes at room temperature and the resulting pellets were washed 6 times in total - 3 times using NETTAM-0.1% BSA and, for the latter 3 washes, NETTAM in the absence of 0.1%

BSA. (The BSA was removed to avoid interference with the interpretation of the specific immuno-precipitated protein product on the subsequent SDS gel and fluorographic analysis). Finally, the washed pellets were resuspended in 100 μl of the gel sample application buffer (see 3.3 (ii) above), mixed, heated at 95°C for 2 mins, spun at 10 000 g for 3 minutes, and the supernatants (containing IgG, antigen/specific immuno-precipitated protein and Protein A) analyzed on SDS gels.

3.3(v) SDS Polyacrylamide gel electrophoresis and fluorography

The denaturation of samples for SDS polyacrylamide gel electrophoresis using the gel sample application buffer and the gel system used has already been described (see 3.1 (i)). A lane consisting of a set of Rainbow Molecular Weight standards (Amersham Rainbow^{TM} Protein MW Markers, Code RPN 756) was included on each gel. Following completion of the run, the 5-20% SDS-gradient gel was submerged and gently mixed in 2 solutions: destain I, a 50% methanol and 10% acetic acid solution, for 1 hour (with a change in the solution after 30 minutes), followed by destain II, (5% methanol, 78% acetic acid), overnight. On the following day the gel was rinsed in deionized, distilled water for 30 minutes to remove acetic acid prior to soaking the gel in a 1 M sodium salicylate solution for a further 30-45 mins (sodium salicylate, a fluor for converting the ß radiation from the ^{35}S-labelled protein atoms into light energy, precipitates in the presence of acetic acid).

The salicylate-treated gel was then dried onto a sheet of blotting paper or Whatman 3 M paper using a gel drying apparatus at a temperature of 60°C (Dual Temperature Slab Dryer Model SE1150, Hoefer Scientific Instruments, San Francisco) attached to a vacuum pump. The dried gel was allowed to cool before being tightly juxtaposed onto a Kodak X-OMAT AR Diagnostic X-ray film in a metal cassette in a photographic dark room. The sealed cassette or holder was placed in a deep freeze (-70°C) for 2 to 5 days.

The film was developed using a standard automated developing system in the Radiology Department of the Groote Schuur Hospital, Cape Town.

The fluorogram obtained was aligned with the original gel and the positions of the "Rainbow" MW markers which were present on the dried gel were traced onto the fluorogram. Where necessary further exposures of gel to film at -70°C were carried out for shorter or longer periods to obtain optimal fluorographic pictures.

N.B.: Gels intended for ^{35}S fluorography were generally not stained with Coomassie solution as this was found to cause significant quenching of radioactivity and thus poorer quantification (see 3.6(ii)).

3.4 DEMONSTRATION OF IMMUNO-PRECIPITATED PROTEIN PRODUCTS IN CELL LYSATES AND INCUBATION MEDIA

The general protocol for experiments performed to demonstrate the presence of specific immuno-precipitable protein products following the incubation of freshly isolated jejunal enterocyte sheets with [^{35}S]-methionine is summarized in figure 3.3. Details of the methods used in each step are referenced in the flow chart. Specific details regarding the duration of enterocyte incubation, the type of antiserum or antibody preparation used and the immuno-precipitation technique followed will be provided for each fluorogram presented.

3.4 (i) Apolipoprotein B-48

When freshly prepared jejunal enterocyte sheets were incubated in Krebs-Ringers-HCO_3 medium together with fuels, antibiotics and [^{35}S]-methionine (2 ml cell suspension & 15 μCi ^{35}S-methionine) and the subsequent radiolabelled protein products from the cell lysate and medium were reacted with a specific anti-apolipoprotein B antiserum, a single immuno-precipitation (IP) product of MW ~200kDa and corresponding to the known MW of apolipoprotein B-48 was observed. There was no protein product at the larger B-100 position (see Figs. 3.4 & 3.5). The immuno-precipitations were quantitative when a specific antiserum was used since no additional apolipoprotein B-48 could be re-immuno-precipitated from the cell lysate supernatant of the first IP and the apo B-48 band disappeared in the medium supernatant. The "neat" medium (not reacted with antiserum) gave rise to 2 additional bands of protein with MW's of ~44 & 28 kDa, respectively (Fig. 3.4). These corresponded to the MW's of apolipoproteins A-IV and A-I, both known to be products of intestinal synthesis and secretion.

In addition to the aforementioned proteins, immuno-precipitations using the rabbit 5 (or rabbit 2) antisera directed against all proteins associated with hamster plasma

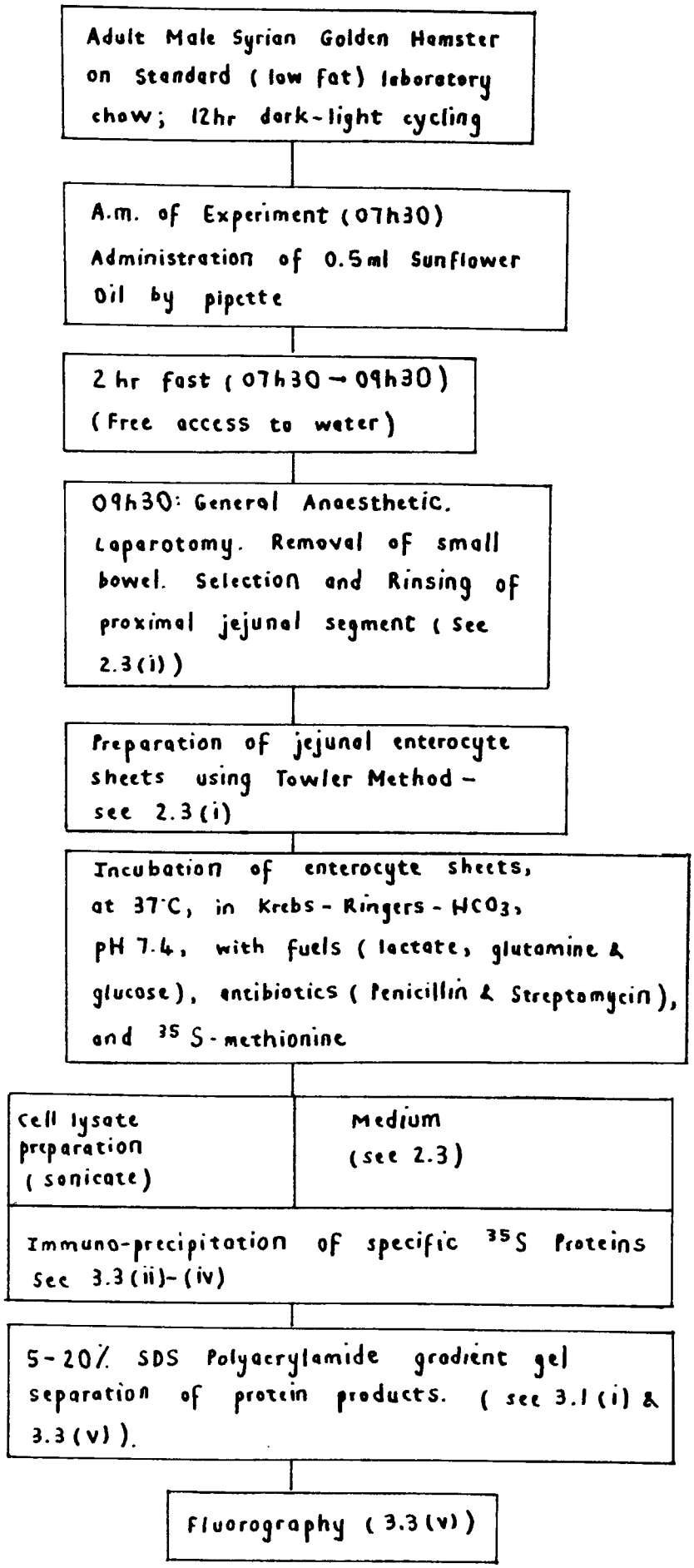


Fig. 3.3. General protocol used to demonstrate the presence of specific radiolabelled protein products in cell lysates and media from freshly isolated hamster jejunal enterocyte sheets incubated with ^{35}S-methionine. Details of the methods used are referenced in each step.

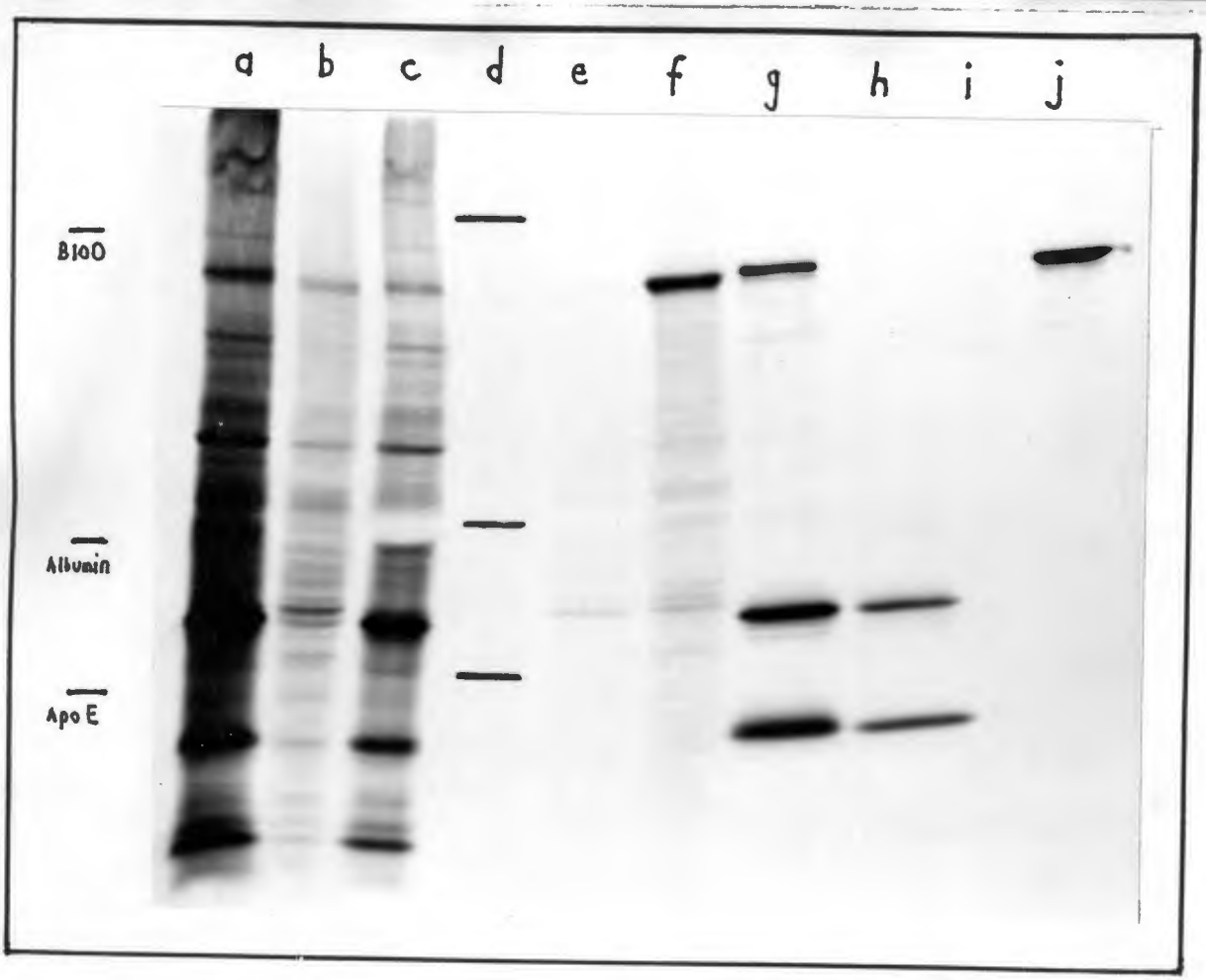


Fig. 3.4. Immuno-precipitation (IP) of apolipoprotein B-48 from a jejunal enterocyte lysate and medium sample.

The protocol outlined in Fig. 3.3 was followed. Freshly isolated jejunal enterocyte sheets were incubated in the presence of ^{35}S-methionine for one hour before the cells and media were separated and analyzed for apo B-48 using a polyclonal, rabbit-derived, anti-rat Apo B antiserum and Protein A as the precipitating agent.

Key to lanes:

a = Cell lysate, starting material for IP.

b = Cell lysate, pre-bound to Protein A ("clearing step").

c = Cell lysate from supernatant after IP.

d = Human VLDL standard. Traced lines from the Coomassie-stained gel show the positions of apolipoprotein B-100, albumin and apolipoprotein E (MWs 350, 67 & 35 kDa, respectively).

e = Cell lysate IP without specific antiserum (non-immune serum used).

f = cell lysate IP with anti-apoB antiserum, showing a single bond at MW ~ 200 kDa, = expected position of Apo B-48. No apo B-100 band.

g = medium, starting material for medium IP.

h = medium supernatant following specific IP, showing disappearance of the putative B-48 band, but retention of 2 bands at the expected positions of apo A-IV (MW ~ 44 kDa) and apo A-I (MW ~ 28 kDa), respectively.

i = medium IP with non-immune serum.

j = medium IP with anti-apo B antiserum showing single IP band corresponding to the expected position of apo B-48.

Fig. 3.5. IP of intestinal enterocyte apo B-48 using "OSAN", a commercially available anti-human apolipoprotein B antiserum (Behring Diagnostica), and IP of protein products using "rabbit 5" antiserum (prepared by immunizing a rabbit with floated hamster lipoproteins $\rho < 1.21$ g/ml).

The protocol of Fig. 3.3 was followed in the main. However, a pulse-chase method of cell incubation was used. Initially, the freshly prepared jejunal enterocyte sheets from a fat-fed hamster were incubated with ^{35}S-methionine for twenty mins. "Cold" methionine (in Krebs-Ringer -HCO_3) was then added to the incubation flasks to a final concentration of 2 mM. The flasks were re-gassed, re-sealed and the incubation continued for an additional sixty minutes. The Protein A-Sepharose method of IP was used.

Key to lanes:

a = cell lysate, t=20' incubation = 0' chase, IP with non-immune serum (NIS).

b = cell lysate, t=0' chase + "OSAN" IP.

c = cell lysate, t=60' chase, + "OSAN".

d = cell lysate, t=0' chase, + rabbit 5 (R5) antiserum.

e = cell lysate, t=60' chase, + R5 antiserum. Note bands at: MW ~ 200 kDa (apo B-48), MW ~ 54-64 kDa (see Chapt. 6), MW ~ 45 kDa (? apo A-IV) and at 28 kDa (? apo A-I).

f = MW standards (see RHS of figure for key)

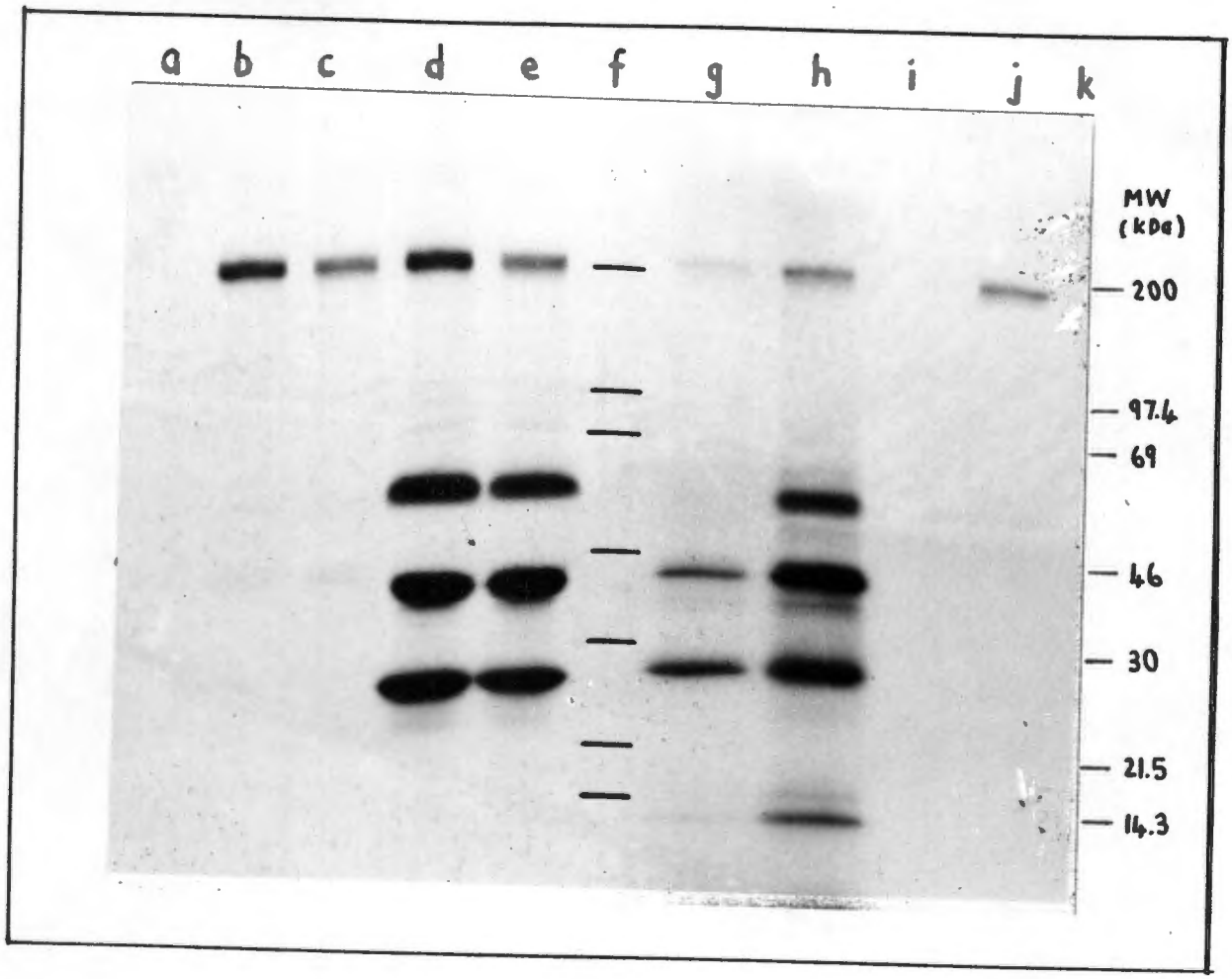
g = medium, t=0' chase ("neat" medium, no IP)

h = medium, t=60' chase ("neat")

i = medium, t=60' chase + IP with NIS

j = medium, t=60' chase + IP with "OSAN"

k = Re-IP of supernatant from IP lane *b* i.e. supernatant from 1st IP + "OSAN" IP. No additional protein precipitated.



3.5

lipoproteins of density < 1.21 g/ml, revealed an additional unknown fourth protein with a broad MW band of ~54-64 kDa (see Fig. 3.5). Further attempts at identification of this material will be discussed in chapter six.

Immuno-precipitations (IPs) using rabbit 5 were not entirely quantitative. About 80-90% of the apolipoprotein B-48 and the putative apolipoprotein A-I (MW ~28 kDa) were precipitated in the first IP (as opposed to the re-IP of the supernatant from the first IP). These relative quantities were assessed by densitometric scanning (see Fig. 3.6).

3.4 (ii) Apolipoprotein A-IV

The presence of this protein, suspected from the previous IPs using R5 antiserum (Figs. 3.5) and the appearance of the neat medium (Fig. 3.4), was confirmed using a specific rabbit-derived, anti-rat apolipoprotein A-IV antiserum. Conditions were also set for optimal quantitative IP of this protein from fat- and non fat-fed hamster enterocyte preparations (see Fig. 3.7).

3.4 (iii) Apolipoprotein A-I

The protein at MW ~28 kDa was specifically immuno-precipitated from the enterocyte lysate of a fat-fed hamster using a specific rabbit-derived, anti-rat apolipoprotein A-I antiserum. However, the quantity of protein obtained using the specific anti-AI antiserum was smaller than that obtained with the rabbit 5 antiserum although the MWs of both antisera corresponded exactly (see Fig. 3.8). The rabbit 5 antiserum was therefore deemed to be more quantitative (volume for volume) and in the re-IP experiment previously discussed (Fig. 3.6) was indeed shown to immuno-precipitate 80% of apo A-I in the first IP compared with a second re-IP.

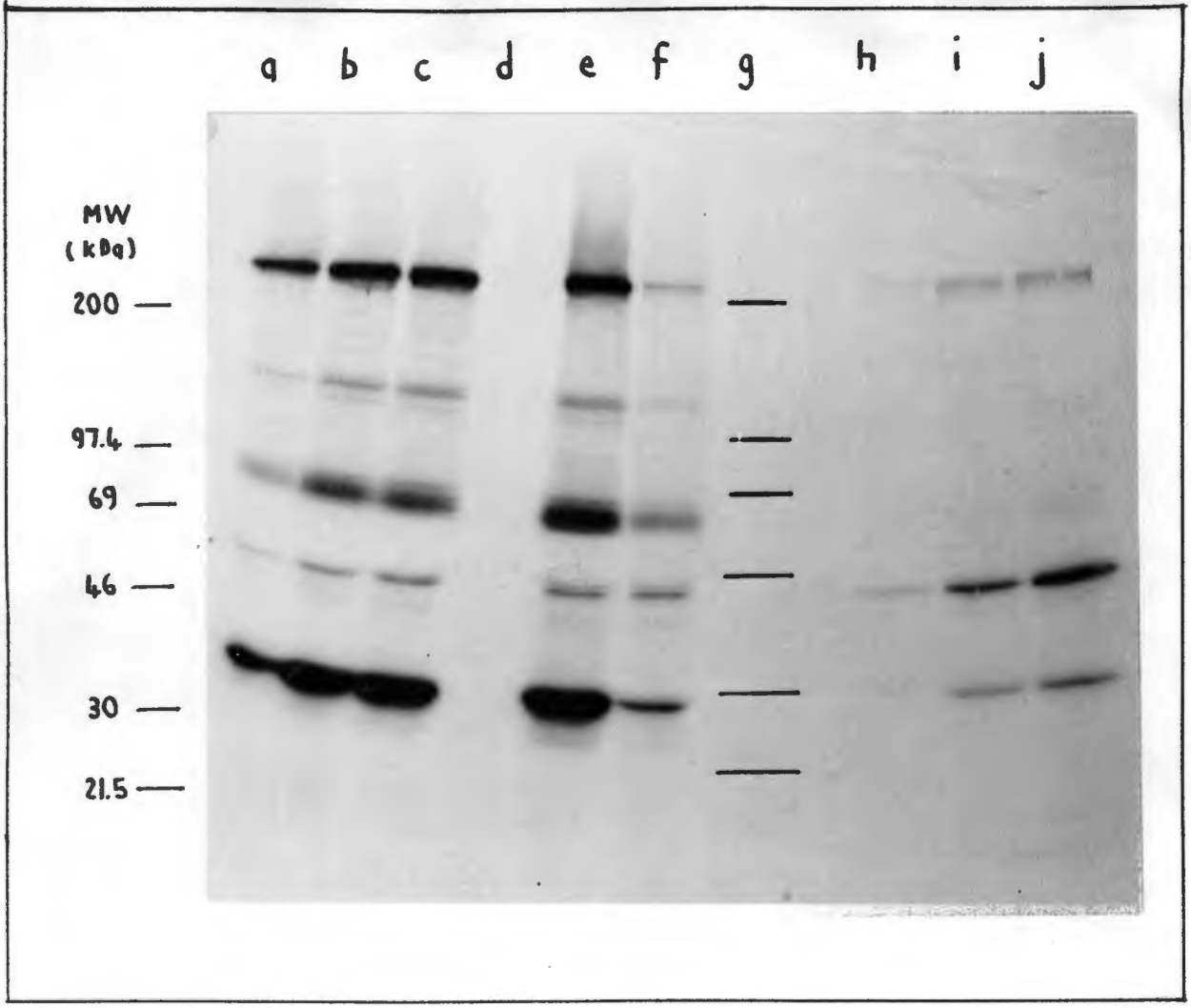


Fig. 3.6. IP using rabbit 5 antiserum of enterocyte and medium proteins following the incubation of jejunal enterocytes, prepared from a fat-fed hamster, with [^{35}S] methionine.

Incubation of 4 flasks of cells with analysis at 20', 40', 60' and 80' incubation time, respectively. Protein A-Sepharose technique of IP.

Key to lanes:

a = cell lysate + rabbit 5 (R5) antiserum, t=20'.
b = cell lysate + R5 antiserum, t=40'.
c = cell lysate + R5 antiserum, t=60'.
d = cell lysate + NIS, t=80'.
e = cell lysate + R5 antiserum, t=80'.
f = cell lysate, supernatant from IP (lane *e*), re-IP with R5 antiserum.
g = MW stds.
h = medium, t=20', "neat". (No IP).
i = medium, t=60'.
j = medium, t=80'.

The R5 IP was not quantitative at the 1st attempt, although 80-90% of the apoB (MW ~ 200 kDa) and apo A-I (MW ~ 28 kDa) immuno-precipitated, did so in the first IP (compare lanes *e* & *f*). The relative quantities precipitated in the 1st as opposed to the 2nd IP were assessed by densitometric scanning of the 2 lanes using a "Cliniscan" densitometer (Helena Laboratories), slit width of light beam, 8.5 mm, and wavelength = 610 nm.

Fig. 3.7. IP of Apolipoprotein A-IV.

Cell lysates were prepared according to the protocol of Fig. 3.3. However, both a low fat-fed animal (not administered sunflower oil on the A.M. of the experiment, but also previously on standard chow and also fasted for 2 hrs prior to anaesthesia) as well as an acutely fat-fed animal were used. The IP was performed using a rabbit-derived, polyclonal anti-rat apolipoprotein A-IV antiserum and the Protein A-Sepharose technique for immuno-precipitation.

Key to lanes:

a = cell lysate (starting material), low fat-fed animal, following a 20' incubation with ^{35}S-methionine.

b = cell lysate (starting material), fat-fed animal, following a 20' incubation with ^{35}S-methionine.

c = MW markers.

d = "*a*" cell lysate material + IP using 50 μl anti-A-IV antiserum.

e = "*b*" cell lysate material + NIS.

f = "*b*" cell lysate + IP using 25 μl anti-A-IV antiserum.

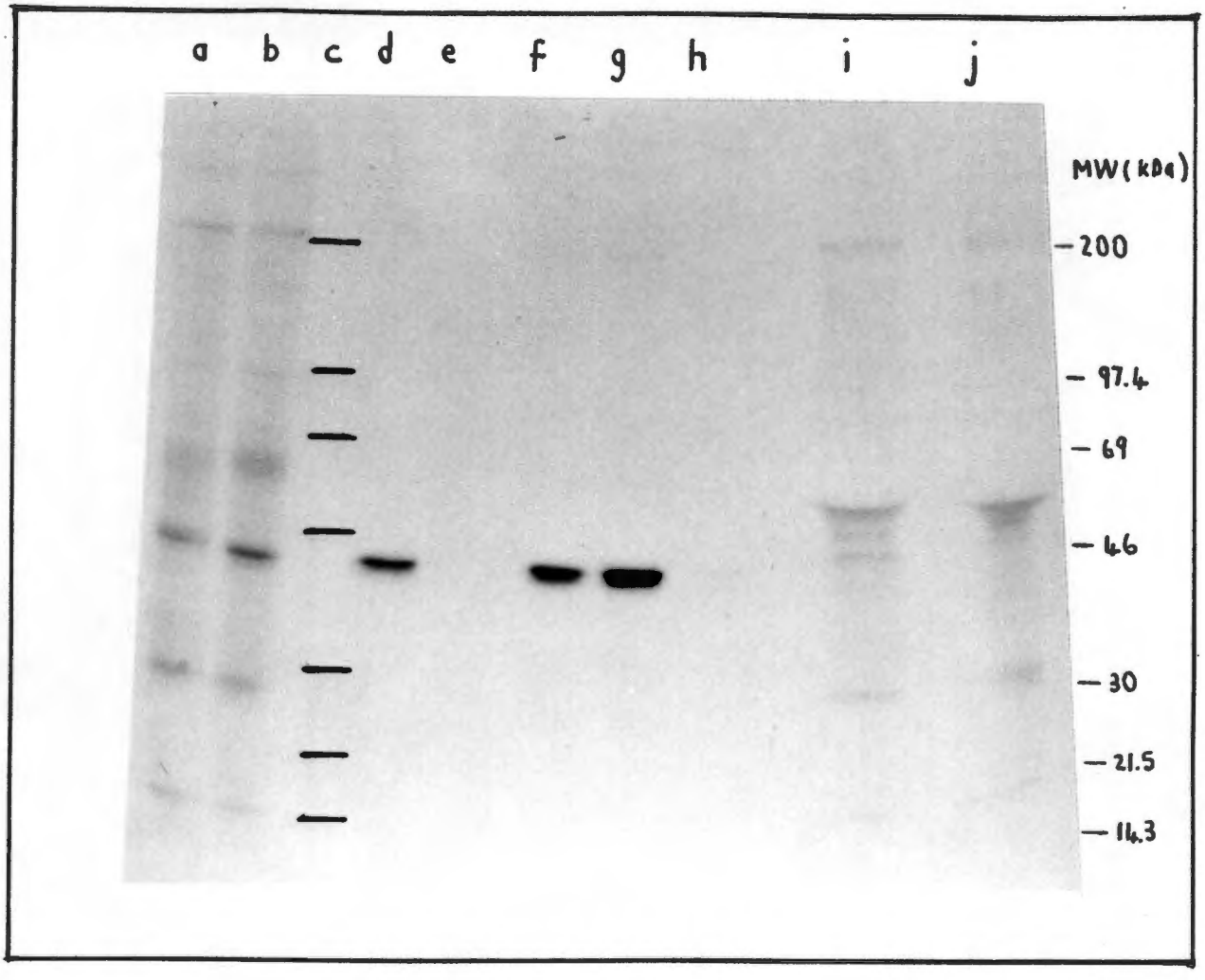
g = "*b*" cell lysate + IP using 50 μl anti-A-IV antiserum.

h = Re-IP of supernatant from "*f*" IP.

i = cell lysate supernatant from IP of lane "*d*".

j = cell lysate supernatant from IP of lane "*g*".

Note: (i) IP was quantitative when 50 μl anti-A-IV antiserum was reacted with 200 μl of the cell lysate preparation but was incomplete when 25 μl were used.
rom the whole cell lysate supernatant (compare lanes *a* & *i* and *b* & *j*, i.e. lysate material before and after IP).



3.7

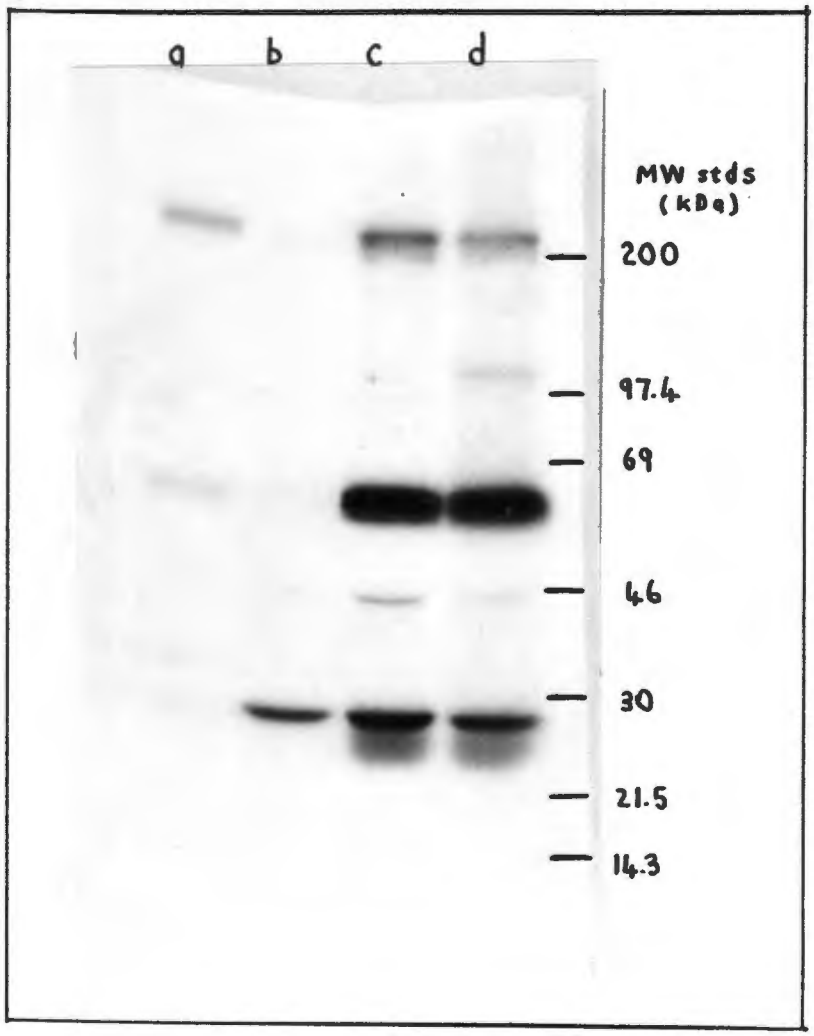


Fig. 3.8. IP of apo A-I using a polyclonal rabbit-derived, anti-rat apo A-I antiserum. Jejunal enterocyte sheets from a fat-fed hamster were incubated for 20' with ^{35}S-methionine (2 ml cell suspension and 15 μCi of ^{35}S-methionine).

IPs of the resulting cell lysate were carried out using:

a, a polyclonal, rabbit-derived, anti-rat apo B antiserum;

b, a polyclonal, rabbit-derived, anti-rat apo a-I antiserum;

c, "rabbit 5" antiserum (to floated hamster apolipoproteins $\rho < 1.21$ g/ml) and,

d, "rabbit 2" antiserum (also directed against total floated lipoprotein-associated hamster proteins $\rho < 1.21$ g/ml). While a specific band corresponding to the expected position of apolipoprotein A-I (MW ~ 28 kDa) was immunoprecipitated in lane *b*, the quantity of protein was much less than the band of corresponding MW obtained when rabbits 5 or 2 antisera were used (lanes *c* & *d*).

3.4 (iv) Conclusion

Freshly isolated hamster jejunal enterocytes synthesized apolipoproteins B-48, A-IV and A-I when incubated in vitro in a supportive medium containing [^{35}S] methionine as the radiolabelled precursor amino acid. Two of these proteins, B-48 and A-IV, could be quantitatively immuno-precipitated from the cell lysates prepared from such incubations; all three radiolabelled proteins were also clearly visible in the untreated media samples (i.e. without specific immuno-precipitation). A fourth labelled protein product is as yet unidentified (see below).

With the development of these specific techniques for quantifying the radiolabelled protein products, it now appeared possible to measure the relative quantities of apolipoproteins synthesized, secreted and degraded in the in vitro system following dietary perturbations performed on the intact animal during life. (See Chapter 4). Some subsidiary questions had first to be addressed, however.

3.5 BIOSYNTHETICALLY LABELLED APOLIPOPROTEINS IN THE INCUBATION MEDIUM: CELL LYSIS OR CELL SECRETION IN VITRO?

The appearance of proteins in the incubation medium of enterocytes may have been due to cell lysis or cell secretion. It is contended that the evidence presented thus far would favour a secretion rather than a lytic model.

Firstly, when incubation conditions were characterized and optimized (Chapt. 2.4), it was found, using assays of actate dehydrogenase and alkaline phosphatase in the cell lysates and incubation media, that only a tiny minority of cells died or were damaged in vitro. Such overall cell stability and integrity over a five-hour incubation period is unlikely to result in significant release of intracellular protein into the medium through cell lysis. If protein did appear in the medium under such conditions, physiological secretion rather than lysis would be a more likely explanation.

Secondly, the nature of the proteins released into the medium as demonstrated in the fluorograms of Chapt. 3.4 (especially Fig. 3.4) strongly suggests cell secretion. The process appears highly selective for 3 proteins which are known, in any case, to be secretory products of the mammalian small intestine - viz. the apolipoproteins B-48, A-IV and A-I. If lysis were operating the medium protein profile would be expected to resemble that of the whole cell lysate and perhaps to show increasing resemblance with increasing incubation times. Neither of these phenomena is seen in the fluorograms.

In order to definitively resolve the question of secretion versus lysis, 2 experiments (of which a representative example is presented here) were performed using sodium azide, a small water-soluble compound which inhibits oxidative phosphorylation by binding reversibly to cytochrome oxidase.

In 1988, R. Persson *et al.* showed that by incubating isolated rat hepatocytes (with ^{35}S-methionine) in the presence of graded concentrations of sodium azide (2 → 10 mM), they could proportionally arrest the transport of secretory proteins. By studying glycosylation patterns of the retained protein, they were able to show the steps in the secretion pathway (e.g. transfer of protein from medial to trans Golgi complex) which were more or less sensitive to ATP depletion. The use of sodium azide in their study had the additional advantage over other hydrophobic uncouplers of oxidative phosphorylation of not affecting cell membrane permeability as well. At 10 mM sodium azide concentration the secretion of albumin from viable isolated hepatocytes was completely blocked.

In my experiments, enterocytes were prepared from fat-fed hamsters according to the protocol in Fig. 3.9. (A non fat-fed animal not administered sunflower oil at 07h30 was included in the experiment as a control). 1 ml aliquots of the cell suspension from the fat-fed animal were incubated (each with 8 μCi ^{35}S-methionine) in small Erlenmeyer flasks. Following a twenty minute incubation period, cold methionine was added to 2 of the flasks (to a final concentration of 2 mM) and sodium azide, 10 mM final concentration, was added to one of the flasks. A further 60' "chase" incubation period followed. At the end of this period the cell lysates and media samples were analysed on SDS-polyacrylamide gels and fluorography. (No specific immuno-precipitations were carried out) The results are shown in Figs. 3.10A & B. The first fluorogram (A) was exposed for 1 day, the second (B) for 5 days.

In the incubation system "chased" with cold methionine but no azide, significant quantities of the apolipoproteins B-48, A-IV and A-I appeared in the incubation medium. However, in the system "chased" in the presence of 10 mM azide, much smaller quantities appeared in the medium (lane j versus lane i). In the azide-treated system apo A-IV even appeared to accumulate in the cells (compare the band in the A-IV position, lane f versus lane g in the fluorograms of Fig.3.10).

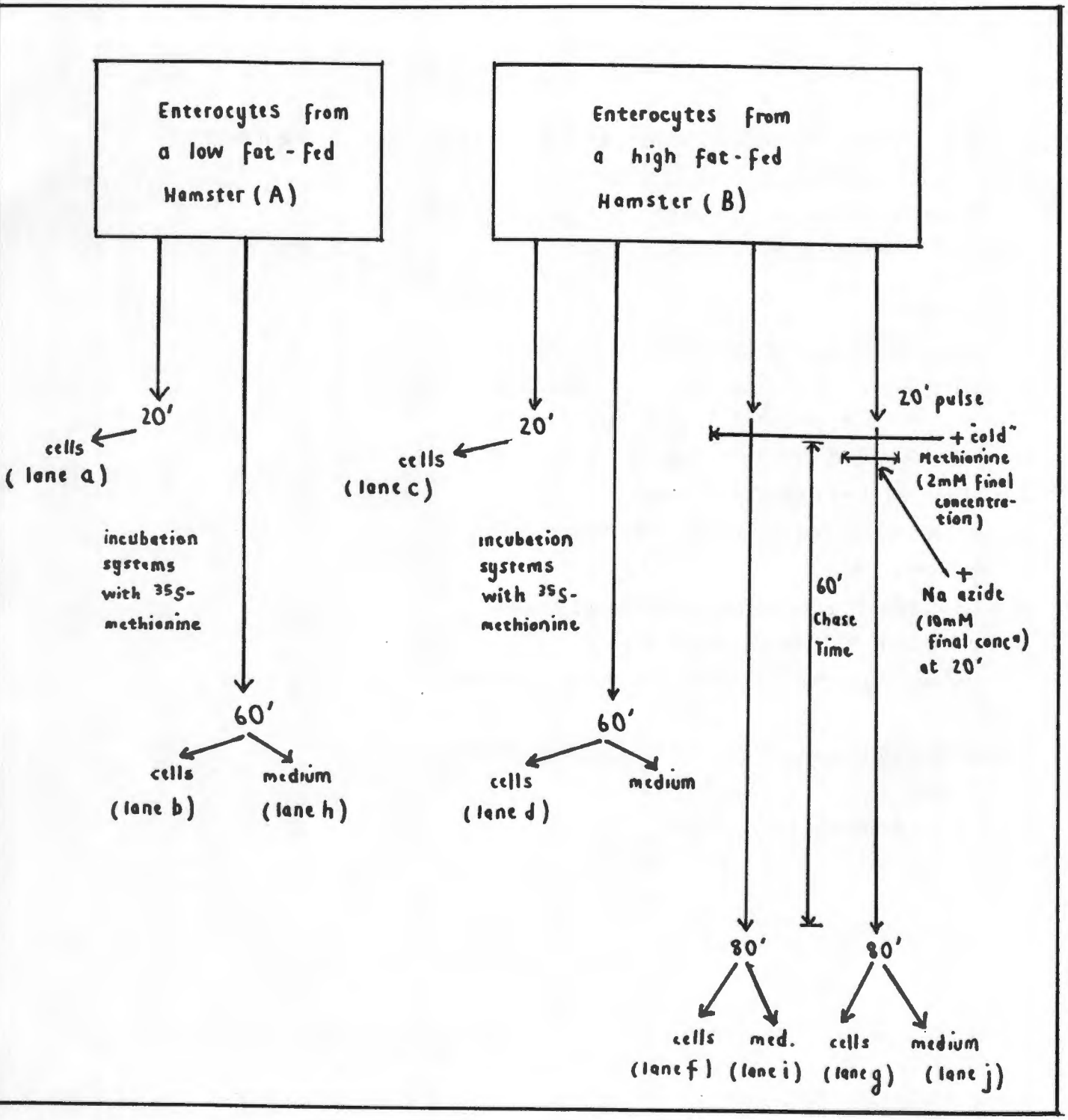


Fig. 3.9. Protocol for an experiment to determine whether sodium azide (10 mM final concentration) inhibited the release of apolipoproteins into the medium from incubated jejunal enterocyte sheets.

Fig. 3.10 A & B. Fluorograms showing azide inhibition of apolipoprotein release into the medium of incubated jejunal enterocyte sheets.

A. = fluorogram following 1 day exposure of gel to X-ray film.

B. = after 5 days exposure.

Key to lanes:

a = cell lysate, A, low fat-fed animal, 20' incubation

b = cell lysate, A, low fat-fed animal, 60' incubation

c = " " , B, high fat-fed " , 20' incubation

d = " " , B, " " " " , 60' incubation

e = MW stds. (Key on LHS of fluorograms)

f = cell lysate, B, after 20' pulse & 60' chase, in absence of azide

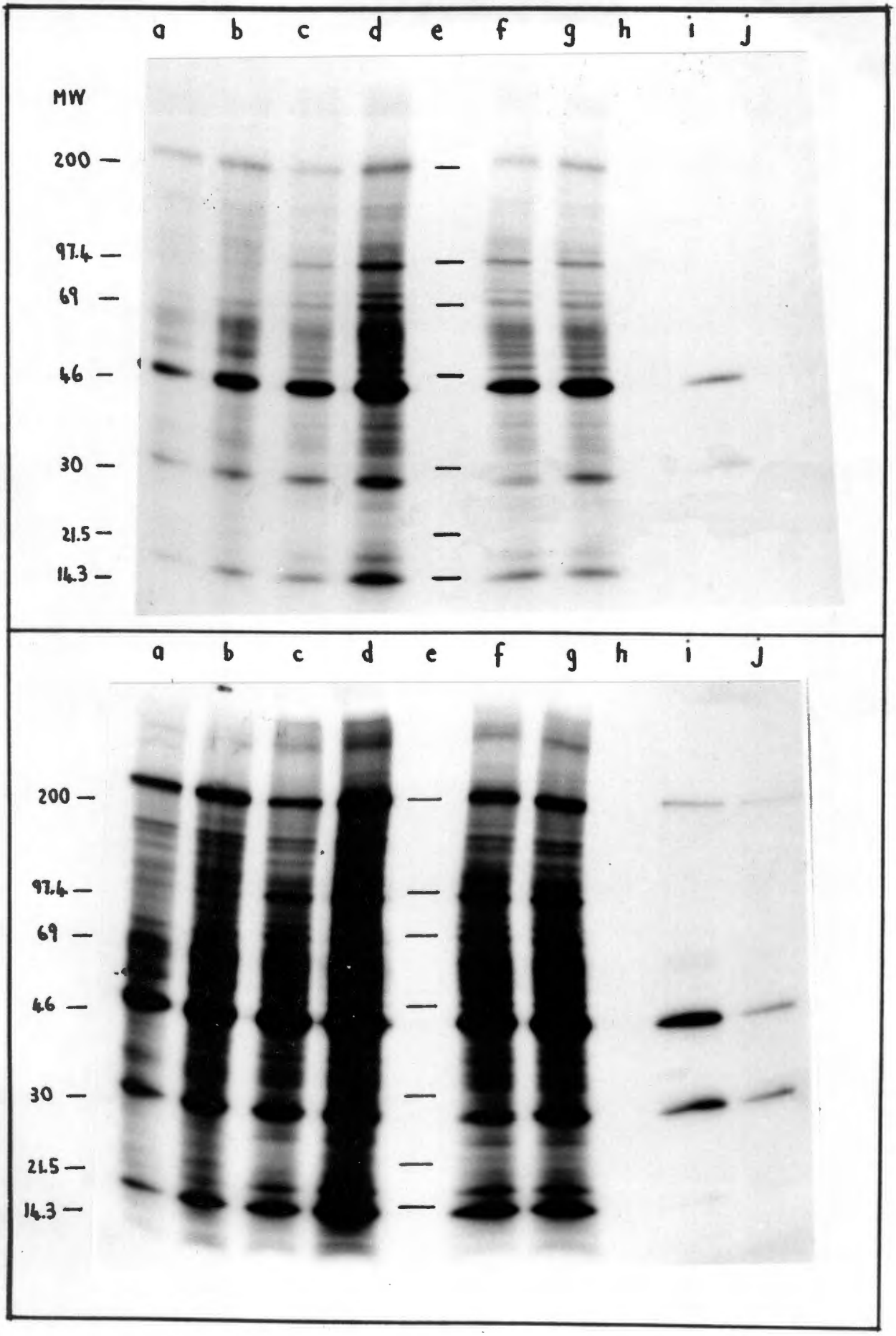
g = cell lysate, B, after 60' chase in presence of azide

h = medium of A, after 60' incubation

i = medium of B, after 20' pulse & 60' chase, in absence of azide

j = medium of B, after 20' pulse & 60' chase, in presence of azide.

See text for comments on the results.


a b c d e f g h i j
MW
200
97.4
69
46
30
21.5
14.3

3.10 A

a b c d e f g h i j
200
97.4
69
46
30
21.5
14.3

3.10 B

This result shows that the release of proteins into the media of my enterocyte suspensions was azide-sensitive and, therefore, ATP-dependent. This is in keeping with what is known about physiological secretion (Persson *et al.* 1988). Cell lysis is not ATP-sensitive and is, in fact, likely to be exacerbated by ATP depletion.

Finally, additional evidence for secretion as opposed to lysis is also provided by the comparison of the media of the fat-fed versus the non fat-fed hamster enterocytes (lanes h and i, Fig. 3.10): the medium of the non fat-fed animal's enterocyte suspension contained no visible proteins even on the more exposed fluorogram; the medium from the fat-fed animal's cells contained prominent apolipoprotein bands. Thus the appearance of apolipoproteins in the medium appeared to depend on whether fat loading or fat absorption had taken place or not and this is consistent with some of the evidence (presented in Chapt. 1.8) of increased apolipoprotein secretion with lipid feeding. (The question of the effects of high versus low fat diets on apolipoprotein synthesis, secretion and degradation will be more comprehensively addressed in Chapter 4).

In conclusion, it can be assumed that incubated jejunal enterocytes synthesized and secreted radiolabelled apolipoproteins into the incubation medium.

3.6 TECHNIQUES FOR QUANTIFYING THE RADIO-LABELLED, IMMUNO-PRECIPITABLE, PROTEIN PRODUCTS

Although complete immuno-precipitation of apolipoproteins B-48 and A-IV was demonstrated, an important aim of this project was to perform quantitative comparisons of the synthesis, secretion and degradation of the apolipoproteins under various dietary perturbations in different animals. Since the incorporation of amino acids by different preparations of enterocytes was unpredictably variable, a more precise expression of the results of such experiments was required, and a way of standardizing the results was needed in order to make valid comparisons of different animal experiments.

(i) Trichloro-acetic acid precipitation (TCA)

Since the immuno-precipitation of apolipoproteins B-48 and A-IV (using specific antisera) yielded single protein bands on the fluorograms, one method of quantifying the amount of radiolabelled protein immuno-precipitated would simply be to count the TCA-precipitable, immuno-precipitable radioactivity in the final (resuspended) Protein A (-Sepharose) pellet. So, following solubilization (including heating and spinning) of the Protein A-Sepharose pellet in gel sample application buffer (see Chapt. 3.3(ii)), a small quantity - usually 10 μl - was applied (in duplicate) onto filter discs and the TCA-precipitable, immuno-precipitated radioactivity determined using the method of Mans and Novelli (1961) (see Chapt. 2.3 section (iii)). TCA-precipitable, IP-radioactivity from non-immune serum control IPs was subtracted from the TCA-precipitable IP radioactivity of specific protein IPs to give the true IP counts. Results were then related to the total TCA precipitable radioactivity of the system (cells and media).

Thus: percentage synthesis of a specific apolipoprotein

$$= \frac{\text{immuno-precipitated, TCA-precipitable dpm in cells and media (expressed per } \mu g \text{ cell protein) at Time } x}{\text{Total TCA-precipitable radioactivity (cells \& media) per } \mu g \text{ cell protein at Time } x} \times 100$$

The synthesis, of a particular protein was therefore expressed in terms of its abundance relative to the total quantity of radioactive protein synthesized. In all instances the protein dpm were normalized to the measured amount of cell protein in each incubation, since the quantity of cell protein actually incubated (i.e. number of cells incubated) varied from animal to animal and from flask to flask in the same animal experiment (although variation in the latter was far smaller than that of the former). The method, therefore, of direct measurement of IP radioactivity by TCA-precipitatioan of the final IP material was valid as long as fluorographic analysis could demonstrate a "clean" IP of a single protein. It could not be used, for example, when rabbit 5 antiserum (which immuno-precipitated multiple apolipoproteins and lipoprotein-related proteins) was used.

3.6 (ii) Quantification using the fluorogram

This technique was more tedious than the direct TCA precipitation method but was nevertheless useful when multiple proteins were present in the same sample or gel lane, e.g. analysis of proteins in the media or the products of "rabbit 5" immuno-precipitations. It depended on the establishment of a linear relation between the amount of radioactivity present in the protein and the degree of "blackness" imparted onto the X-ray film (i.e. the amount of silver deposited by the ß radiation from the ^{35}S protein and its conversion to light energy in the presence of the fluor, sodium salicylate). The original method for eluting silver grains from autoradiograms and quantifying the amounts by spectrophotometry was described by Suissa in 1983.

Standardization was achieved in the following way: a solution of ^{14}C-methylated α-casein was obtained from Dr Jane Arnold. This protein sample was separated on duplicate SDS-polyacrylamide gels with increasing amounts of the protein loaded in each of 5 lanes (e.g. lane *a*, 10 μl; lane *b*, 20 μl, lane *c*, 50 μl, etc.). One of the gels was stained with Coomassie-blue and dried. The casein appeared as a double band at around MW ~32 kDa. The double band from each of the five lanes was excised and completely oxidized using a Packard Tricarb Sample oxidizer. The $^{14}CO_2$ released was trapped using a strong alkali solution (Carbosal, Packard) and the total radioactivity obtained counted in a Packard Liquid Scintillation Counter using a scintillation system (Permafluor V, Packard) for ^{14}C. A blank segment of the dried gel not stained with Coomassie was also excised, oxidized and used as a background subtraction.

The second (duplicate) gel was not stained with Coomassie-blue since staining was found to reduce the density of the bands on the fluorogram by 25% compared with an unstained gel. The gel was fixed, dried and a fluorogram of the radio-labelled ^{14}C-casein bands was obtained. The film was cleaned with 96% ethanol, dried and wiped with an antistatic cloth. The darkened bands on the film (corresponding to the Coomassie-stained bands from the first gel) were excised, weighed and submerged in a solution consisting of 1 ml of 1 M NaOH for at least 3 hours in order to elute the silver from the film slices (see Fig. 3.11). An appropriate "blank" portion of the fluorogram (corresponding to the background darkness of the film) was treated in the same way and served as the blank subtraction.

Once all the silver grains had eluted from the film slice, glycerol was added to the solution to a final concentration of 30% and the sample mixed well before the absorbance of the solution was measured in a spectrophotometer at 500 nm. Absorbance readings were all corrected for the mass of fluorographic slice excised. A standard graph was then plotted of the radioactivity in the excised, oxidized gel bands versus the

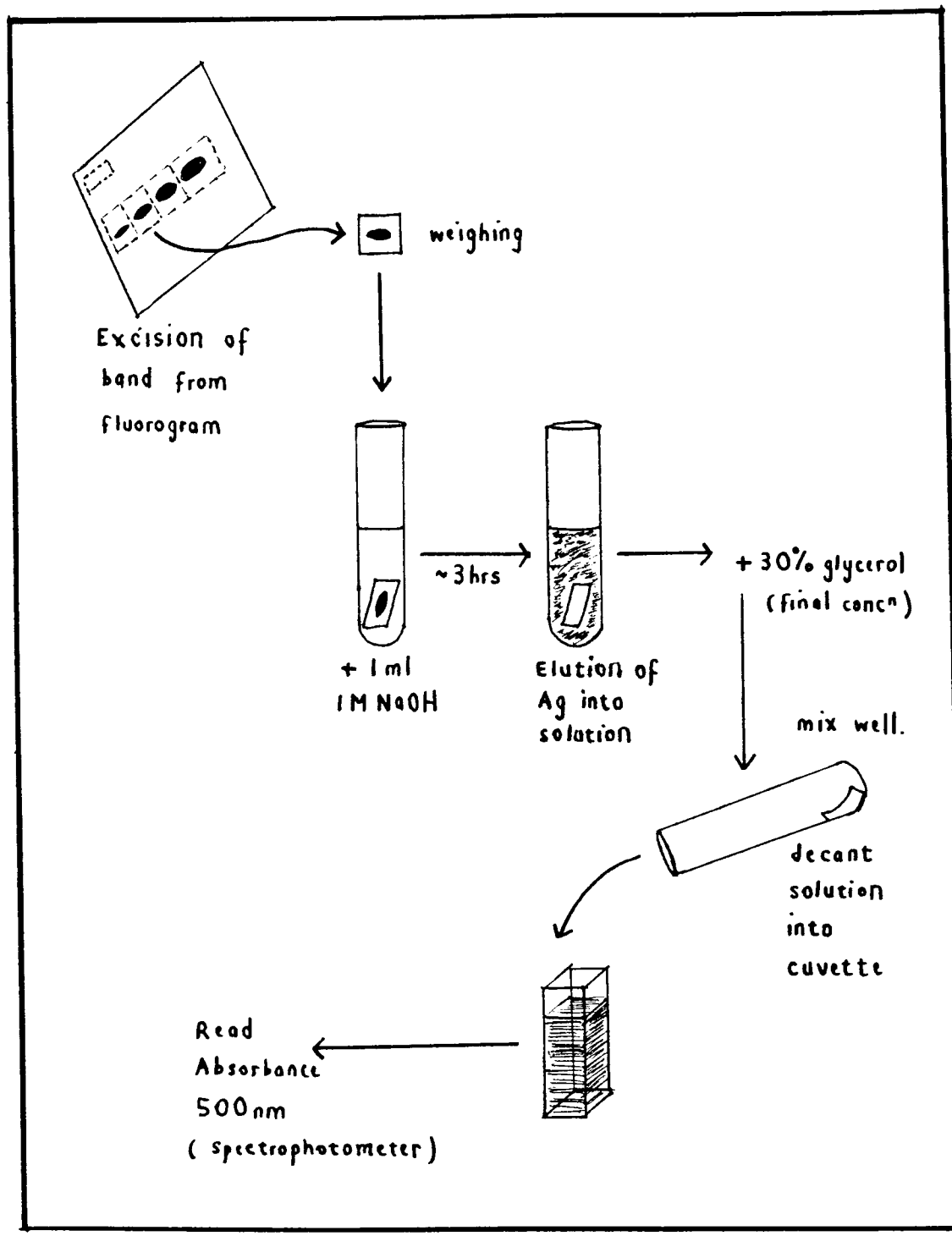


Fig. 3.11. Quantification of fluorographic (protein) bands by elution of silver using 1 M NaOH. A "background" area of the fluorogram was similarly processed to give the "blank subtraction".

absorbance obtained from excision and silver extraction of the corresponding bands on the fluorogram (see Fig. 3.12). The relationship was linear from around 1000 to 10 000 dpm of ^{14}C protein. Thus, in subsequent experiments in which this method of quantification was used, the second dried, unstained gel strip containing the 5 ^{14}C casein bands was exposed to the same X-ray film and for the same duration as the gel from the experiment. By excising the bands on the fluorogram of the proteins of unknown radioactivity from the experiment as well as the fluorographic bands of the standard ^{14}C-casein samples, the radioactivities in the experiment could be derived fromm the standard graph.

This method was valid provided the radioactivity in the experiment proteins fell within the range of radioactivity of the standard casein bands.

N.B. A ^{14}C labelled protein was chosen as the standard for 2 reasons: firstly, ^{14}C has a similar energy of radiation as ^{35}S which was used in the experiments (both are "soft" ß-emitters, i.e. decay by negatron emission and are counted using the same "window" settings in a liquid scintillation counter). Secondly, ^{14}C is more stable than ^{35}S ($t_{½}$ ^{14}C = 5760 years compared with ^{35}S, 87.2 days). So the ^{14}C standards would be expected comfortably to last for the duration of the project.

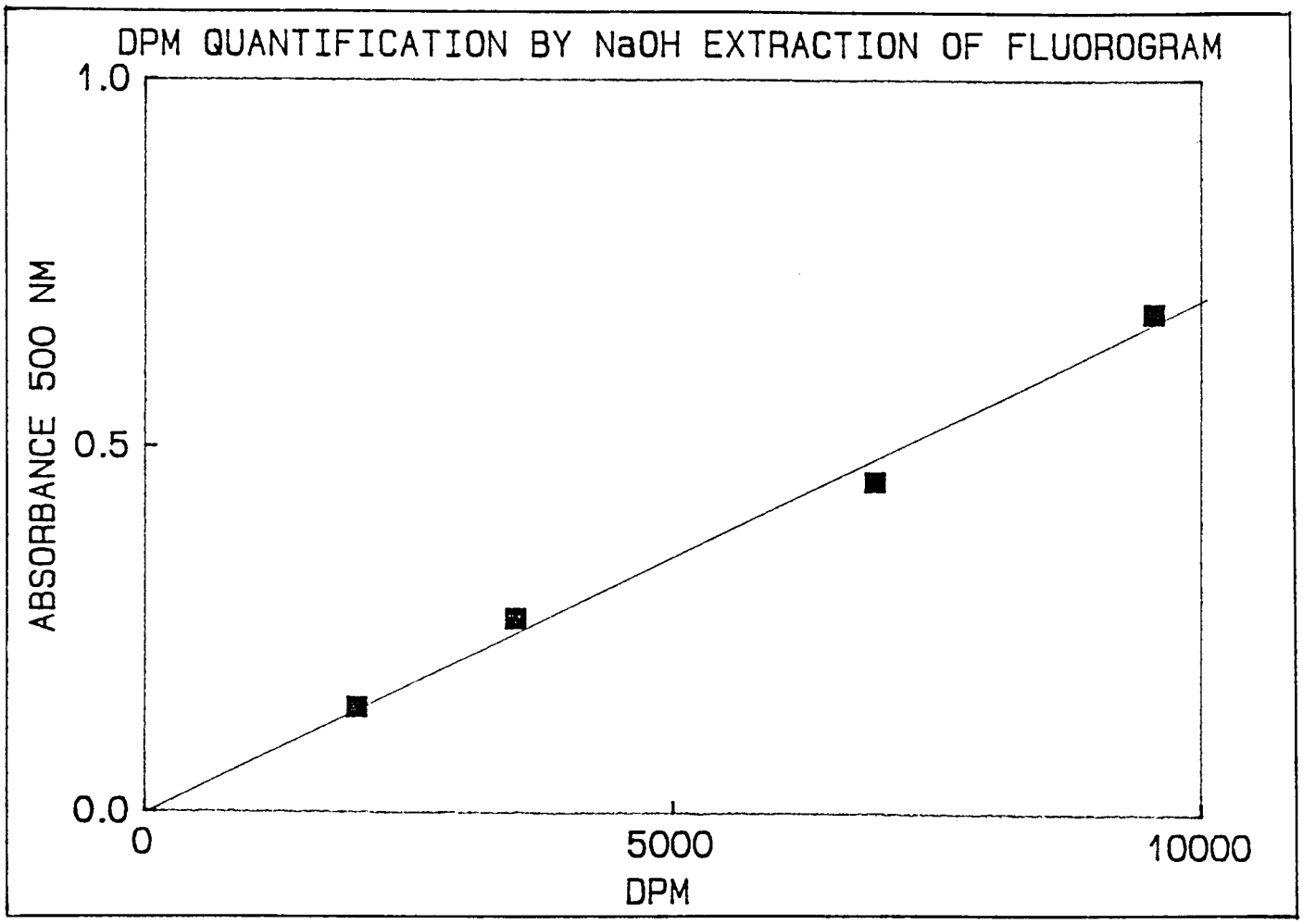


Fig. 3.12. The linear relationship between the quantity of ^{14}C radioactivity in the protein standard (^{14}C-casein) and the amount of silver eluted from the corresponding fluorographic band.

3.7 LIPOPROTEIN-ASSOCIATED VERSUS "FREE" APOLIPOPROTEIN SECRETION

Radio-labelled apolipoproteins have thus far been immuno-precipitated from Triton X-100 solubilized cell homogenates or from whole (neat) media samples. No attempt was made to determine whether the apolipoproteins in the media were present in a free form or in association with lipoprotein particles. While it would seem unlikely that the large relatively hydrophobic apolipoprotein B-48 molecule could exist in any form other than in association with lipid (i.e. as part of a lipoprotein), it is possible that the smaller apolipoproteins A-IV and A-I may have been secreted as free apoproteins.

Following a sixty minute pulse-labelling of jejunal enterocytes derived from an acutely fat-fed hamster with ^{35}S-methionine, the medium was removed, adjusted to a density of 1.25 g/ml using KBr (according to the formula, section 3(ii)), and underlayered in a 5 ml Beckman SW 65 Ti centrifuge tube containing saline-EDTA (ρ = 1.006 g/ml) (see Fig. 3.13B, "lipoprotein flotation ρ < 1.006 g/ml"). The sample was then centrifuged at 100 000 g (38 000 rpm, SW 65 Ti rotor) for 18 hours at 15°C in a Beckman L5-65 ultracentrifuge machine. At the end of the centrifugation the floated material (ρ < 1.006 g/ml) was aspirated from the top of the tube.

Both the aspirated material (ρ < 1.006 g/ml) as well as the infranatant (ρ > 1.006 g/ml) were then denatured using a gel solubilizing buffer (see section 3.3(v)), heated for 2 minutes, cooled, spun, and the supernatant (solubilized proteins) separated on a 5-20% gradient SDS reducing polyacrylamide gel electrophoresis. Coomassie blue staining of the dried gel showed no bands corresponding to the expected MW positions of apolipoproteins B-48 (~200 kDa), A-IV (~44 kDa) or A-I (MW ~ 28 kDa), respectively, in both the > 1.006 and < 1.006 g/ml density fractions. Fluorographic analysis (see 3.3(v)), however, demonstrated the 3 bands in both fractions of the media. The pattern

of bands in the fractions was similar to that shown in fig. 3.4 (lane *g*) with apparently more material in the infranatant compared with the floated material.

This single experiment indicated that the apolipoproteins B-48, A-IV and A-I were found in the medium associated with particles both denser and less dense than 1.006 g/ml. Material less dense than 1.006 g/ml would be expected to comprise chylomicrons and intestinal VLDL while that denser than 1.006 g/ml would include nascent intestinal HDL (see review, section 1.4) as well as free apolipoproteins. The experiment design did not permit differentation between free apolipoproteins and apolipoproteins associated with particles greater than ρ 1.006 g/ml.

It is possible that HDL-like lipoprotein particles may arise during the centrifugation process through "budding off" from large chylomicrons. Apolipoproteins may therefore have been found in denser lipoprotein particles that were artefactually produced. Apolipoprotein B-48 has also been described as a constituent apoprotein of nascent HDL (Hamilton *et al.* 1976). The origin of apolipoproteins in the denser than 1.006 g/ml fraction is therefore unclear.

3.8 ATTEMPTED ISOLATION OF INTRACELLULAR LIPOPROTEINS

A tight-fitting Dounce homogenizer was used in the preparation of an initial jejunal homogenate from an acutely fat-fed hamster (see protocol, Fig. 3.13A). Techniques of differential centrifugation were then employed to prepare microsomal fractions. These were based on standard methods previously developed for preparing subcellular fractions from rat liver homogenates (Griffiths 1981) but were also adapted for use in enterocytes (e.g. Magun *et al.* 1988; Mahley *et al.* 1971).

In releasing the microsomal contents by disruption of the microsomal membranes, 2 methods were attempted: (1) a chemical method, utilizing Na_2CO_3 at high alkaline pH, to dissolve the microsomal membranes, first described by Fujiki *et al.* in 1982 and utilized by Howell & Palade (1982) to resolve hepatic Golgi fractions into membrane and content subfractions; and (2), sonication, to resuspend and disrupt the spun microsomal pellet (on ice). While the latter method appeared more likely to result in damage to the lipoprotein particles, I found that the microsomal pellet remained firmly stuck down to the bottom of the ultracentrifuge tube following the 100 000 g spin for 1 hour when using the Na_2CO_3 technique alone, with insufficient material available for subsequent column chromatographic analysis. In practice, a combination of Na_2CO_3 treatment and sonication was used (Fig. 3.13A).

The released material (microsomal content fraction) was separated from the microsomal membranes, and material less dense than 1.006 g/ml or 1.21 g/ml was prepared by flotation-ultracentrifugation (Fig. 3.13B). The floated material was subsequently analysed by gel exclusion column chromatography (Rudel *et al.* 1986). A 4% cross-linked agarose column (Sepharose CL-4B, Pharmacia) was used. It measured 30 cm in length, with an internal diameter of 0.7 cm, a cross-sectional area of 0.385 cm^2, a bed volume of 12 cm^3, and a 35 μm exclusion filter at the base (Biorad 838-1252). The elution buffer ("SEAP") comprised NaCl 0.015 M, NaH_2PO_4 0.002 M, NaN_3 0.01 g%

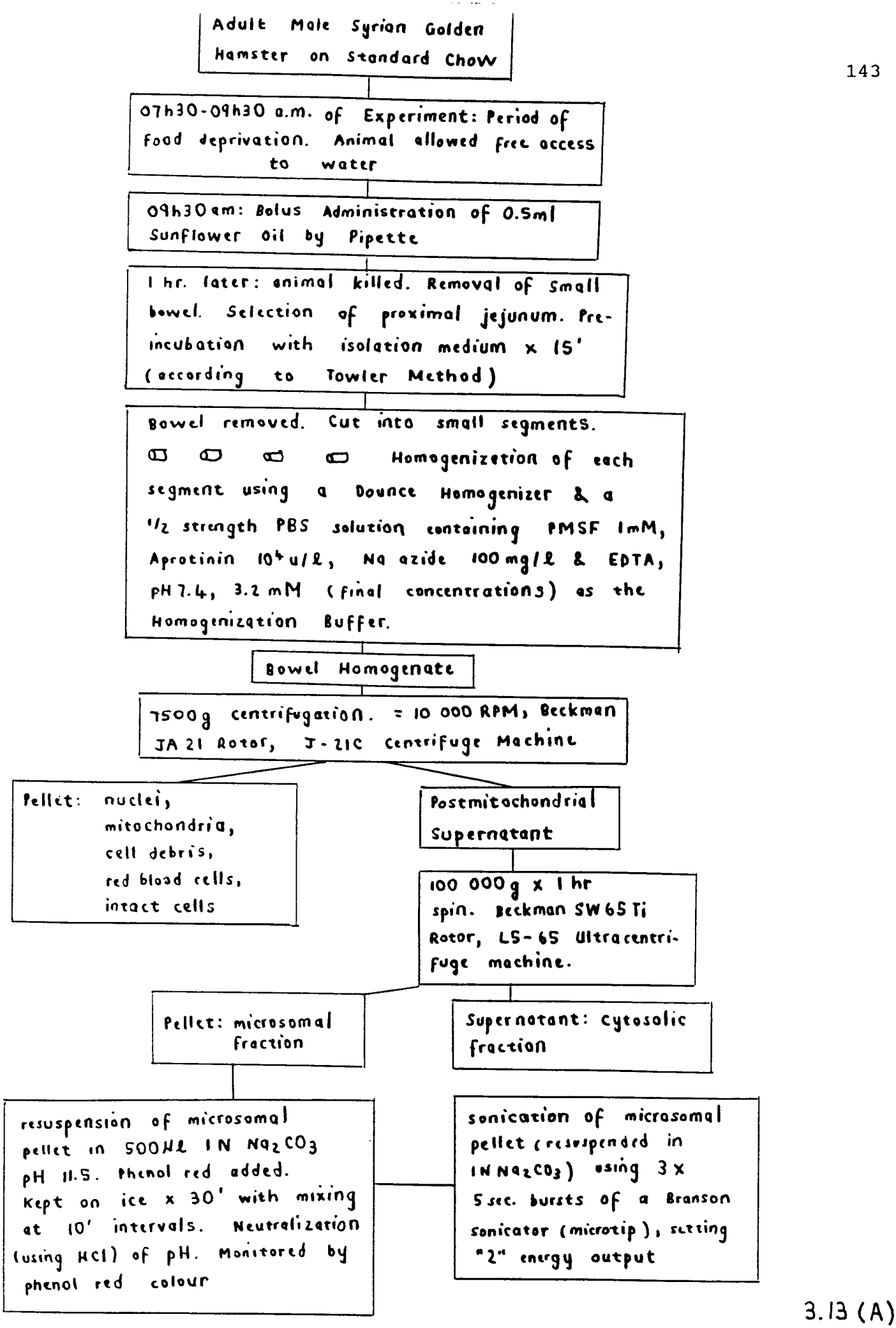


Fig. 3.13. (A & B). Protocol for the preparation of nascent lipoproteins from jejunal enterocytes of a fat-fed animal

Resuspended microsomal pellet
(sonicated)

10 000 RPM x 10' spin (= 7500g)
Beckman JA-21 rotor, J-21C
Centrifuge Machine

Pellet:
microsomal
membranes

Supernatant:
containing released
microsomal contents

For
lipoprotein
flotation
$\rho < 1.21$:

Density of supernatant adjusted
from $\rho = 1.006$ to 1.25 using
KBr & formula (see section 3.1)
underlayered thus:

KBr-EDTA solution: $\rho = 1.21$

released microsomal contents $\rho = 1.25$

100 000 g = 38 000 rpm spin. SW 65 Ti rotor (k factor = 63)
LS-65 Ultracentrifuge (Beckman) x 44hrs
(Temp = 15°C)

For
lipoprotein
flotation
$\rho < 1.006$ g/ml:
("VLDL"/ chylo-
microns)

Saline-EDTA: $\rho = 1.006$

released microsomal contents $\rho = 1.25$
(for underlayering)

100 000 g = 38 000 rpm spin. SW 65 Ti rotor x 18 hrs
LS-65 Ultracentrifuge (Beckman)
(Temp = 15°C)

End of Centrifugation: pipette aspiration of floated material ($d < 1.006$ OR < 1.21 g/ml)

→ analysis of floated material on a 4% cross-linked Agarose Column (sepharose CL-4B, Pharmacia).

3.13(B)

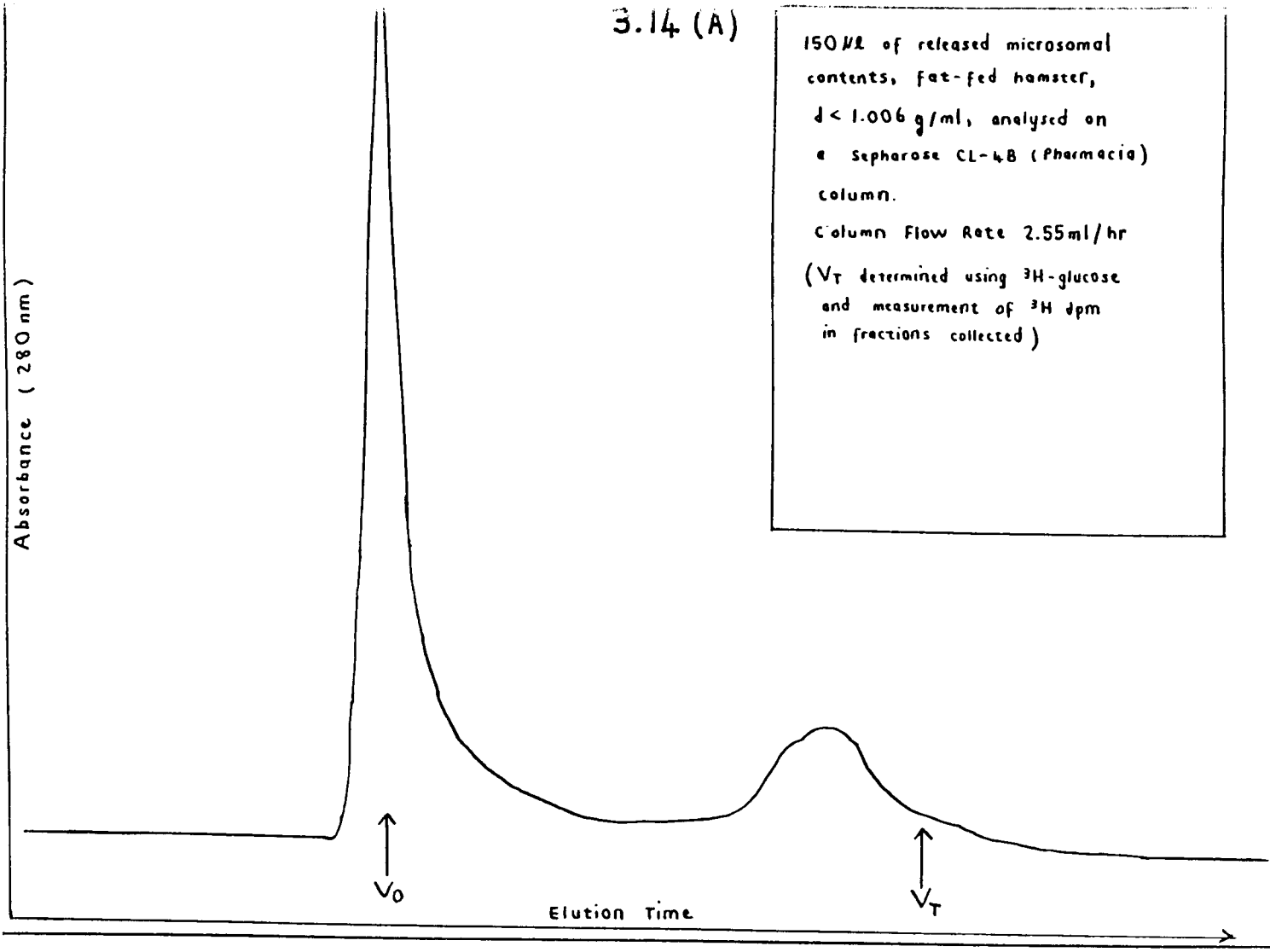
and EDTA, 3.2 mM, pH 7.34. The column was run using hydrostatic pressure with a flow rate of 2.55 ml/hr. A sample volume not exceeding 1% of total volume (V_T) was loaded for each run and the absorbance of the eluent was recorded using an ultraviolet monitor (LKB 2158 Uvicord SD monitor and recorder) at 280 nm. Fractions were collected at 15 minute intervals.

The typical column profile of floated material ($\rho < 1.006$ g/ml) derived from jejunal microsomes of a fat-fed hamster is shown in Fig. 3.14A. A large peak corresponding to the void volume (V_O) of the column and a much smaller peak close to the total volume (V_T) are seen. Chylomicrons and VLDL (MW $> 10 \times 10^6$ daltons) would be expected to elute in the V_O and the apparently high absorbance of this peak is likely to relate to the light-scattering properties of the particles rather than to a true absorbance. The second peak near the V_T of the column (MW $\leq$ 85 000 daltons) could be caused by free apolipoproteins as well as other proteins such as albumin which may adhere to the chylomicrons.

The second column profile (Fig. 3.14B) of jejunal microsomal contents ($\rho < 1.21$ g/ml) from a fat-fed animal has been compared with a superimposed profile of hamster plasma lipoproteins ($\rho < 1.21$ g/ml) prepared from the same animal. (The plasma HDL peak was identified by running purified ^{125}I-labelled human HDL through the same column under the same conditions as for the hamster plasma and microsomal contents, and measuring the ^{131}I radioactivity in the eluted fractions). Two peaks are seen on the second profile (Fig. 3.14B): one, a small peak coinciding with the V_O (MW $> 10 \times 10^6$ daltons), and a second larger peak nearer the V_T but smaller than the peak for hamster plasma HDL. This peak could represent nascent intestinal HDL (HDL_n), free apolipoproteins, or other "contaminating" proteins that are known to attach to HDL (e.g. cholesteryl ester transfer protein or CETP, & albumin).

3.14 (A)

150 µl of released microsomal contents, fat-fed hamster, $d < 1.006$ g/ml, analysed on a Sepharose CL-4B (Pharmacia) column.
Column Flow Rate 2.55ml/hr
(V_T determined using ^3H-glucose and measurement of ^3H dpm in fractions collected)

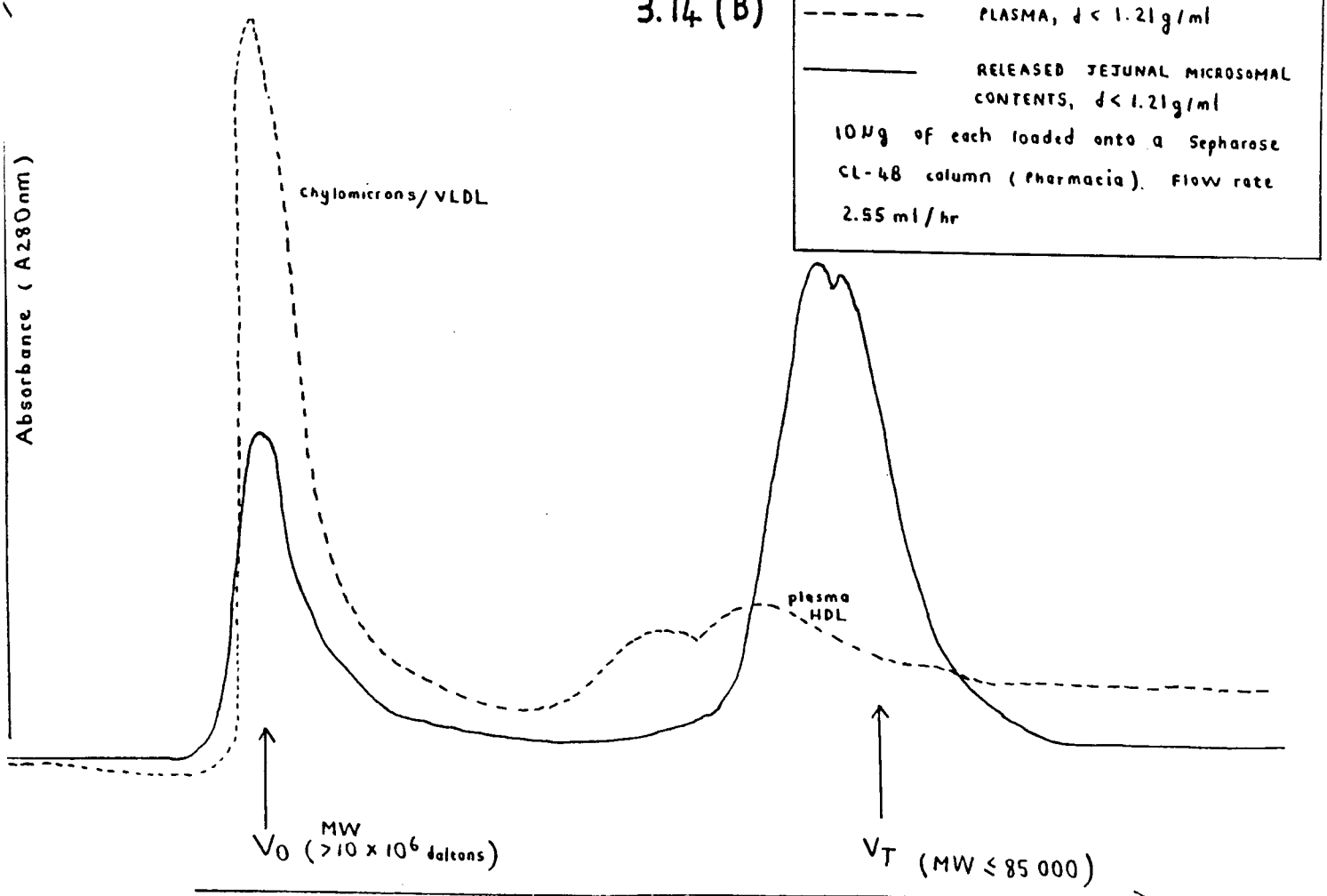


3.14 (B)

------ PLASMA, $d < 1.21$ g/ml

——— RELEASED JEJUNAL MICROSOMAL CONTENTS, $d < 1.21$ g/ml

10 µg of each loaded onto a Sepharose CL-4B column (Pharmacia). Flow rate 2.55 ml/hr



Degradation of the larger chylomicrons probably also occurred in the longer (ρ < 1.21 g/ml) ultracentrifugation procedure and some of the material seen in the V_T may be accounted for by apolipoprotein constituents or smaller particles that have "budded off" from the chylomicrons during the 48 hr spin. The shorter centrifugation time of the ρ < 1.006 g/ml spin (18 hrs as opposed to 44 hrs) might have left more chylomicrons intact, hence the larger V_0 peak in the first profile (Fig. 3.14A).

Unfortunately, the quantity of material obtained precluded any subsequent SDS-polyacrylamide gel analysis of the proteins eluted in the pooled fractions corresponding to the 280 nm absorbance peaks.

The results of these preliminary experiments therefore indicated that particles of chylomicron/VLDL size as well as smaller particles (possibly nascent HDL) were released from the microsomes of jejunal enterocytes prepared from a fat-fed hamster. However, verification of the preparative procedures (especially the effects of sonication on the lipoprotein particles) and positive identification of the known apolipoproteins in the column fractions has yet to be carried out.

3.9 IDENTIFICATION OF OTHER PROTEINS POSSIBLY RELATED TO LIPOPROTEIN METABOLISM

Enterocyte lysates incubated with ^{35}S-methionine were also tested with a variety of antibody or antisera preparations directed against various proteins related to lipoprotein metabolism, one of these was Acylation Stimulating Protein (ASP), a small 14 kDa protein present in human plasma which rises dramatically following an oral fat load. This protein was first described by Cianflone *et al.* in 1989 and appears to be a potent stimulator of triglyceride synthesis in human skin fibroblasts and adipocytes. The exact tissue of origin of this protein is unknown but attempted immuno-precipitations of this protein from incubated hamster jejunal enterocytes using an anti-ASP IgG obtained from Dr Cianflone were unsuccessful.

Immuno-precipitations were also attempted using antisera against "hepatic" Fatty Acid Binding Protein (hFABP), a 14 kDa protein found in both rat hepatic and intestinal tissue, and "intestinal" FABP (gFABP), a ~15 kDa protein found in rat intestine. The role of these proteins in the intracellular transport of fatty acids has previously been discussed (see Chapter 1.2). Unfortunately, neither protein could be immuno-precipitated from my enterocyte lysates.

The negative results obtained more likely reflect the cross-species differences between the rat and hamster proteins rather than the absence of the proteins from the hamster enterocyte preparations. The polyclonal antisera were produced in rabbits and therefore should have reacted with Protein A-Sepharose which was used as the precipitating agent.

3.10 DO HAMSTER SMALL INTESTINAL ENTEROCYTES SYNTHESIZE AND SECRETE CHOLESTERYL ESTER TRANSFER PROTEIN (CETP) / LIPID TRANSFER PROTEIN-1 (LTP-1)?

3.10 (i) Introduction

When cell lysates and medium samples prepared from low and high fat-fed hamster small intestines were reacted with a rabbit-derived, anti-hamster apolipoprotein antiserum, a prominent though variable feature on the resultant fluorographic analysis of the immuno-precipitated products was a broad molecular weight band of around 63 kilodaltons. This did not correspond to the MW of any of the known intestinal apolipoproteins; it was not a major band on the original antigenic mixture of floated hamster lipoprotein-associated proteins (ρ < 1.25 g/ml) used to immunize the rabbit (Fig. 3.2) although it may have been obscured by the large albumin band in the same region; this protein nevertheless appeared highly antigenic but did not cross-react with specific anti-apolipoprotein B or anti-apolipoprotein A-IV antisera, respectively; nor was it present when non-immune rabbit serum was used. When additional "cold" apolipoprotein B (in the form of hamster LDL) was added to the immuno-precipitation mixture, it was not displaced (as was radio-labelled apolipoprotein B). The unknown protein did not correspond to the expected MW of the thrombin-derived apolipoprotein B degradation products (B-48 → "B-26" & "B-22").

An antigenic mixture of all proteins in the ρ < 1.25 fraction would be expected to include not only the integral lipoprotein apolipoproteins but also other proteins or enzymes that might associate with lipoproteins especially high density lipoproteins. These would include lecithin:cholesterol acyltransferase (LCAT) MW 66 kDa, acid phosphatase (54 kDa) and cholesteryl ester transfer protein (CETP), MW 58 - 64 kDa (James *et al.* 1988).

CETP (also known as lipid transfer protein-I or LTP-1) is a relatively heat-stable glycoprotein with a MW of 54 kDa (as derived from cDNA studies) which contains 4 potential sites for N-linked glycosylation (Drayna *et al.* 1987). It mediates the transfer of neutral and negatively charged lipids such as cholesteryl ester, tri-acylglycerol and phosphatidylcholine between plasma lipoproteins but is also believed to have important functions in modulating the activity of the enzyme LCAT and to play an important role in the process of reverse cholesterol transport where cholesterol from extra-hepatic tissues is transported to the liver, secreted into bile and thus cleared from the body (Tollefson & Albers, 1986). Its activity measured in the serum varies considerably among different mammalian species and Stein *et al.* (1990) have noted an apparently inverse relation between the plasma levels of CETP and the relative resistance of different species to the induction of atherosclerosis. Dogs, rats, sheep and cows have a low CETP activity and a high resistance to atherosclerosis while rabbits, humans, chickens and turkeys have a high CETP activity and are prone to develop atheroscelrosis when fed cholesterol.

A study of CETP activity in the hamster revealed values apparently intermediate between those of rats and humans. An increase in plasma activity was obtained when the hamsters were fed 2% cholesterol as opposed to a standard diet and this increase was even more pronounced when butter or margarine was added to the cholesterol in the diet. The increase in hamster plasma CETP activity correlated with the rise in the plasma cholesterol and triacylglycerol levels.

Another interesting study by Faust and Albers in 1988 demonstrated that the human enterocyte cell line, CaCO-2, secreted functional CETP (LTP-1) vectorially into the basolateral medium when grown as monolayers on a permeable culture membrane. Furthermore, this secretion was regulated in a dose-dependent manner by the addition of free fatty acids bound to albumin in the apical ("luminal") medium. The LTP-1 activity in the basolateral medium could also be inhibited by a goat anti-human LTP-1 antiserum.

The human hepatoma cell line, HepG2, was also shown in the same study to secrete LTP-1 but this was not subject to regulation by medium free fatty acids. It was thus postulated that dietary fat might upregulate LTP-1 levels in the postprandial state and that the intestine was a major source of regulatable LTP-1.

In 1988, however, another study failed to detect CETP mRNA in the rabbit intestine and this finding was confirmed in a comprehensive study by the group of Tall and co-workers in 1991 who analyzed the levels of CETP mRNA in various mammalian tissues including the hamster (Jiang *et al.* 1991). They found that adipose, heart and muscle tissue were the major sources of CETP mRNA and that high cholesterol diets increased the CETP mRNA abundance in these tissues. The levels of CETP mRNA in adipose tissue and heart correlated with the plasma CETP concentration. Small intestine CETP mRNA showed a much lower activity compared to these tissues although high fat and cholesterol feeding significantly increased the intestinal levels (but still well below those of adipose tissue and heart).

3.10 (ii) Tunicamycin, Pulse-chase Experiments and Attempted Immuno-precipitation with an Anti-LTP-1 Antiserum

Freshly isolated intestinal epithelial cell sheets from the proximal small bowel of an adult male hamster fed a mixture of rice and sunflower oil (20% oil w/w) in the preceding 18 hrs were pulse-labelled with ^{35}S-methionine for twenty minutes at 37°C, either in the absence or presence of tunicamycin, an inhibitor of N-linked glycosylation (10 μg/ml final concentration). At the end of the twenty minute pulse period, cold methionine (final concentration 2 mM) was added to both systems and a subsequent 60 minute chase incubation at 37°C was carried out. The cell lysates were immuno-precipitated using the rabbit-5 anti-hamster apolipoprotein antiserum while the media samples at 60 mins chase time were analysed directly on SDS-PAGE. The ^{35}S-labelled protein products were demonstrated by fluorography and molecular weights determined relative to a set of

Rainbow MW standards on the original gel.

3.10 (iii) Results

A band of MW 54 kDa appeared more prominent below the 59-67 kDa band (mid. MW 64 kDa) in the tunicamycin-treated versus the control experiment suggesting that this protein was indeed glycosylated through N-linked sugars. The prominent additional band following tunicamycin treatment corresponded to the cDNA-derived MW for CETP (see Fig. 5.1). A goat-derived anti-human LTP-1 peptide antiserum directed against a 26 amino acid peptide corresponding to the active site at the carboxy-terminus of the molecule, was received from Dr J. Adolphson in Dr Albers' laboratory. (The amino acid sequence of hamster CETP bears close homology with that of the human protein and the 26 amino acids in the active site differ in the 2 species in the last amino acid only) (Fig. 3.15). Protein G (which binds goat immunoglobulin) was used as the immuno-precipitating agent. Unfortunately, no specific protein product could be precipitated. A positive control for the Protein G was provided by a concomitant successful immuno-precipitation of apolipoprotein A-IV using another anti-A-IV-specific, goat-derived antiserum (Fig. 3.16).

Thus, although some features of the 59-64 kDa protein present in my enterocyte lysates were consistent with those of CETP, an attempted immuno-precipitation using a specific anti-LTP-1 antiserum was unsuccessful. The true identity of this protein therefore remains obscure.

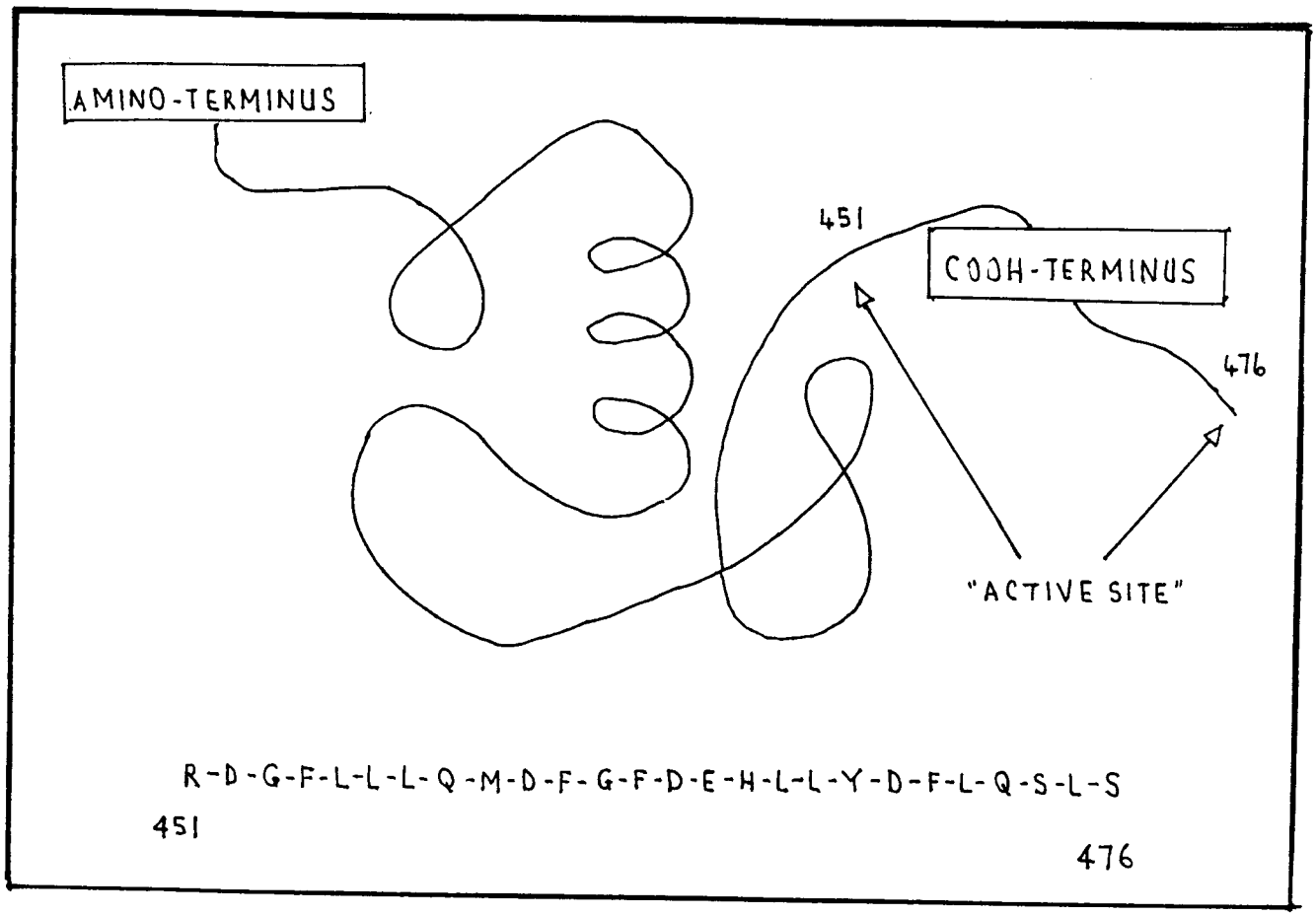


Fig. 3.15. The putative active site of lipid transfer protein-1 (LTP-1). The amino acid sequence of hamster LTP-1 bears close homology with that of the human protein and the 26 amino acids in the active site differ in the 2 species in the last amino acid only. (Ref: Dr J. Adolphson, NW Lipid Research Laboratory, Seattle, personal communication).

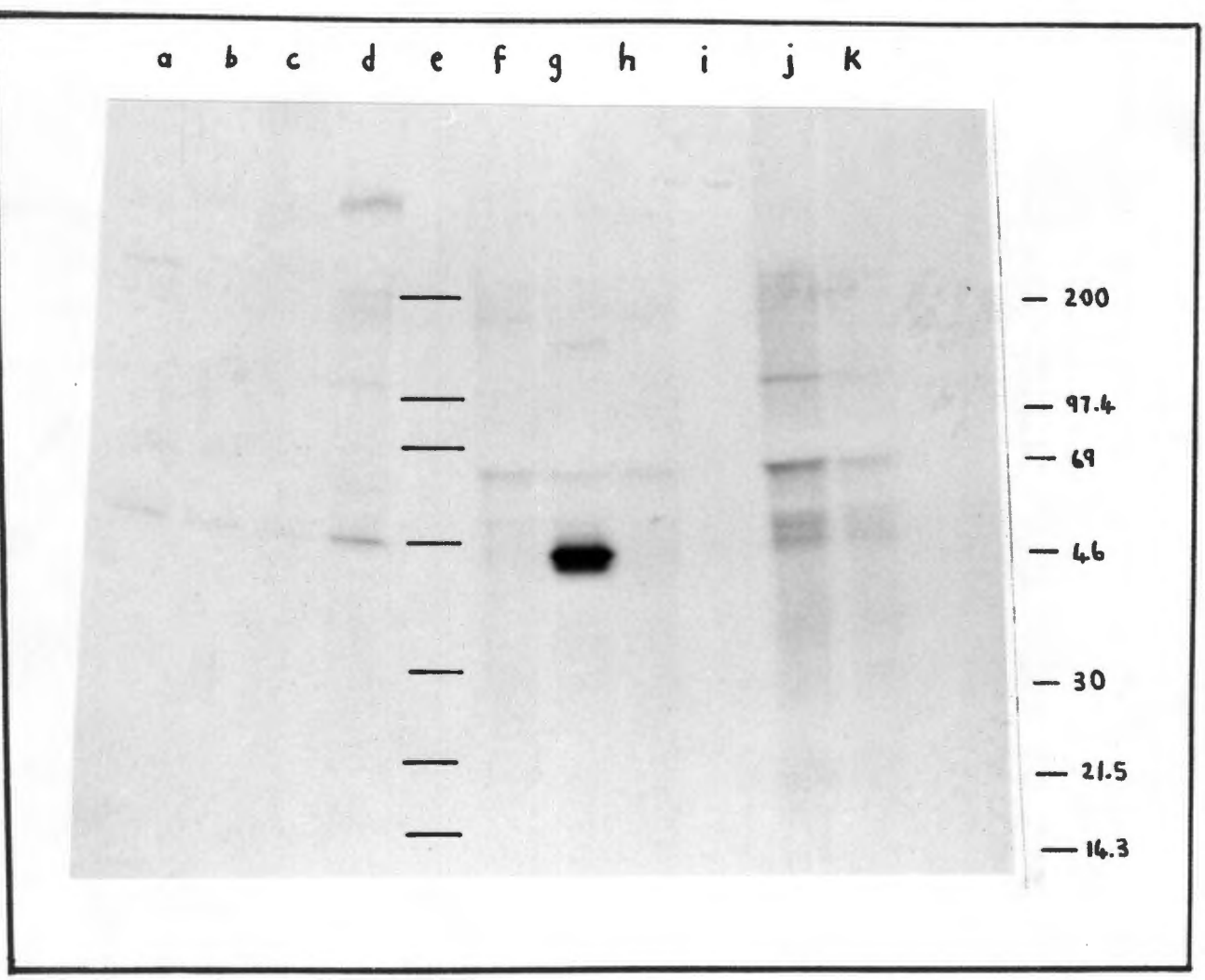


Fig. 3.16. Attempted IPs of LTP-1.

3.11 CONCLUSION

Using specific antisera and the Protein A or Protein A-Sepharose techniques of immuno-precipitation, freshly isolated hamster jejunal enterocyte suspensions have been shown to synthesize and secrete apolipoproteins B-48, A-IV and A-I during *in vitro* incubations. Methods have been devised to quantify the radio-labelled immuno-precipitable protein products. Column chromatographic analysis of the nascent intracellular lipoprotein particles has suggested that particles of chylomicron/very low density lipoprotein and nascent high density lipoprotein size were produced by enterocytes from a fat-fed animal. The system offers certain clear advantages as a method for studying the regulation of apolipoprotein synthesis, secretion and degradation following various dietary perturbations performed on intact animals.

CHAPTER FOUR

STUDIES OF THE SYNTHESIS, SECRETION AND DEGRADATION OF APOLIPOPROTEINS B-48, A-IV AND A-I: EFFECTS OF DIETARY LIPID

4.1. DISCUSSION

From the previous review of the experimental evidence to date concerning the regulation of apolipoprotein biosynthesis and secretion (chapt. 1.8), the picture that appeared to emerge for apolipoprotein B-48 and A-I was one in which neither acute nor chronic triacylglycerol (TAG) flux had any apparent effect on the synthesis of these apolipoproteins although the synthesis of apolipoprotein A-IV was significantly increased. Further evidence pointed to the existence of a large intracellular pool of Apo B-48 (and of Apo A-I) which was mainly membrane-bound rather than lipoprotein-associated, and which could not easily be depleted with either acute or chronic dietary TAG stimulation.

Evidence gathered in various laboratories from studies on hepatocytes has suggested that the mechanism accounting for the increased secretion of apo B (and, therefore, apo B-containing lipoproteins) caused by lipid (oleate) is that of inhibition of the early intracellular degradation of apo B by some kind of "protective" effect on the protein. The availability of apo B for lipoprotein assembly may therefore be regulated at the level of varying rates of degradation rather than changes in the rates of synthesis. No studies of apolipoprotein degradation have been reported in the intestine; indeed the systems of study mostly used, e.g. the *in vivo* pulse-labelling technique of Glickman described in chapt. 2.2, did not permit such studies.

The technique of incubating freshly isolated enterocyte sheets from the hamster small intestine does, however, enable controlled studies of synthesis, secretion and degradation of the apolipoproteins to be performed. The dietary perturbations e.g. low fat feeding, acute bolus administration of TAG, overnight high fat feeding and chronic high fat feeding, were performed in advance of *in vitro* experimentation on intact animals; once active absorption was in progress, the animals were killed and enterocyte sheets prepared and incubated. The fundamental assumption in this system was that the cells *in vitro* continued to behave in a manner analogous to that of the *in vivo* state just prior to killing of the animal.

4.2.(i) PULSE-CHASE EXPERIMENTS: EXPRESSION OF RESULTS

The method of pulse-chase labelling enabled studies of degradation to be performed in the incubated sheets of cells. Following a labelling period of twenty minutes, during which the cell sheets were incubated in the presence of ^{35}S-methionine (8 μCi for every 1 ml cell suspension), "cold" (non-radioactive) methionine was added to a final concentration of 2 mM, and a ten minute "equilibration" incubation time was allowed for the specific activity of the radio-labelled amino acid to become significantly diluted in the medium, in the intracellular pool and, indeed, in the methionylacyl transfer RNA pool, (the true precursor pool for [^{35}S]-methionine-labelled proteins). From then on a "chase" period of 90 minutes was effected, during which the rate of appearance of proteins in the medium (secretion) and the overall loss of protein radioactivity from cells and media i.e. the rate of protein degradation, were measured in respect to total protein as well as specific (immuno-precipitated) apoproteins.

Fig. 4.1 shows the total ^{35}S methionine-labelled protein dynamics of a typical pulse-chase experiment. Each time point on the graph represents the mean TCA-precipitable radioactivity from a duplicate set of incubation flasks. The enterocyte sheets from all the flasks were derived from the proximal small bowel (jejunum) of a single animal.

In initiating the "chase" period, the "old" radioactive medium was not changed but simply diluted with excess cold methionine at the point arrowed (20 mins after the start of the incubation). (The start of the chase period - T=0' on the X-axis - was defined 10 mins after the addition of the cold methionine). A medium change would have necessitated centrifuging the cells, removing the old medium, and perhaps even washing the cells once or twice before resuspending them in fresh medium. This was attempted on two occasions, but the viability of the cells appeared to be compromised by such procedures. In any case, when great care was taken to centrifuging and washing was the same, i.e. no significant differences in residual labelled protein was

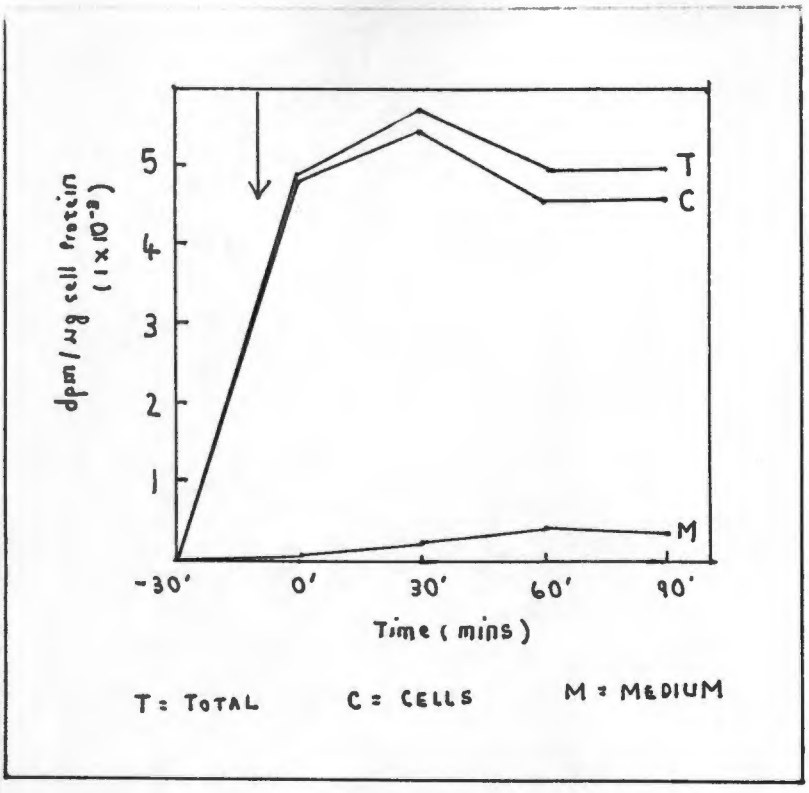


Fig. 4.1. TCA-precipitable ^{35}S-methionine labelled material in cells, media and total, following a typical pulse-chase experiment. Each point on the graph represents the mean TCA-precipitable radioactivity from a duplicate set of incubation flasks at a given time. The total TCA-precipitable radioactivity was obtained by adding those of the cells and media, respectively. The enterocyte sheets used for all the time points were derived from the jejunum of a single animal.

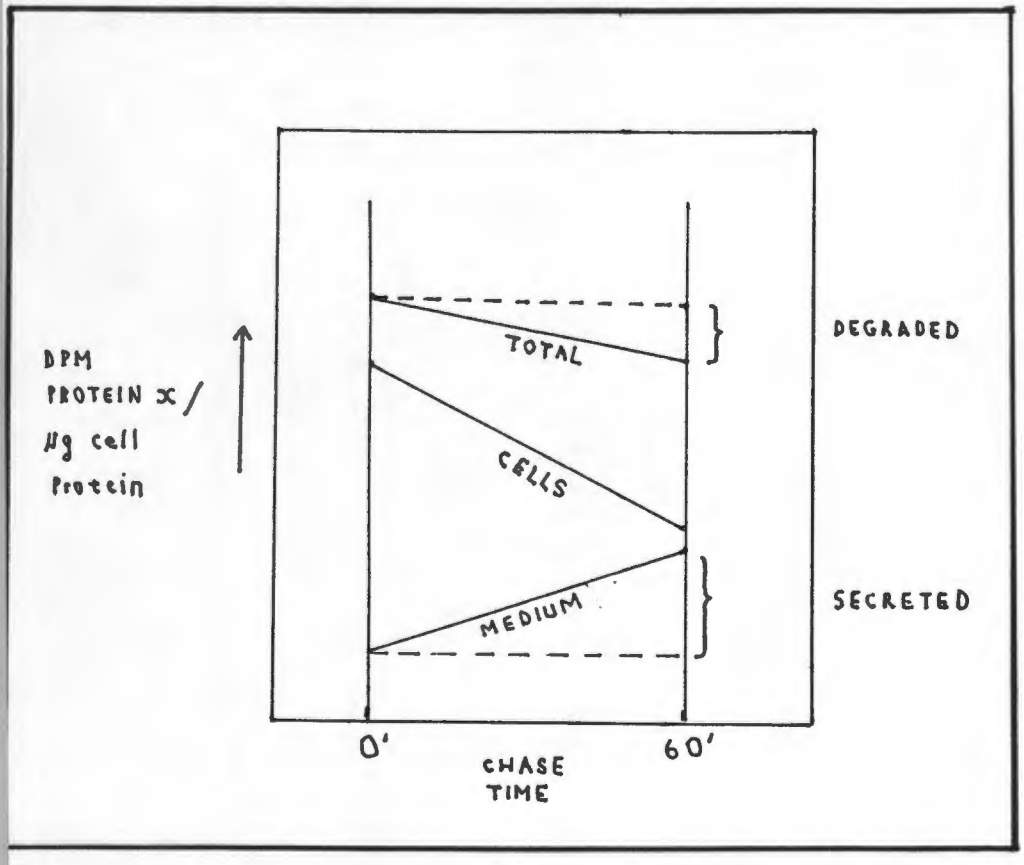


Fig. 4.2. A schematic representation of the definitions of "secretion" and "degradation" for a hypothetical (secreted) protein studied using the pulse-chase method.

observed between the 2 techniques. The less "traumatic" method was therefore favoured.

It should be noted that the protein-synthesis inhibitor, specific for eukaryotic systems, cycloheximide, was not used during the chase periods. Although this inhibitor has been used in pulse-chase experiments involving cells in culture, its use has been criticized because of its potential role in inhibiting the synthesis of proteins normally responsible for the degradation of other proteins, so that unphysiological and abnormally long protein half lives have been obtained in the presence of this agent.

Protein degradation (in the entire system i.e. general protein degradation, or of a specific apolipoprotein) was expressed quantitatively in the following way:

$$\text{Degradation of total protein in the system} = \frac{\text{Total TCA precipitable dpm in cells \& media, of T=0' - T=60'}}{\text{Total TCA-precipitable dpm in cells \& media, T=0'}} \times 100$$

OR

$$\text{Degradation of a specific apolipoprotein} = \frac{\text{Specific Immuno-precipitated TCA-precipitable dpm in cells \& media, of t=0' - T=60'}}{\text{Specific immuno-precipitated, TCA-precipitable dpm in cells \& media, T=0'}} \times 100$$

i.e. degradation was expressed as a percentage per hour. The immuno-precipitations of specific apolipoproteins from the cells and media were performed separately. N.B.: all TCA/IP dpm were corrected per μg cell protein.

Synthesis of a particular protein was expressed as:

$$\frac{\text{Immuno-precipitated, TCA-precipitable dpm in cells \& media. T=0' chase}}{\text{Total TCA-precipitable dpm in cells media, T=0'}} \times 100$$

Synthesis was therefore expressed as the proportional contribution of a particular protein to the total TCA-precipitable counts in the system at T=0' chase.

Finally, secretion was expressed as:

$$\frac{\text{IP precipitable, TCA ppt. apolipoprotein dpm in medium, (T=60' - T=0')}}{\text{Total IP ppt, TCA-ppt apolipoprotein dpm in cells, T=0'}} \times 100$$

i.e. that proportion or percentage of protein, present in the cells at T=0', that entered the medium after a 60' chase period.

The definitions of secretion and degradation are represented schematically for a hypothetical (secreted) protein in Fig. 4.2.

4.2.(ii) STANDARDIZATION OF PROTEIN SYNTHESIS EXPERIMENTS

As we shall see, the rates of incorporation of radio-labelled amino acid into general protein varied markedly from one enterocyte preparation to the next. One possible factor that might have accounted for this variation was the relative sizes of the intracellular methionine pools in the different preparations. An enterocyte preparation with a relatively small intracellular methionine pool would result in a high specific activity of the radio-labelled methionine and, therefore, high rates of incorporation into protein; conversely, a preparation with a high intracellular methionine pool would dilute the specific radioactivity to a greater extent and thus produce lower rates of incorporation into protein.

In an attempt to standardize for this potential source of variation, a "high methionine" protein synthesis incubation was also performed in each experiment; in these flasks, the enterocyte suspensions were incubated with ^{35}S-methionine in the presence of 0.5 mM cold methionine in the medium. (This concentration was lower than the 2 mM methionine used in the pulse-chase technique). If variations in the intracellular methionine pool existed prior to incubation, these would be considerably reduced by the

dilutional effect of the large amount of cold methionine added since any endogenous variation in the intracellular pool would be swamped by the exogenous methionine. The specific activities of the radiolabelled amino acid would therefore be similar in all enterocyte preparations. The incorporation into protein of the "high methionine" systems was also corrected in each flask by measuring the microgram quantity of cell protein present, so that direct comparisons of the protein synthesis activity in different preparations could be made.

4.3. EXPERIMENT AIMS

The experiments attempted to answer some of the questions addressed in chapt. 1.7 using the established system of freshly isolated incubated jejunal enterocytes, viz:

(1) Are the processes of lipid assembly and apolipoprotein biosynthesis linked or co-ordinated? Is TAG flux a specific stimulus for apolipoprotein biosynthesis?

(2) If TAG flux does stimulate apolipoprotein synthesis, does the composition of the TAG (saturated versus unsaturated fat) produce differing quantitative effects on the rates of apolipoprotein synthesis and secretion?

(3) If an acute dietary TAG challenge does not stimulate or "up-regulate" apolipoprotein biosynthesis, does more sustained feeding (designed to deplete the intracellular pool of apolipoproteins by prolonging the TAG flux and therefore increasing the utilization of apolipoproteins for lipoprotein packaging) do so? How does low fat feeding ("baseline" control) compare with an acute TAG bolus administration, overnight high fat feeding and chronic (6 week) high fat feeding?

(4) Are apolipoproteins degraded? If so, to what extent? Are there differences in the degradation rates for low fat-fed as opposed to high fat-fed animals?

4.4 DIETS

The adult male Syrian Golden hamsters were maintained on a standard laboratory chow ("Epol" pellets) prior to entering the experimental dietary regimes. This standard chow contained about 4% fat w/w (mainly plant or polyunsaturated fat). Cooked, polished rice was chosen at the baseline low fat diet since it contains the lowest fat content of almost any foodstuff (0.1% fat, w/w, Geigy Scientific Tables). It was not administered for periods greater than 20 hours since it was not a "balanced" nutritious diet although it was eagerly consumed by the animals. The "single lipid meal" consisted of the bolus administration of sunflower oil (0.5 ml) fed to the animals by pipette. This material was also accepted by the hamsters who are known for their fondness of sunflower seeds. The animals were sacrificed two hours later to allow for gastric emptying, peristalsis and the establishment of an active transmucosal flux of TAG. The bowels removed from these animals were, in fact, obviously engorged with fat and the plasma was lipaemic, indicating active lipid transport from the intestine. In all experiments the TAG content of the enterocyte lysates and the plasma TAG concentrations were determined using a commercially available TAG assay kit (Triglycerides - GPO, Human GmbH, Germany). This enzymatic colorimetric test involved the determination of TG after enzymatic hydrolysis with lipases. It was linear up to a TG concentration of 1000 mg/dl (11.4 mmol/l). A typical standard graph is shown in Fig. 4.3. Experiment sample volumes for the TG assay were chosen in such a way that they fell within the range of the standards.

The "high unsaturated fat" feed consisted of a mixture of rice and sunflower oil (~ 20% oil w/w) i.e. 80 g rice + 20 ml oil, mixed in a foodmixer to produce a soppy pulp which was homogeneous enough that the animals could not selectively eat the rice or the oil separately. The "high saturated fat" feed consisted of a mixture of rice and lard (80g rice + 20 g lard), also mixed to a palatable pulp. The chronic (6 week) high fat diet was devised by Dr A.D. Marais. The salt mix was based on a report published by

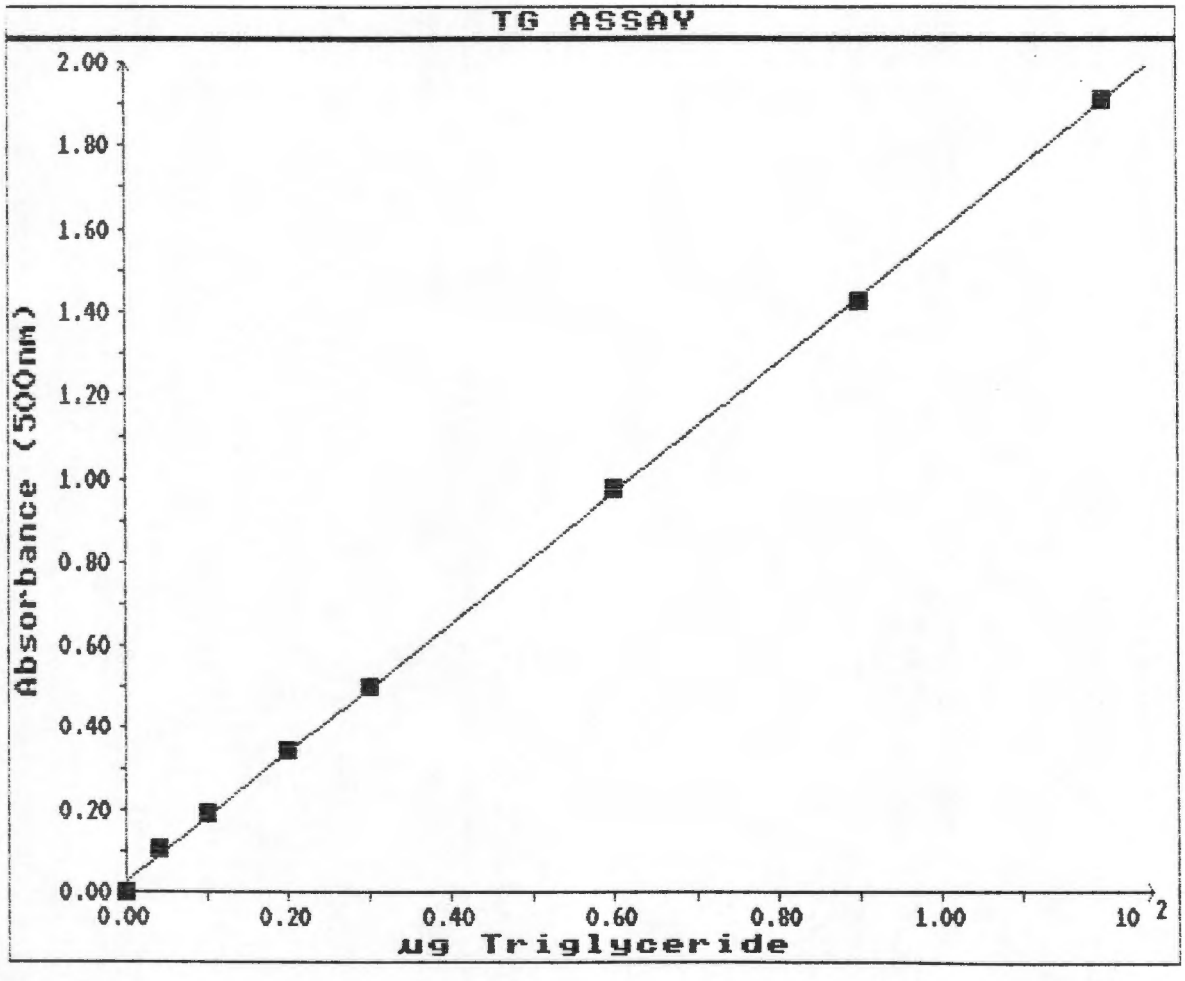


Fig. 4.3. A typical standard graph for the enzymatic-colorimetric TAG assay.

Cameron-Clarke and Manchester (1985). The diet comprised (w/w) oats (29%), maize (29%), skimmed milk powder (29%), Brewer's yeast (0.36%), multivitamin & mineral capsules (opened to release the powder), (0.71%), sunflower oil (9.26%), and a salt mix consisting of sodium chloride, calcium lactate, disodium hydrogen phosphate, iron citrate, magnesium sulphate, calcium phosphate, potassium iodide and potassium hydrogen phosphate (2.1%). The total fat content was approximately 10% (w/w), mainly polyunsaturated, and contributed 20% of the total calorific content of the diet.

The composition of the various diets, their calorific contents and fatty acid composition are shown in Table 4.1.

It should be noted that the high fat diets were obviously not isocaloric with the low fat diets. However, the aim of the experiments was to compare high fat with low fat feeding in terms of apolipoprotein synthesis, secretion and degradation. A high fat diet would inevitably contain more calories since fat has a higher energy content per gram than protein or carbohydrate. Secondly, the calorific differences between the diets (per unit mass of the foodstuff) would be compensated, at least in part, by an established observation about the eating habits of hamsters - they tend to adjust their intake according to their energy requirements (Borer 1985). A caged animal will eat approximately twice as much carbohydrate as fat to maintain the same energy intake in a 20 hour feeding period, say. This was confirmed by my own observation that they consumed more than double the quantity of rice compared with the rice:20% oil mixture in an equivalent period. The total caloric consumption was therefore similar. The fatty acid composition of lard and sunflower oil was verified by gas chromatography with the assistance of Dr J. Cohen at the Medical Research Council Nutritional Research Institute (RIND), Parow, South Africa.

A

	LOW FAT DIET	BOLUS FAT ADMINISTRATION	HIGH UNSATURATED FAT	HIGH SATURATED FAT
FOODSTUFF	RICE	SUNFLOWER OIL	RICE: SUN-FLOWER OIL (80:20 w/w)	RICE: LARD (80:20 w/w)
WATER	72.6%	0.2%	58%	58%
PROTEIN	2%	0	1.6%	1.6%
CARBOHYDRATE	24.2%	0	19%	19%
FIBRE	0.1%	0	<0.1%	<0.1%
FAT	0.1%	99.8%	20%	20%
CALORIFIC CONTENT	109 kcal/ 100g	882 kcal/ 100g	267 kcal/ 100g	267 kcal/ 100g

B

	SUNFLOWER OIL	LARD
RATIO: SAT. : UNSAT	1:9	2:3
% UNSAT. FAT	≅ 90% MAINLY LINOLEIC (C18:2) & OLEIC (C18:1)	≅ 58% MAINLY OLEIC (C18:1)
% SAT. FAT	10% (STEARIC & PALMITIC)	42% MAINLY PALMITIC (C16:0) & STEARIC (C18:0)

Table 4.1. A & B. Composition of experimental diets. The figures were calculated using information from Geigy Scientific Tables, Vol. One (8th Ed.), 1981. Ciba-Geigy Ltd., Basle, Switzerland, pp 251 & 254.

The enterocyte sheets were, in any case, supplied with exogenous fuels in the incubation medium, so "endogenous pre-loading" of the cells with fuels *in vivo* would not, generally have been likely to account for differences in their *in vitro* behaviour.

N.B.: Food was always provided in excess so that the animals were never starved during the feeding period. However, a fasting period of 2 hours following the last meal and prior to sacrifice was adopted in all the experiment protocols. Animals were always allowed free access to water.

4.5 MEASUREMENTS OF THE RATE OF BIOSYNTHESIS OF APOLIPOPROTEINS B-48, A-IV AND A-I UNDER CONDITIONS OF LOW AND HIGH FAT FEEDING

4.5.(i) Protocol and Aims

This set of preliminary experiments was designed to determine:

(i) whether acute dietary TAG challenge stimulated or "up-regulated" apolipoprotein biosynthesis, and

(ii) whether more sustained feeding, by prolonging the TAG flux and thus the utilization of apolipoproteins for lipoprotein assembly and secretion, depleted the intracellular pool of apolipoproteins and therefore stimulated apolipoprotein biosynthesis.

Four dietary regimes were followed: A low fat (rice only) control, a single bolus administration of TAG (0.5 ml of sunflower oil pipette-fed to the animal), a 20 hr ("overnight") feed consisting of a rice:20% sunflower oil mixture, and a chronic feeding regime, comprising 6 weeks on a 10% w/w fat diet (see 4.4). the dietary perturbations were performed on a duplicate set of animals.

The experiment protocol is outlined in figure 4.4. Enterocytes prepared from these animals were incubated for 20' in the presence of ^{35}S-methionine. The cells and media were immuno-precipitated using specific anti-apoB, anti-A-I, and anti-A-IV antisera and the fluorograms were quantified using the NaOH-silver elution technique (see 3.6ii). Results were expressed as

$$\frac{\text{dpm specific apolipoprotein (cells \& media)}_{T=20'}}{\text{Total TCA ppt. dpm (cells \& media)}_{T=20'}} \times 100$$

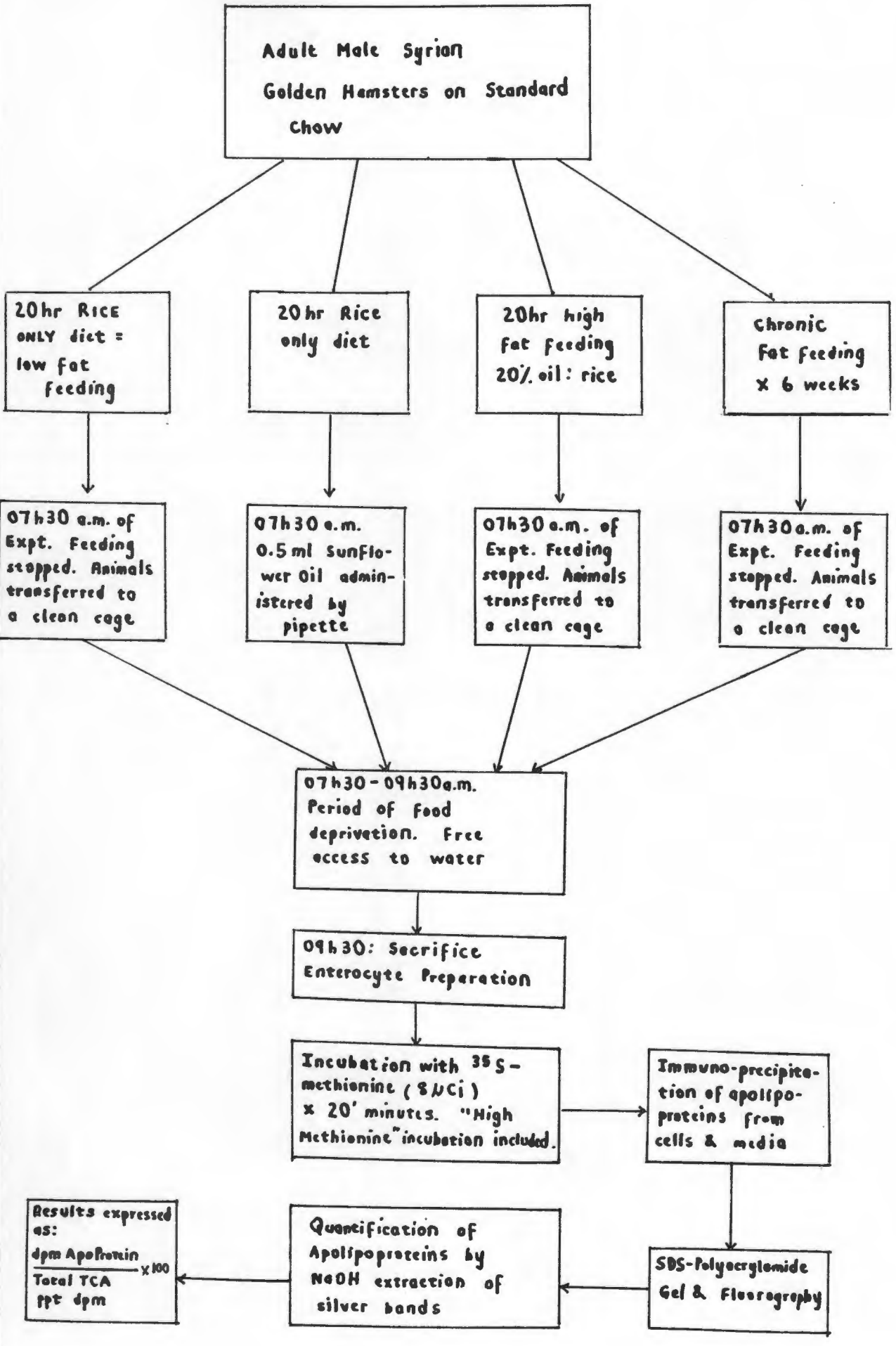


Fig. 4.4. Experimental protocol for determining the effects of acute dietary TAG challenge, overnight fat feeding, and sustained lipid feeding for six weeks, on the synthesis of apolipoproteins B-48, A-IV and A-I, respectively. A low fat-fed control was included in the protocol.

4.5.(ii) Results

Two animal experiments were performed for each dietary treatment.

Low fat feeding (Rice only) (expts. 1 and 2)

Expt. One: Mass of animal = 143.9 g

Average quantity of cell protein incubated (per incubation flask) = 0.390 mg

Cell TAG content (per incubation flask) = 50 μg

Cellular TAG content (μg)/cell protein (μg) = 0.146

Plasma TAG concentration at time of killing of animal = 181 mg/dl

Rate of general protein synthesis, T=20' = 8083 dpm/μg cell protein

"High Methionine" system (20' incubation): 551 dpm/μg protein

Apo B-48 as % of total radio-labelled protein synthesis (after 20' incubation) : 1.32%

Apo A-IV : 7%

apo A-I : 2.0%

Expt. Two: Mass of animal = 145.3 g

Average quantity of cell protein incubated = 0.492 mg

Cell TAG content (per incubation flask) = 13 μg

Cell TAG content (μg)/cell protein (μg) = 0.235

Plasma TAG concentration at time of killing of animal = 177 mg/dl

Rate of general protein synthesis, T = 20' = 11 307 dpm /μg cell protein

"High Methionine" system (20' incubation): 292 dpm/μg cell protein

Apo B-48 as % of total radiolabelled protein synthesis: 1.24%

Apo A-IV : 4.5%

Apo A-I: 2.6%

Single fat meal (bolus administration of 0.5 ml sunflower oil following 20 hr rice only diet) (expts. 3 and 4)

Expt. Three: Mass of animal = 167.4 g

Average quantity of cell protein incubated (per incubation flask) = 1.017 mg

Cell TAG content (per incubation flask) = 270 μg

Cellular TAG content (μg)/cell protein (μg) = 0.271

Plasma TAG concentration = 1401 mg/dl

Rate of general protein synthesis, T=20' = 3596 dpm/μg cell protein

"High Methionine" system (20' incubation) = 256 dpm/μg protein

Apo B-48 as % total protein synthesis: 0.73%

Apo A-IV : 3.9%

Apo A-I: 3.9%

Expt. Four: Mass of animal - 132.6 g

Average quantity of cell protein incubated (per incubation flask) = 1.937 mg

Cell TAG content (per incubation flask) = 603 μg

Cellular TAG content/cell protein = 0.334

Plasma TAG concentration = 164 mg/dl

Rate of general protein synthesis, T=20' = 4019 dpm/μg cell protein

"High Methionine" system (20' incubation) : 153 dpm/μg cell protein

Apo B-48 as % total protein synthesis: 1.46%

Apo A-IV: 4%

Apo A-I: 1.1%

20 Hour high fat feeding (Rice: sunflower oil mixture)

(Expts. 5 & 6)

Expt. Five

Mass of animal: 134.2 g

Average quantity of cell protein incubated (per incubation flask) = 0.300 mg

Cellular TAG content (per incubation flask) = 318 μg

Cellular TAG content/cell protein = 1.03

Plasma TAG = 269 mg/dl

Rate of general protein synthesis, T=20', = 12 769 dpm/μg cell protein

Apo B-48 as % total protein synthesis: 0.77%

Apo A-IV: 6.0%

Apo A-I: 2.6%

Expt. Six:

Mass of animal: 116.5 g

Average quantity of cell protein incubated (per incubation flask) = 0.850 mg

Cellular TAG content (per incubation flask) = 1763 μg

Cellular TAG content/cell protein = 2.06

Plasma TAG concentration: 204 mg/dl

Rate of general protein synthesis, T=20', = 7291 dpm.μg cell protein

"High Methionine" system (20' incubation): 293 dpm/μg cell protein

Apo B-48 as % total protein synthesis: 0.73%

Apo A-IV: 4.8%

Apo A-I: 2.8%

Chronic fat feeding (10% fat diet, for 6 weeks)

(Expts. 7 & 8)

Expt. Seven

Mass of animal: 188.7g

Average quantity of cell protein incubated (per incubation flask) = 1.381 mg

Cellular TAG content (per incubation flask) = 1008 μg

Cellular TAG content/cell protein = 0.634

Plasma TAG = 254 mg/dl

Rate of general protein synthesis, T=20', = 5411 dpm/μg cell protein

"High Methionine" system (20' incubation): 350 dpm/μg cell protein

Apo B-48 as % of total protein synthesis: 1.4%

Apo A-IV: 3.8%

Apo A-I: 1.3%

Expt. Eight

Mass of animal: 178.6 g

Average quantity of cell protein incubated (per incubation flask) = 1.744 g

Cellular TAG content (per incubation flask) = 708 μg

Cellular TAG content/cell protein = 0.425

Plasma TAG concentration = 235 mg/dl

Rate of general protein synthesis, T=20' : 10 567 dpm/μg cell protein

"High Methionine" system (20' incubation) 348 dpm/μg protein

Apo B-48 as % total protein synthesis: 0.71%

Apo A-IV: 4.5%

Apo A-I: 1.5%

4.5.(iii) Discussion of Results

The animal-to-animal variation even within a given dietary treatment was large. Compare, for example, the tenfold difference in the post-prandial plasma TAG content between the 2 animals both fed a single lipid meal. Reasons for these large differences probably relate to varying rates of gastric emptying of a lipid meal. Peculiarities of the hamsters' digestive tract and its feeding habits are likely to account for these differences (see discussion, section 4.8). Nevertheless, the rates of synthesis of the apolipoproteins B-48, A-IV and A-I do not differ significantly from one dietary group to the next (see Tables 4.2 A,B & C).

These results are consistent with previous findings regarding the synthesis rates of apolipoprotein B-48 and A-I under conditions of low and high dietary fat intake. They are not in keeping, however, with data on Apo A-IV where a significant increase in the synthesis of this protein following acute and chronic dietary fat feeding has been reported (Apfelbaum *et al.* 1987). Possible reasons for this seemingly anomalous result will be discussed in section 4.8.

LOW FAT FEEDING	SINGLE LIPID MEAL	20hr HIGH FAT FEEDING	6 WEEKS FAT FEEDING
1.32%	0.73%	0.77%	1.40%
1.24%	1.46%	0.73%	0.71%

A

LOW FAT FEEDING	SINGLE LIPID MEAL	20 hr HIGH FAT FEEDING	6 WEEKS FAT FEEDING
7.0%	3.9%	6.0%	3.8%
4.5%	4.0%	4.8%	4.5%

B

LOW FAT FEEDING	SINGLE LIPID MEAL	20 hr HIGH FAT FEEDING	6 WEEKS FAT FEEDING
2.0%	3.9%	2.6%	1.3%
2.6%	1.1%	2.8%	1.5%

C

Table 4.2. The synthesis of (A) apolipoprotein B-48, (B) apolipoprotein A-IV, and (C) apolipoprotein A-I (expressed as the % contribution of the radio-labelled, immunoprecipitated apoprotein to the total TCA-precipitable labelled protein synthesis), under four dietary treatments (2 experiments per dietary group).

4.6 EFFECTS OF DIETARY FAT SATURATION ON SYNTHESIS OF APOLIPOPROTEINS B-48 AND A-I

4.6.(i) Aims and Protocol

In the preceding set of experiments, unsaturated dietary fat did not alter the rates of synthesis of the apolipoproteins B-48, A-IV and A-I compared with low fat controls. Given, though, the epidemiological and experimental evidence implicating saturated as opposed to unsaturated lipids in the genesis of hypercholesterolaemia, and in the light of previous experimental work suggesting that chylomicrons prepared from saturated fat-fed animals were selectively enriched in Apo B compared with unsaturated fat fed controls (Renner *et al.* 1986), a series of experiments was performed comparing the rates of synthesis of apolipoproteins B-48 and A-I encountered after administration of saturated and unsaturated fat diets. The experiment protocol is outlined in figure 4.5. The diets have been discussed in section 4.4 (Table 4.1). Only the 20 hr ("overnight") mode of feeding was employed. Because of a shortage of anti-apo A-IV antiserum, only the rates of B-48 and A-I synthesis were studied.

4.6.(ii) Results

Low fat feeding (expts. 9 & 10)

(Rice only)

Expt. Nine

Mass of animal: 153.06 g

Average quantity of cell protein incubated per flask: 1228 μg

General rate of protein synthesis, T=20': 247 dpm/μg cell protein

Apolipoprotein B-48 as % total protein synthesis: 2.19%

Apo A-I: 2.14%

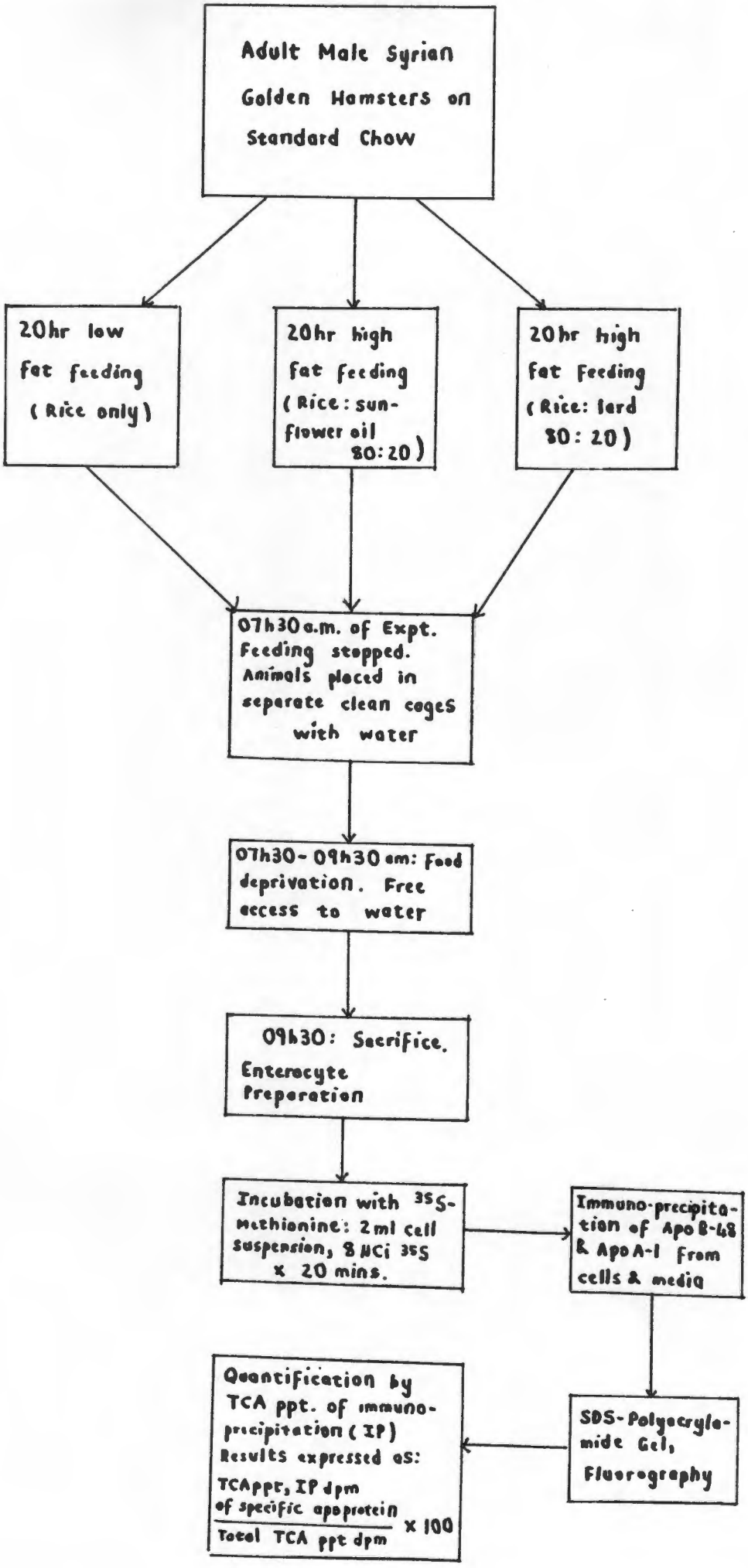


Fig. 4.5. Experiment protocol for determining whether dietary fat saturation has any effect on the synthesis of apolipoproteins B-48 and A-I respectively.

Expt. Ten:

Mass of animal: 173.65 g

Average quantity of cell protein incubated per flask: 1248 μg

General rate of protein synthesis, T=20': 138 dpm/μg cell protein

Apo B-48 as % total protein synthesis: 1.38%

Apo A-I: 0.50%

20 hr High unsaturated fat feeding (Rice-sunflower oil)

(Expts. 11 & 12)

Expt. Eleven:

Mass of animal: 166.15 g

Average quantity of cell protein incubated per flask: 1284 μg

General rate of protein synthesis, T=20': 269 dpm/μg cell protein

Apo B-48 as % total protein synthesis = 0.90%

Apo A-I: 0.90%

Expt. Twelve:

Mass of animal: 171.80 g

Average quantity of cell protein incubated per flask: 1400 μg

General rate of protein synthesis, T=20': 2285 dpm/μg cell protein

Apo B-48 as % total protein synthesis: 0.45%

Apo A-I: 1.60%

20 hr High saturated fat feeding (Rice:lard)

(Expts. 13 & 14)

Expt. Thirteen:

Mass of animal: 177.68 g

Average quantity of cell protein incubated per flask: 1256 μg

General rate of protein synthesis, T=20': 344 dpm/μg cell protein

Apo B-48 as % total protein synthesis: 1.24%

Apo A-I: 1.04%

Expt. Fourteen:

Mass of animal: 153.16 g

Average quantity of cell protein incubated per flask: 1397 μg cell protein

Apo B-48 as % total protein synthesis: 1.03%

Apo A-I: 1.08%

4.6.(ii) Discussion

Fat saturation, in this set of experiments, did not affect the synthesis of apolipoproteins B-48 and A-I in freshly isolated jejunal enterocytes from hamsters fed a saturated or unsaturated fat-enriched diet (see Tables 4.3A & B), compared with controls. Davidson *et al.* (1987) also found no change in Apo B-48 or Apo A-I synthesis following sustained feeding (six weeks) of a 30% saturated or unsaturated fat diet. The study by Renner *et al.* in 1986 showing a selective enrichment of saturated fat-containing rat chylomicrons by apolipoprotein B did not specifically measure the protein synthesis rate; the origin of the additional apo B (whether it was derived from recent *de novo* intestinal synthesis, from a pre-existing intracellular pool, or from apolipoprotein exchanges with other particles) was therefore not established.

A

LOW FAT FEEDING	UNSAT. FAT FEEDING	SAT. FAT FEEDING
2.19	0.90	1.24
1.38	0.45	1.03

B

LOW FAT FEEDING	UNSAT. FAT FEEDING	SAT. FAT FEEDING
2.14	0.90	1.04
0.50	1.60	1.08

Table 4.3. (A) Apo B-48, and (B) apo A-I, as %s of total radio-labelled protein synthesis under conditions of low dietary fat intake, high unsaturated fat intake and high saturated fat intake, respectively. (Duplicate expt. results per dietary group).

4.7 STUDIES OF APO B-48 AND APO A-IV SYNTHESIS, SECRETION AND DEGRADATION UNDER CONDITIONS OF LOW AND HIGH FAT FEEDING

4.7.(i) Protocol

The pulse-chase technique (see 4.2(i)) was used to study the degradation and secretion of apolipoproteins B-48 and A-IV under conditions of low and high fat feeding. The experiment protocol and the methods used in calculating and expressing the results of synthesis, secretion and degradation studies, are outlined in figures 4.6 and 4.7, respectively.

4.7.(ii) Results

Apolipoprotein B-48 was specifically immuno-precipitated by a polyclonal, rabbit-derived anti-human apolipoprotein B antiserum ("OSAN", Behring) and the immuno-precipitated product had a molecular weight approximately the same as that of the 200 kDa MW standard on a 5-20% reducing SDS polyacrylamide gel. Apolipoprotein A-IV was quantitatively immuno-precipitated using a polyclonal rabbit-derived anti-rat apo A-IV antiserum and had a MW of 44.5 kDa on a 5-20% reducing SDS polyacrylamide gel. All immuno-precipitations (IPs) resulted in a single protein band (of the expected MW for that protein) on the subsequent gel and fluorogram. Thus, in the calculations of rates of synthesis, degradation and secretion, the TCA-precipitable, IP counts (dpm) (minus the non-immune serum control IP dpm) were considered to originate exclusively from the specific IP protein product.

Results: Histology

Figs. 4.8 and 4.9 show transverse sections through the villi of the proximal jejunum of 2 animals fed the low and high fat diets, respectively. The sections were stained with

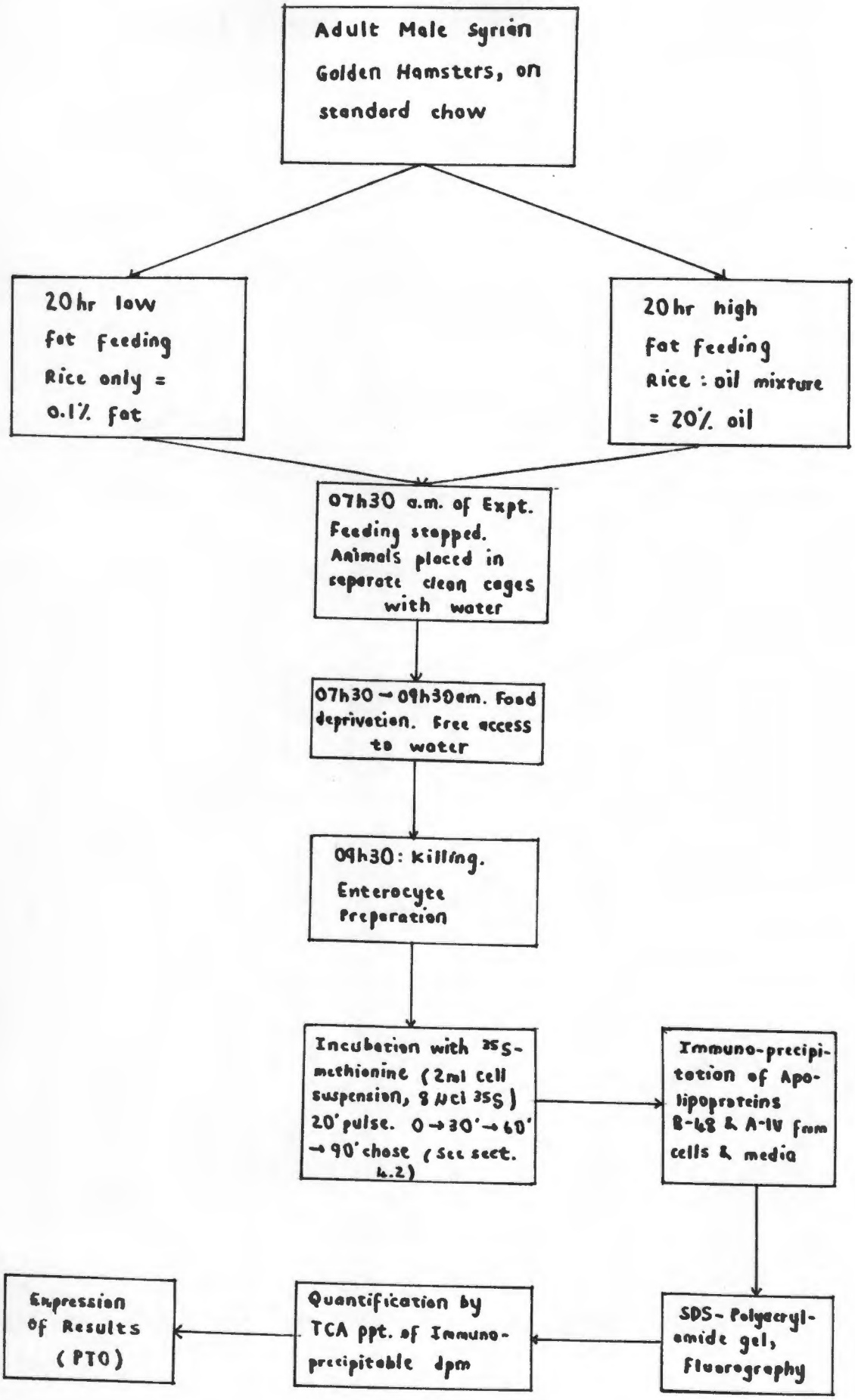


Fig. 4.6. Protocol for studying the synthesis, degradation and secretion of apolipoproteins B-48 and A-IV under conditions of low and high fat feeding.

Expression of Results

Synthesis of a specific apolipoprotein (as a % of total protein synthesis) =

$$\frac{\text{IP, TCA-ppt dpm in cells \& media, T=0' chase}}{\text{Total TCA-ppt dpm, cells \& media, T=0' chase}} \times 100$$

Degradation of a specific apolipoprotein (% degraded per hr) =

$$\frac{\text{specific IP, TCA-ppt dpm in cells \& media, (T=0') − (T=60')}}{\text{specific IP, TCA-ppt dpm in cells \& media, T=0'}} \times 100$$

Secretion of a specific apolipoprotein (% secreted per hr) =

$$\frac{\text{specific IP, TCA-ppt dpm in medium, (T=60') − (T=0')}}{\text{IP, TCA-ppt. dpm in cells, T=0'}} \times 100$$

TCA-ppt = trichloro-acetic acid precipitable

IP = immuno-precipitated

Fig. 4.7. Expression of results of apolipoprotein synthesis, degradation and secretion.

Haematoxylin and Eosin and the fat-fed preparation showed a large number of vacuolated and distended intestinal epithelial cells indicating previous lipid engorgement.

Results: Experiments

Low fat feeding (expts. 15-18)

Expt. Fifteen

Mass of animal: 177 g

Length of small bowel: 37 cm

Length of proximal jejunum chosen for enterocyte preparation: 17 cm

Mass of bowel chosen: 1.182 g

Average quantity of cell protein incubated: 96 μg

Average cellular TAG content, per incubation flask: 39 μg

Cell TAG/protein ratio: 0.41

Plasma TAG concentration: 107 mg/dl

General rate of protein synthesis (T=0' chase) 4874 dpm/μg cell protein

"High Methionine" incubation (20' pulse labelling) 995 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase time): 2.9% nett synthesis

Total protein "secreted"/hr: 7.6%

Apo B-48: synthesis (as % total protein synthesis at T=0' chase): 4.1%

degradation (0'-60' chase time): 65%hr

"secretion" (0'-60' chase time): 0%/hr i.e. undetectable in medium

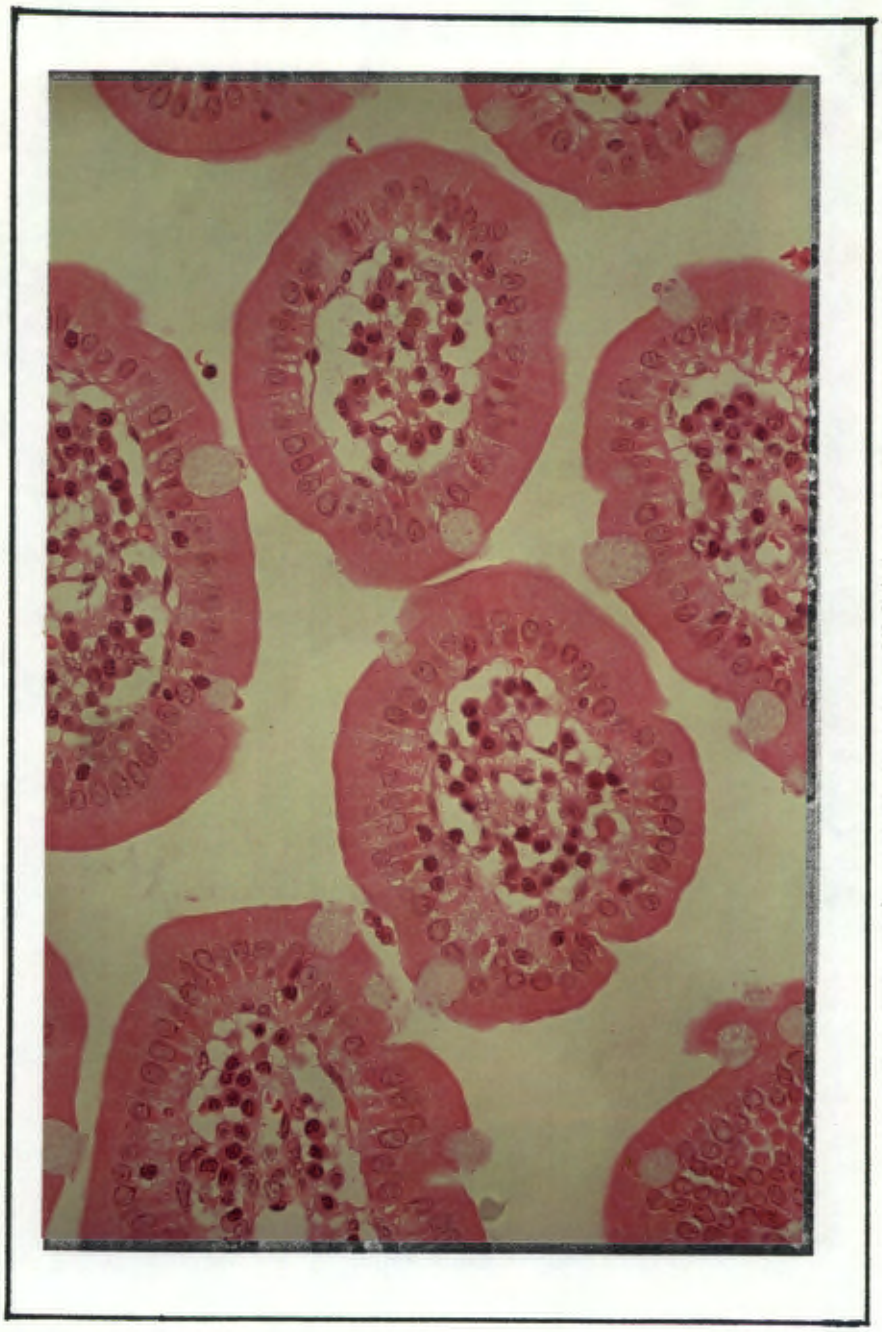


Fig. 4.8. A cross-section of jejunal villi from a low fat-fed hamster (Haematoxylin-Eosin staining).

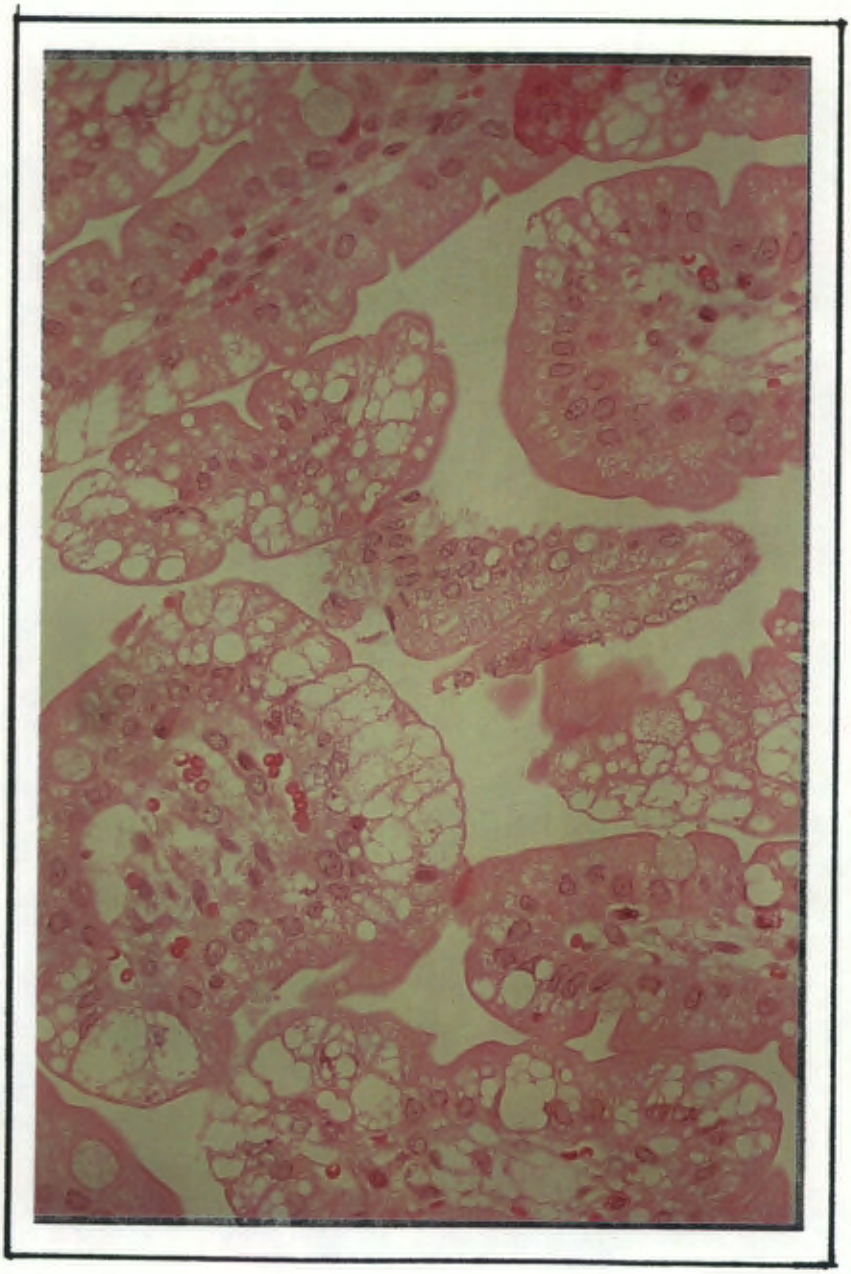


Fig. 4.9. A cross-section of jejunal villi from a high fat-fed hamster (Haematoxylin-Eosin staining) showing numerous distended, vacuolated cells indicating lipid engorgement. The lipid has been washed out by the fixing and staining procedure.

Apo A-IV: synthesis: 0.4%

degradation: 60% nett synthesis of A-IV observed (i.e. more apo A-IV present after 60' chase than at T=0' chase)

"secretion": 0%/hr

Figures 4.10 and 4.11 depict the fluorograms of the pulse-chase labelling for apolipoprotein B-48 and A-IV, respectively.

Expt. Sixteen

Mass of animal: 171 g

Length of small bowel: 44.5 cm

Length of proximal jejunum chosen for enterocyte preparation: 17 cm

Mass of bowel chosen: 1.154 g

Average quantity of cell protein incubated: 875 μg

Average cellular TAG content, per incubation flask: 144 μg

Cell TAG/protein ratio: 0.16

Plasma TAG concentration: 127 mg/dl

General rate of protein synthesis (T=0' chase): 712 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 15 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 4.9% of nett synthesis

Total protein "secreted"/hr: 22.4%

Apo B-48 synthesis (as % total protein synthesis at T=0' chase): 2.2%

degradation (0'-60' chase): 43.7%

"secretion" (0'-60' chase): 0%

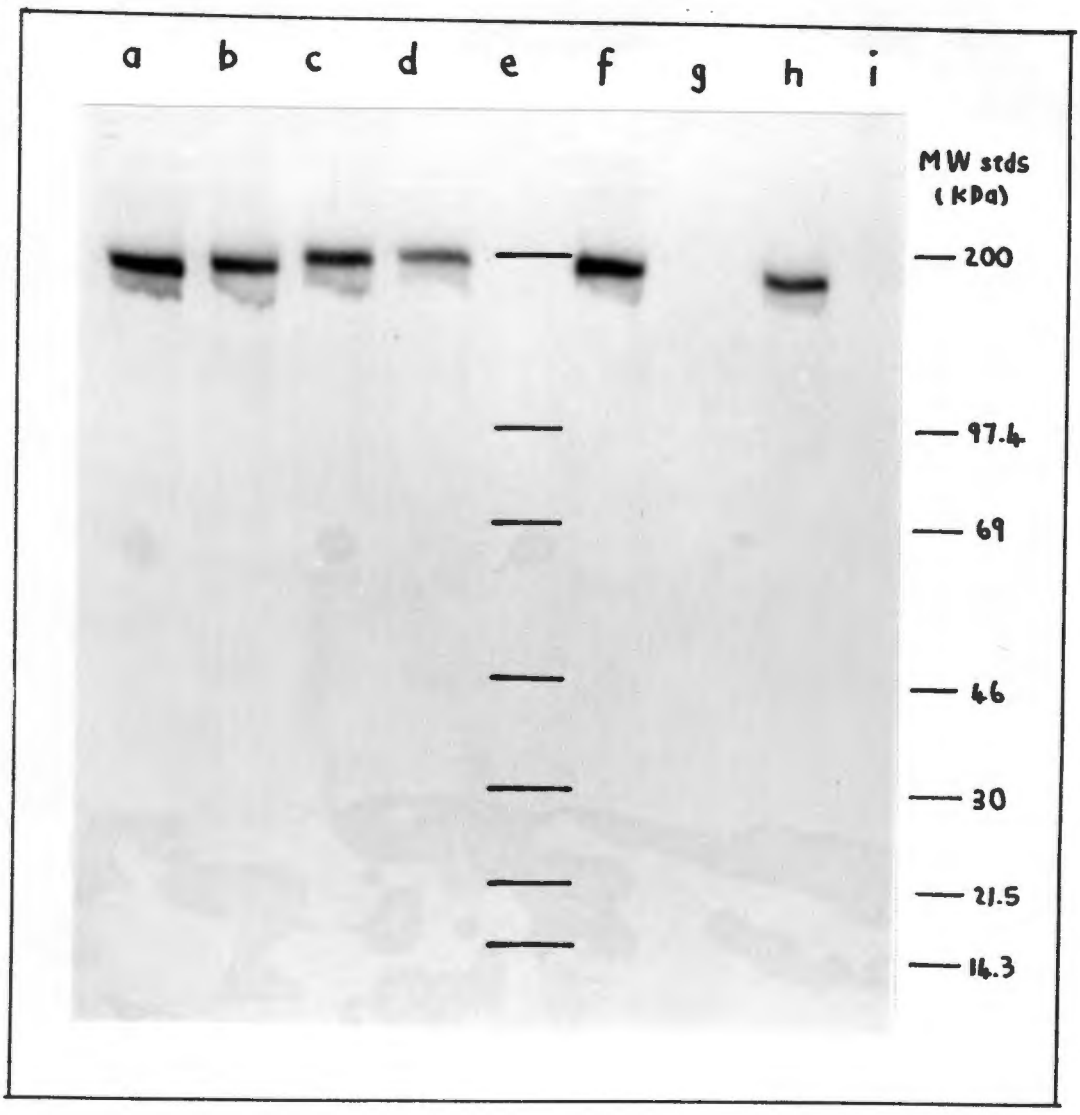


Fig. 4.10. Fluorogram showing pulse-chase labelling of apolipoprotein B-48 (MW ~ 200 kDa) following imnmuno-precipitation (IP) from enterocytes prepared from a low fat-fed animal (expt. 15) and incubated with ^{35}S-methionine.

OSAN = a commercially available rabbit-derived anti-human apolipoprotein B antiserum.

Key to lanes:

a = 0' chase, cells & medium, combined IP using "OSAN"

b = 30' " , " , " " " "

c = 60' " , " , " " " "

d = 90' " , " , " " " "

e = MW standards

f = 0' chase, cells only; IP with "OSAN"

g = 0' " , medium only, " " "

h = 60' " , cells only, " " "

i = 60' " , medium only, " " "

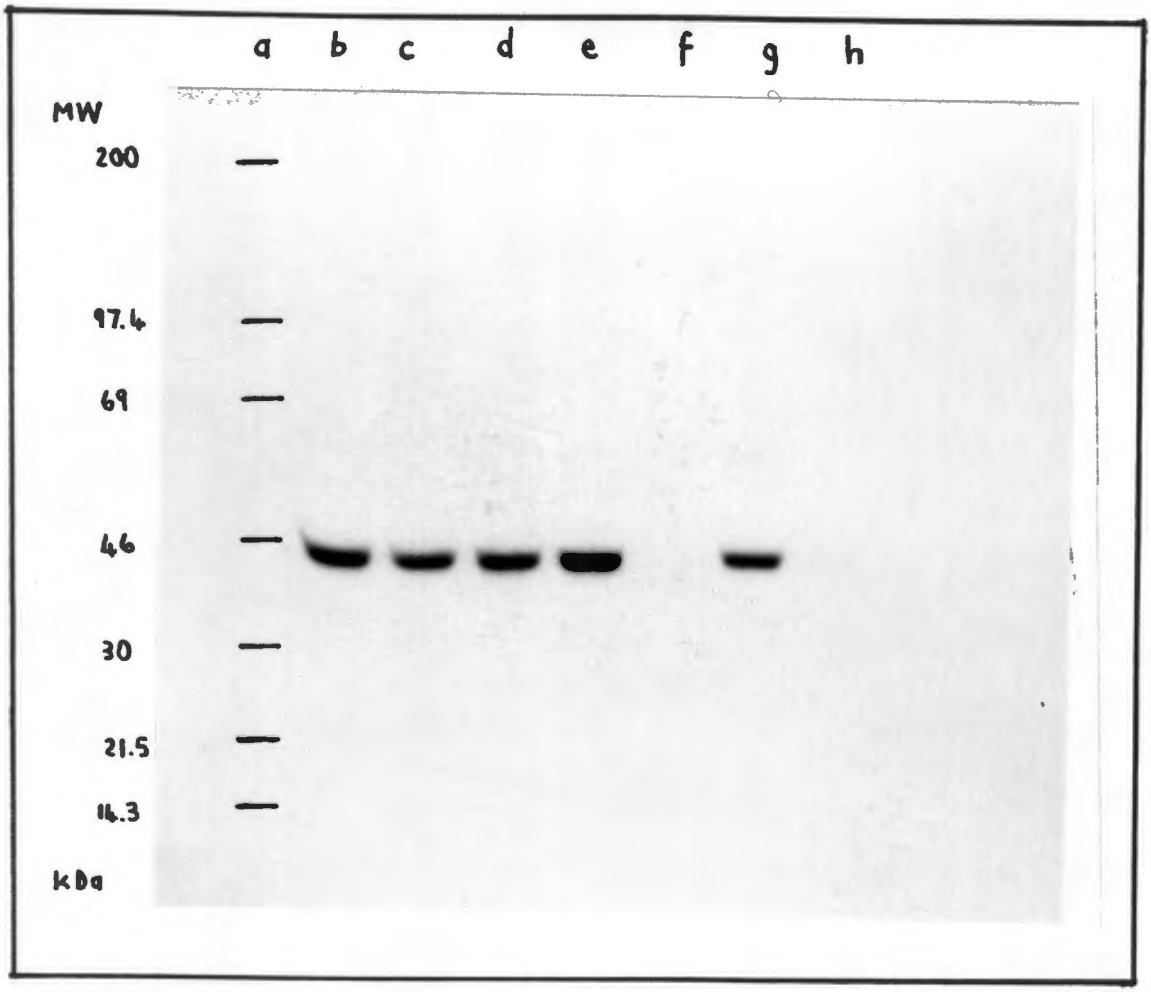


Fig. 4.11. Fluorogram showing pulse-chase labelling of apolipoprotein A –IV (MW - 44.5 kDa) following immuno-precipitation (IP) from enterocytes prepared from a low fat-fed animal (expt. 15), and incubated with ^{35}S-methionine.

Key to lanes:

a = MW stds.

b = 30' chase, cells & medium combined, IP of apo A-IV

c = 60' " " " " " " " "

d = 90' " " " " " " " "

e = 0' chase, cells only, IP of apo A-IV

f = 0' " , medium only, " " " "

g = 60' " , cells only, " " " "

h = 60' " , medium only, " " " "

Apo A-IV synthesis: 3.9%

degradation: 69.7%

"secretion": 0%

Expt. Seventeen

Mass of animal: 165 g

Length of small bowel: 43 cm

Length of proximal jejunum chosen for enterocyte preparation: 17 cm

Mass of bowel chosen: 1.147 g

Average quantity of cell protein incubated: 1000 μg

Average cellular TAG content, per incubation flask: 114 μg

Cell TAG/protein ratio: 0.11

Plasma TAG concentration: 76 mg/dl

General rate of protein synthesis (T=0' chase): 1128 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 18 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 4.3%

Total protein "secreted"/hr: 8.8%

Apo B-48 synthesis (as % total protein synthesis at T=0' chase): 0.9%

degradation (0'-60' chase): 80.8%

"secretion" (0'-60' chase): 0%

Apo A-IV synthesis: 1.6%

degradation: 78%

secretion: 0%

Expt. Eighteen

Mass of animal: 221 g

Length of small bowel: 43 cm

Length of proximal jejunum chosen for enterocyte preparation: 17 cm

Mass of bowel chosen: 1.350 g

Average quantity of cell protein incubated: 1260 μg

Average cellular TAG content, per incubation flask: 198 μg

Cell TAG/protein ratio: 0.16

Plasma TAG concentration: 128 mg/dl

General rate of protein synthesis (T=0' chase): 2143 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 30 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 13.2% nett synthesis

Total protein "secreted"/hr: 25%

Apo B-48

synthesis (as % total protein synthesis at T=0' chase): 2.6%

degradation (0'-60' chase): 0%/hr

"secretion" (0'-60' chase): 13%

Apo A-IV

synthesis: 2.5%

degradation: 9% nett synthesis

"secretion": 20%/hr

Results: High fat feeding experiments (19-22)

Expt. Nineteen

Mass of animal: 166 g

Length of small bowel: 45 cm

Length of proximal jejunum chosen for enterocyte preparation: 15 cm

Mass of bowel chosen: 1.120 g

Average quantity of cell protein incubated: 1510 μg

Average cellular TAG content, per incubation flask: 517 μg

Cell TAG/protein ratio: 0.34

Plasma TAG concentration: 327 mg/dl

General rate of protein synthesis (T=0' chase): 7797 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 34 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 13.2% nett synthesis

Total protein "secreted"/hr: 7.4%/hr

Apo B-48 synthesis (as % total protein synthesis at T=0' chase): 2.2%

degradation (0'-6-' chase): 3.5%

"secretion" (0'-6-/ chase): 28.9%

Apo A-IV synthesis: 3.7%

degradation: 35.1%

"secretion": 16.9%

Expt. Twenty

Mass of animal: 199 g

Length of small bowel: 46.5 cm

Length of proximal jejunum chosen for enterocyte preparation: 17 cm

Mass of bowel chosen: 1.234 g

Average quantity of cell protein incubated: 1390 μg

Average cellular TAG content, per incubation flask: 1710 μg

Cell TAG/protein ratio: 1.23

Plasma TAG concentration: 274 mg/dl

General rate of protein synthesis (T=0' chase): 3713 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 30.5 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 13.7% /hr

Total protein "secreted"/hr: 10.3% /hr

Apo B-48 synthesis (as % total protein synthesis at T=0' chase): 1.3%

degradation (0'-60' chase): 34.6% /hr

"secretion" 25.9 /hr%

Apo A-IV synthesis: 4.5%

degradation: 17.6% /hr

"secretion": 27.6% /hr

Figure 4.12 shows the fluorogram of pulse-chase labelling of apolipoprotein A-IV in expt. 20 (high fat feeding).

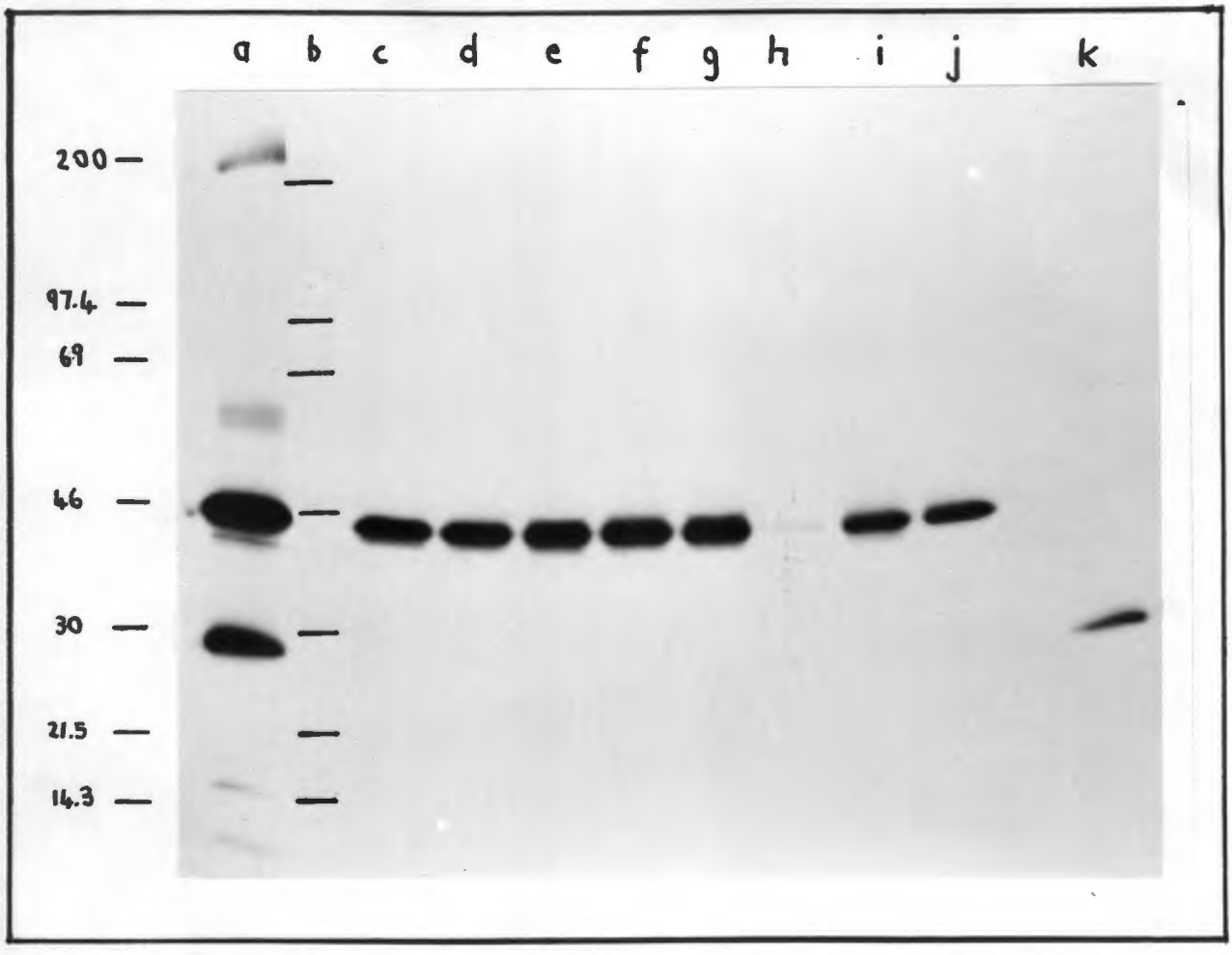


Fig. 4.12. Fluorogram showing pulse-chase labelling of apolipoprotein A-IV (MW ~ 44.5 kDa) following immuno-precipitation (IP) from enterocytes prepared from a high fat-fed animal (expt. 20) and incubated with ^{35}S-methionine.

Key to lanes:

a = medium (neat) t = 90'

b = MW standards

c = 0' chase, cells & medium, combined IP with anti-A-IV antiserum

d = 30' chase, cells & medium, combined IP with anti-A-IV antiserum

e = 60' chase, cells & medium, combined IP with anti-A-IV antiserum

f = 90' chase, cells & medium, combined IP with anti-A-IV antiserum

g = 0' cells, anti-A-IV IP

h = 0' medium, " " "

i = 60' cells, " " "

j = 60' medium, " " "

(*k* = anti-A-I IP)

Expt. Twenty-one

Mass of animal: 197 g

Length of small bowel: 45.5 cm

Length of proximal jejunum chosen for enterocyte preparation: 11.5 cm

Mass of bowel chosen: 0.787 g

Average quantity of cell protein incubated: 97 μg

Average cellular TAG content, per incubation flask: 98 μg

Cell TAG/protein ratio: 1.00

Plasma TAG concentration: 241

General rate of protein synthesis (T=0' chase): 3876 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 47 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 13.1% /hr

Total protein "secreted"/hr: 0.19% /hr

Apo B-48 synthesis (as % total protein synthesis at T=0' chase): 0.6%

degradation (0'-60' chase): 17.6%

"secretion" (0'-60' chase): 41.2%

Apo A-IV synthesis: 4.1%

degradation: 74% /hr

"secretion": 38% /hr

Expt. Twenty-two

Mass of animal: 218 g

Length of small bowel: 44.5 cm

Length of proximal jejunum chosen for enterocyte preparation: 18 cm

Mass of bowel chosen: 1.310 g

Average quantity of cell protein incubated: 921 μg

Average cellular TAG content, per incubation flask: 1608 μg

Cell TAG/protein ratio: 1.75

Plasma TAG concentration: 942 mg/dl

General rate of protein synthesis (T=0' chase): 3923 dpm/μg cell protein

"High Methionine" incubation (20' pulse-labelling): 44 dpm/μg cell protein

Total protein degradation/hr (0'-60' chase): 1.5% /hr

Total protein "secreted"/hr: 5.1% /hr

Apo B-48	synthesis (as % total protein synthesis at T=0' chase): 1.3%
	degradation (0'-60' chase): 7.4% /hr
	"secretion" (0'-60' chase): 28% /hr
Apo A-IV	synthesis: 1.9%
	degradation: 14% nett synthesis observed
	"secretion": 32% /hr

Figure 4.13 shows the fluorogram of both apo B-48 and apo A-IV pulse-chase labelling in expt. 22.

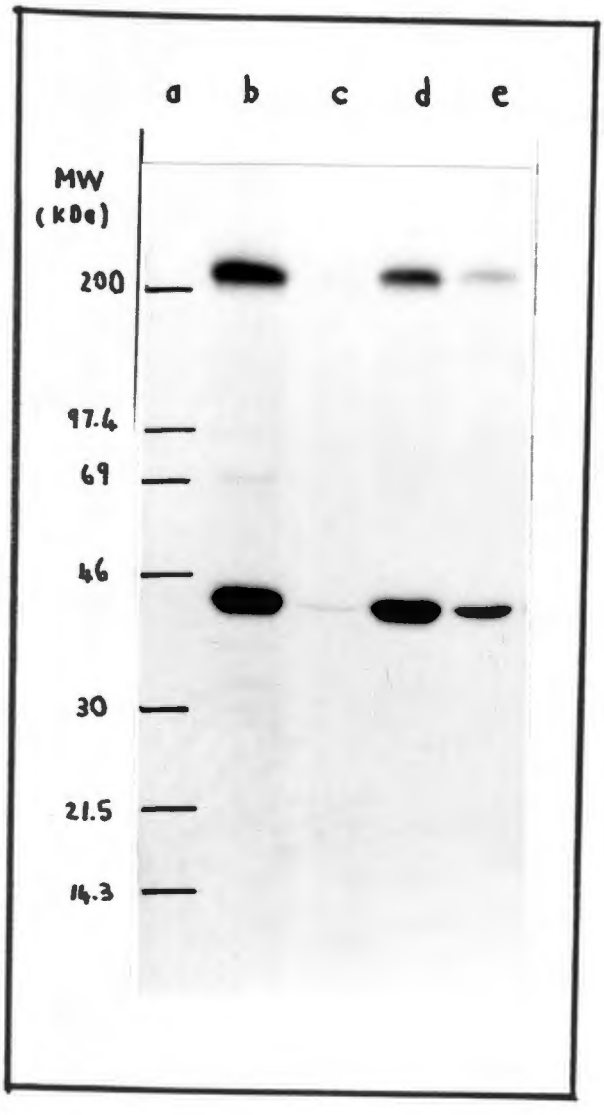


Fig. 4.13. Fluorogram showing pulse-chase labelling of apolipoproteins B-48 and A-IV (MW ~ 200 & 45 kDa, respectively) following IP from enterocytes prepared from a high fat-fed animal (expt. 22) and incubated with ^{35}S-methionine. The IPs have been combined on the same gel lane.

Key to lanes

a = MW stds.

b = cell IP, B-48 & A-IV, t = 0' chase

c = medium IP, " " " "

d = cell IP, " " t = 60' chase

e = medium IP, " " " " "

Comments

In expt. 15 (low fat-fed animal), there was no measurable protein degradation with respect to general proteins (an apparent nett 2.9% synthesis during the "chase" period was obtained), yet 65% of the Apo B-48 synthesized during the pulse-labelling was degraded in the first hour of the chase. Thus apo B-48 did not behave as an "average" cell protein: its turnover rate was much greater. The fluorograms confirmed the specificity of the immuno-precipitation with a single protein of the expected MW for the given apolipoprotein being precipitated on each occasion.

In expt. 16 (low fat-fed animal), once again the general protein degradation in the system was not measurable (an apparent nett synthesis of 4.9% was recorded in the 1 hr chase period) yet the degradation of apolipoprotein B-48 was measured at 55% per hour of chase incubation time. A similar result (4.3% general protein degradation compared with 81% of apo B-48 degradation per hour) was obtained in expt. 17.

It is noteworthy that expt. 18 produced a result rather different from those of the other 3 low fat-fed animal experiments in that apo B-48 was not degraded at all in the chase period (0% degraded/hr) (a nett synthesis of 13.2% was obtained for general proteins during the 1 hr chase period). Also, unlike the preceding 3 low fat-fed expts., secretion of apolipoprotein B-48 (13%/h) was observed in expt. 18. No apo B-48 had been detected in the medium of the first 3 low fat-fed experiments.

The results obtained with apolipoprotein A-IV were more varied with respect to the degradation rates of this protein in the 4 low fat-fed expts. In expt. 15 a 60% nett synthesis was observed, in expt. 16 a 70% nett degradation; a similar nett degradation of 78% was obtained in expt. 17, while a nett synthesis of apolipoprotein A-IV of 9% was obtained in expt. 18. Unlike the first 3 expts., in which apo A-IV was not detected

in the incubation medium after a 60' chase period (i.e. secretion = 0%/hr), in the case of expt. 18, 20% of the apo A-IV previously in the cell at the start of the chase period, was secreted into the incubation medium after a 60' chase time. Expt. 18 therefore appeared inconsistent with the three other low fat-fed expts., and, in fact, more closely resembled the results obtained with the high fat-fed expts.

In all 4 high fat-fed expts. the degradation rates of apolipoprotein B-48 were lower than those obtained in 3 out of the 4 low fat-fed expts.: expt. 19 (B-48 degradation = 3.5%; general protein degradation = 13.2%); expt. 20 (apo B-48, 34.6% degraded/hr; general proteins: 13.7% per hour); expt. 21 (apo B-48, 17.6% degraded/hr; general proteins, 13.1% per hr), and expt. 22 (apo B-48, 7.4% degraded/hr compared with 1.6% degradation per hr in the case of general cell proteins). In all the high fat-fed animal expts., apo B-48 secretion into the chase incubation medium was observed: 29%, 26%, 41% and 28% secreted/hr in expts. 19 to 22, respectively: all these values were above the "general" protein secretion observed in each experiment.

The degradation rates for apolipoprotein A-IV in the case of the high fat-fed experiments were 35.1%, 17.6%, 74% and 14% nett synthesis, for expts. 19 to 22, respectively. The results for apo A-IV degradation are therefore less consistent and more varied. However, apo A-IV was consistently found to be secreted in the case of the 4 high fat-fed expts. (secretion rates of 17, 27.6, 38 and 32% secretion per 60' chase incubation time, respectively, for the 4 high fat-fed expts.).

The TAG content of the cells (expressed as a ratio relative to the cell protein content) was generally higher in the high fat-fed (HFF) compared with the low fat-fed (LFF) expts. LFF: ratios of 0.41, 0.16, 0.11 and 0.16 compared with HFF ratios of 0.34, 1.23, 1.00 and 1.75 respectively, indicating higher mucosal TAG content and, therefore, mucosal TAG flux in the HFF expts. (except for expt. 19). The plasma TAG

concentrations of the HFF animals were all greater than those of the LFF animals (327, 274, 241 and 942 mg/dl, HFF, versus 107, 127, 76 and 128 mg/dl for the LFF animals).

A review of the 22 experiments presented in this chapter shows:

(i) 7 experiments involving animals fed the same low fat (rice only) diet for 20 hours (expts. 1, 2, 9, 10, 15-17);

(ii) 8 experiments in which all the animnals were fed a high unsaturated fat diet (rice:oil, 80:20 w/w) for 20 hours (expts. 7, 8, 11, 12, 19-22).

Within these 2 groups of experiments, the dietary regimes followed were exactly the same.

In all the experiments the % protein synthesis for each apolipoprotein studied was expressed in the same way viz. as that proportion of radioactive TCA-precipitated material present in the specifically immuno-precipitated apolipoprotein relative to the total TCA-precipitated material, following a twenty minute radio-labelling period. The only difference between expts. 1, 2, 7, 8, 9, 10, 11, 12 and expts. 15-22 was that in the former group the incorporation of radio-labelled amino acid into protein was stopped by transferring the incubation flask from a 37°C orbital shaker/waterbath onto ice; in the latter group it was abolished by the addition of excess "cold" amino acid (2 mM methionine, final concentration) i.e. to effect the "chase", with a ten minute equilibration period.

The "percentage synthesis" results of these experiments are therefore directly comparable, and a statistical review of the results is presented in Tables 4.4 (A)-(C).

Degradation and secretion studies could only be studied using the pulse-chase method employed in expts. 15-22. Because of the atypical nature of expt. 18 (apolipoprotein

secretion despite the enterocytes being derived from an apparently low fat-fed animal), I have regarded it as an outlier and have excluded it from the statistical analyses.

The rates of synthesis of the apolipoproteins showed considerable variation even amongst animals within the high or low fat-fed groups, but the overall result indicated that there were no significant differences in the synthesis rates of apolipoprotein B-48 or A-IV between the 2 dietary groups. This finding is consistent with other studies in the literature of apo B-48, but it contradicts previous findings of increased apo A-IV synthesis seen with acute (or chronic) dietary lipid feeding (e.g. Apfelbaum *et al.* 1987).

4.4 A

APO B-48 % synthesis			
LFF		HFF	
expt no.		expt no.	
1	1.32%	5	0.77%
2	1.24	6	0.73
9	2.19	11	0.90
10	1.38	12	0.45
15	4.1	19	2.2
16	2.2	20	1.3
17	0.9%	21	0.6
		22	1.3
$\bar{X} \pm SD$	n = 7 1.90 ± 1.08	$\bar{X} \pm SD$	n = 8 1.03 ± 0.56
unpaired t test	p = 0.07	NSS	

Table 4.4. A & B. Statistical analyses of Apo B-48 synthesis & degradation expts. (NSS = not statisticall y significant, LFF = low fat-fed; HFF = high fat-fed).

4.4 B

APO B-48 DEGRADATION			
LFF		HFF	
expt		expt	
15	65%/hr	19	3.5%/hr
16	44	20	35
17	81	21	18
		22	7.4
$\bar{X} \pm SD$	n = 3 63.3 ± 18.6		n = 4 16.0 ± 14.1
unpaired t test	p = 0.012	(p < 0.05)	

Wilcoxon Two Sample Test: p = 0.034 (< 0.05)

4.4 C

APO B-48 Secretion % apo B-48 "secreted"/ hr.			
LFF		HFF	
Expt. No.		Expt. No.	
15	0	19	29%
16	0	20	26
17	0	21	41
		22	28

obviously significantly different

4.4 D

APO A-I % synthesis			
LFF		HFF	
expt. no.		expt. no.	
1	2.0%	5	2.6%
2	2.6	6	2.8
9	2.14	11	0.90
10	0.90	12	1.60%
n = 4		n = 4	
$\bar{X} \pm SD$	1.81 ± 0.91		1.98 ± 0.89
unpaired t test	p = 0.80 NSS		

Table 4.4. C & D. Apo B-48 secretion & Apo A-I synthesis analyses.

4.4 E

APO A-IV % synthesis			
LFF		HFF	
expt.		expt.	
1	7.0 %	5	6.0 %
2	4.5	6	4.8
15	0.4	19	3.7
16	3.9	20	4.5
17	1.6	21	4.1
		22	1.9
n = 5		n = 6	
$\bar{X} \pm SD$	3.48 ± 2.58	4.12 ± 1.36	
unpaired t test	p = 0.58	NSS	

4.4 F

apo A-IV % degradation / hr			
LFF		HFF	
expt		expt.	
15	60% nett synthesis	19	35% degrad^n
16	70% degrad^n	20	18% degrad^n
17	78% degrad^n	21	74% degrad^n
		22	14% nett synthesis
n = 3		n = 4	
Results not calculable because of nett synthesis observed in expts 15 & 22			

4.4 G

apo A-IV "secretion" % / hr			
LFF		HFF	
expt.		expt.	
15	0 %	19	17 %
16	0	20	28
17	0	21	38
		22	32
n = 3		n = 4	
results obviously significantly different			

Table 4.4. E-G. Results of Apo-AIV synthesis, degradation & secretion.

4.8 ANIMAL-TO-ANIMAL EXPERIMENT VARIATION: POSSIBLE EXPLANATIONS

A pervasive problem in these animal experiments has been the variation in the results from animal to animal (or from one enterocyte preparation to the next). Observe, for example, the large variations in many of the synthesis, secretion and degradation results presented. Standardization of results was attempted as far as possible (e.g. by use of the "high methionine" system to correct for possible variations in the intracellular methionine pools from one animal's enterocytes to the next; also, the expression of results relative to the cell protein content, thus correcting for variations in the cell yield for the different intestinal segments and for varying quantities of cells incubated). Despite these measures, however, animal to animal variation (within a given dietary group) remained an unsolved problem.

Some of these difficulties encountered may be explained by the peculiarities of the hamster digestive tract (see chapt.2.1). An important question in all dietary experiments is the timing of the killing of the animal following the dietary perturbation. This is especially important in the acutely fat-fed animals (bolus lipid administration); it is probably less important in the animals fed for 20 hrs on a given diet and is unlikely to be a significant factor in the chronic adaptation diets of six weeks duration. Ideally one would wish to obtain the enterocytes from the proximal jejunum during the period of maximal transmucosal TAG flux. In my experiments a 2 hr period of food deprivation was used following the last observed meal to allow for gastric emptying into the proximal small bowel. However, the hamster has a forestomach (see Fig. 2.1) in which food may be stored for anything from 10 minutes to an hour or even longer (Borer, 1985) and this could unpredictably delay gastric emptying.

The problem is further complicated by the hamsters' internal cheek pouches which they use to store and transport (non-liquid) food. In my experiments the animals were

initially maintained on a standard laboratory "chow" ("Epol" pellets) which contained approximately 4% of mainly polyunsaturated fat, w/w. At the onset of the 20 hr feeding regimes they were transferred to new sawdust-free cages containing only the new experimental dietary food. Their cheek pouches were inspected (as far as could be done) for any pellets but it is conceivable that some hoarding may have been missed. It is feasible that the tiny quantity of fat present in a stray pellet may contribute to alterations in apolipoprotein synthesis and degradation (see later, for a discussion of a model for apolipoprotein B-48 fluxes in the enterocyte).

Returning to the hamster forestomach, it is known that this organ is capable of fermenting cellulose to produce fatty acids (Borer, 1985). Thus even sawdust may contribute additional fatty acids and it is conceivable that the odd "low fat" experiment (see experiment 18, for example) may have contained more fat or fatty acids from pellet hoarding or forestomach fermentation than one might have believed at the time.

Whole animal experiments are generally known to produce wide variations in results (see, for example, the studies of Davidson *et al.* (1985, 1986) on rat intestinal apolipoprotein biosynthesis). Nevertheless, despite the variation, a picture - at least of apolipoprotein B-48 fluxes in the intestine - does appear to emerge from my experiments, and is supported by previously published data especially concerning degradation studies of apolipoprotein B in liver cell cultures.

4.9 A MODEL FOR INTESTINAL APOLIPOPROTEIN FLUXES?

The rates of apolipoprotein B-48 synthesis were unaltered by dietary lipid (compared with the low fat fed controls), yet the secretion of this protein increased, as one might have expected with increased chylomicron packaging and "export" from the cells. In 3 of the 4 low fat-fed animal experiments, the rates of apolipoprotein B-48 degradation were significantly higher compared to the high fat-fed state. These findings support a model for apolipoprotein B-48 fluxes previously discussed in section 1.8(i) in which the rate of protein synthesis is constant and unaltered by dietary lipid flux (acute or chronic) and in which the rate of protein synthesis is probably always maximally "up-regulated"; this produces a large intracellular pool of pre-formed apolipoprotein B-48 which is always available in the cell should a high lipid load arrive. During periods of low lipid flux a significant proportion of the apolipoprotein B-48 intracellular pool (not utilized in lipoprotein packaging) is degraded intracellularly; with the advent of a large dietary lipid flux, the synthesis rate remains unchanged but the degradation rate of apolipoprotein B-48 is significantly reduced, so that the size of the intracellular pool is perhaps momentarily increased to accommodate the need for increased lipoprotein packaging. With increased utilization of the apolipoproteins for lipoprotein assembly and packaging, the pool is reduced again to its previous size. Hypothetical figures for rates of apolipoprotein B-48 synthesis, degradation and secretion are used to illustrate the model diagramatically (fig. 4.14).

Most of my experiments (excepting for expt. 18) lend support to this model. It is further supported by evidence from the Glickman group who showed no increase in apolipoprotein B-48 synthesis with acute or chronic dietary lipid flux (Davidson *et al.* 1986 and 1987) and who also measured the distribution of apolipoprotein B-48 in 2 intracellular pools or compartments viz. the predominantly membrane-associated, non lipoprotein-bound intracellular pool, and a lipoprotein-associated pool. They found that at any given time most of the apolipoprotein B-48 was membrane-associated; with lipid

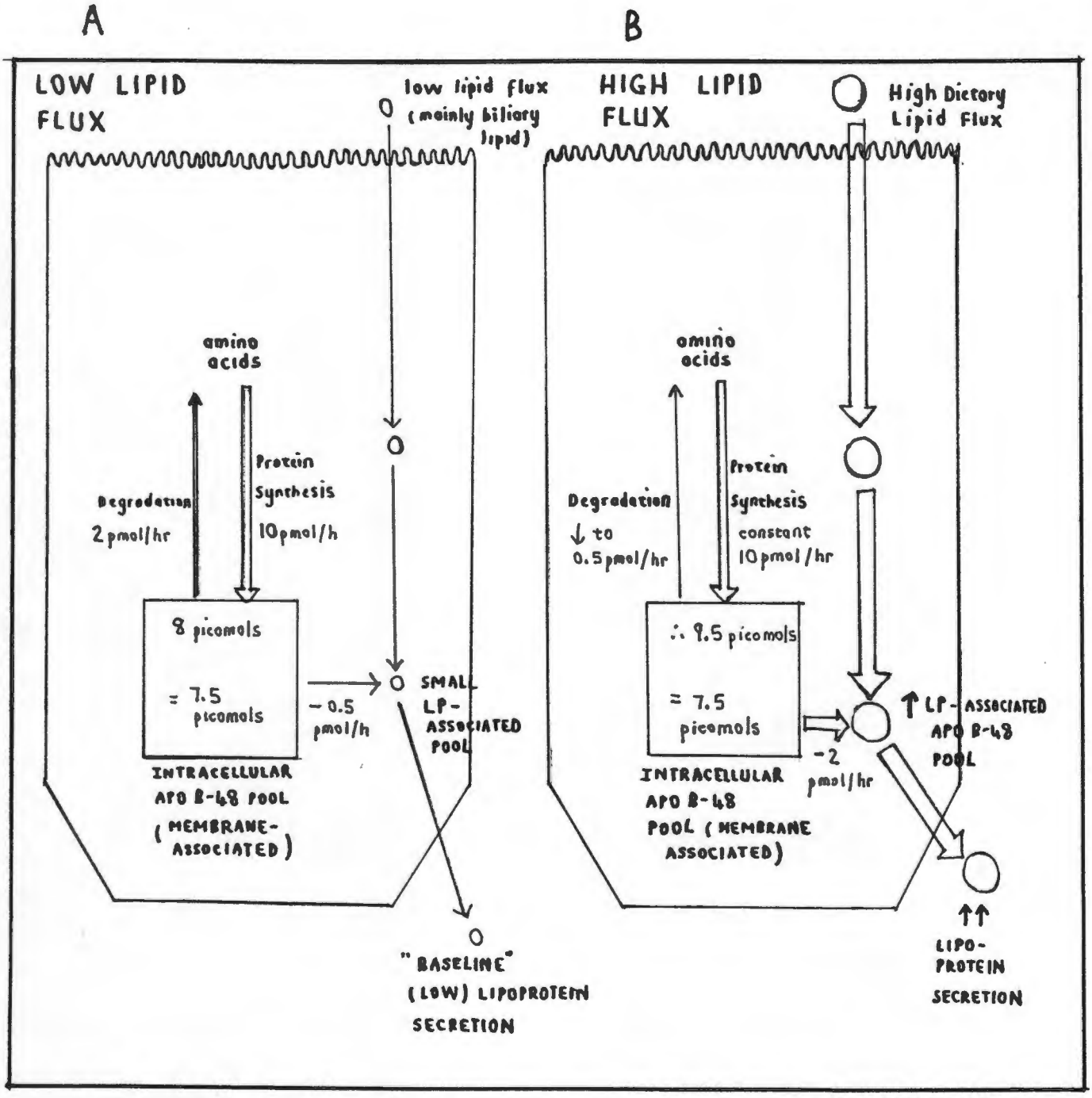


Fig. 4.14. Schematic representation of a model for apolipoprotein B-48 dynamics under conditions of (A) low, & (B), high fat-feeding.

feeding, a small shift occurred from the membrane-associated to the lipoprotein-associated pool, but most of the intracellular apolipoprotein B remained in the membrane-bound pool. The constant presence of this large pool inside the cell meant that apolipoprotein synthesis was not the rate-limiting step in lipoprotein assembly or secretion (Magun *et al.*, 1988).

Measurements of apolipoprotein B-48 degradation in the small intestine have not previously been described in the literature. However, hepatocyte studies of apo B by Borchardt & Davis (1987), Dixon & Ginsberg (1991 and 1993) and White *et al.* (1992) have supported the idea of enhanced apolipoprotein B degradation in the absence of exogenous (medium) fatty acid. With the addition of the fatty acid oleate to hepatocytes of the hepatoma cell line, Hep G2, the synthesis of apoB was unaltered but the secretion of apo B-containing lipoproteins was enhanced by reducing the pre-secretory degradation of the protein. Fatty acids therefore appear to protect nascent apo B from rapid intracellular degradation and a similar mechanism is likely to operate in the enterocyte.

In three of my experiments performed under conditions of dietary lipid depletion, there was no detectable secretion of the apolipoproteins (including B-48) and this coincided with a high rate of B-48 degradation intracellularly. (The enterocytes of the fourth "low fat-fed" hamster - expt. 18 - behaved in a manner that strongly suggested the presence of some lipid in the diet - apo B-48 was secreted into the medium and there was no measurable intracellular degradation of the protein during the chase period).

The Hep G2 studies of Dixon & Ginsberg and White have confirmed that under lipid-poor conditions only a small fraction of newly synthesized apolipoprotein B is actually secreted; most of the molecules are rapidly degraded inside the cells.

In a recent study by Sakata *et al.* (1993), the calpain inhibitor I, N-acetyl-leucyl-leucyl-norleucinal (ALLN) was used to study the possible mechanisms underlying these processes in Hep G2 cells. They showed that ALLN protected apo B from rapid intracellular degradation, thus increasing the intracellular apo B pool (and, therefore, its potential to be secreted), but did not actually accelerate its secretion; oleate, on the other hand, promoted the secretion of the protein from the cells, while the combined effects of oleate and ALLN were additive, resulting in an acceleration and increase in the total apo B secretion.

A possible model proposed by the authors to explain this finding was that oleate facilitated the transport of apo B out of a protease-containing compartment associated with the endoplasmic reticulum (the protease being sensitive to ALLN). By perhaps associating with intracellular lipid, the apo B molecules were rapidly translocated beyond the protease-containing compartment and along the secretory pathway. A direct inhibitory effect of oleate on the proteolysis of apo B could not, however, be excluded by their results (Sakata *et al.* 1993).

In my experiments the fatty acids were supplied by the previous dietary treatments rather than by the *in vitro* introduction of exogenous fatty acids to the medium of the incubated enterocytes i.e. the *in vitro* incubation served only as an assay for radio-labelled apolipoprotein synthesis, degradation and secretion and was not experimentally modified (except during initial optimization expts - see chapt. 2). Pulse-chase studies of apolipoprotein degradation are not feasible in *in vivo* experiments.

Although data for apo A-I degradation are lacking in my experiments (due to a lack of antiserum for immuno-precipitation), the model for apo A-I may be presumed to be similar to that of apo B-48 (see discussion 1.8 (ii)). The experimental work of Magun *et al.* (1988) supports this idea.

A model for apolipoprotein A-IV is more problematic. Previous workers have consistently shown that the synthesis of apolipoprotein A-IV is enhanced by dietary lipid. The mRNA for this protein, the radio-labelled amino acid incorporation into Apo A-IV, the total intracellular apo A-IV content of the cells, the lymphatic content of apo A-IV and even the postprandial plasma concentrations of apo A-IV have all been shown to increase after lipid feeding (see review 1.8 (iii)). The regulation of this protein has therefore generally been accepted to operate at a pre-translational level.

In my experiments, however, despite a quantitative immuno-precipiation of apo A-IV, its rate of synthesis remained unaltered by lipid feeding (although, once again, there was considerable animal to animal variation in the results); its secretion, however, increased with high fat feeding and its degradation was similar in both low and high fat-fed animal enterocytes. If these results are to be believed, the intracellular pool of apolipoprotein A-IV should have fallen significantly following lipid feeding, given increased secretion yet similar rates of synthesis and degradation.

An attempt was made to measure the size of the intracellular apolipoprotein A-IV pool. In the absence of a suitable radio-immunoassay for the purpose, an experiment was devised in which serial immuno-precipitations using a constant quantity of radio-labelled cell lysate from an incubation experiment of a fat-fed and a non fat-fed animal were performed. The amount of antiserum used was progressively increased (1, 2, 5, 10 etc. μl of antiserum). The radio-labelled immuno-precipitable protein products were run on an SDS-polyacrylamide gel, and a fluorogram obtained. The quantity of radio-labelled protein immuno-precipitated by the differing amounts of antiserum were measured by densitometric scanning of the fluorographic bands. The density of the individual bands was expressed relative to the density of the largest band (obtained when the standard 25 μl of antiserum was used). A graph was then plotted of the log 1/antiserum versus the % density of the resultant band for both the non-fat and the fat-fed preparations. Both

graphs yielded very similar gradients (-70 and -73, respectively) indicating no significant differences in the intracellular apo A-IV pool sizes between the LFF and HFF preparations. A coherent model for apo A-IV fluxes in the small intestine based on my experiment results was therefore not possible, since synthesis, degradation and pool size all appeared to remain constant, despite an increase in apo A-IV secretion from the cells.

In 1990 Hayashi *et al.* showed, using the inhibitor of chylomicron assembly and secretion, Pluronic L-81, that the stimulus for increased apo A-IV synthesis was not the actual absorption of MAG & FFA into the cell or its re-synthesis into lipid; rather it was the events of chylomicron assembly and secretion that stimulated apo A-IV synthesis. Perhaps a relative secretion block existed in my *in vitro* system in which chylomicron secretion did occur but not to the extent that it would *in vivo*; perhaps this reduced secretion (due to a lack of calcium intracellularly, say, or to the lack of external stimuli that depended on normal intestinal cellular architecture or an intact systemic blood supply) resulted in a reduced stimulus to apolipoprotein synthesis and a consequent failure to make more apo A-IV in the cell. This would not have been observed in the case of apo B since its regulation was, in any case, not controlled at a pre-translational level and the protective effect of the fatty acids on early apo B degradation may still have been operating even in a collection of enterocyte sheets with a defective lipoprotein secretory mechanism. I shall return to the question of the "validity" of my enterocyte system i.e. how closely it resembles the physiological processes of the cell *in vivo*, in the concluding chapter (chapt. 7).

Finally, I was unable to perform any measurements of the relative mRNA abundance of the apolipoproteins under the various dietary conditions owing to a lack of a suitable probe for the hamster mRNA. Attempts using a rat cDNA probe for the hamster apo B mRNA were unsuccessful.

CHAPTER FIVE

CO-AND POST-TRANSLATIONAL MODIFICATION OF APOLIPOPROTEINS

5.1 INTRODUCTION: N-LINKED GLYCOSYLATION

The polypeptide backbone of human apolipoprotein B undergoes a variety of co-or post-translational modifications. There are 25 cysteine residues in apolipoprotein B-100, 12 of which occur in the first 500 amino acids. 6 out of every 7 of the cysteine residues are involved in intra-molecular disulphide bonds and the amino terminus of the molecule is especially highly cross-linked (Knott *et al.* 1986, Yang *et al.* 1986). N-linked glycosylation has been demonstrated and there is some indirect evidence suggesting the presence of O-linked sugars as well. Palmitate and stearate fatty acylation via covalent thio-ester linkages at cysteine residues has been demonstrated in apo B-100 from the hepatoma-derived cell line, HepG2 (Hoeg *et al.* 1988). Apolipoprotein B-100 also displays 2 intra-molecular thio-ester linkages (between cysteine 51 and glutamine 54, and cysteine 3734 and aspartate 3737, respectively).

From the amino acid sequence of apolipoprotein B-100, 19 potential glycosylation sites for N-linked oligosaccharides have been found (sequence Asn-X-Thr/Ser). Of these 16 have been shown to be glycosylated. Since apolipoprotein B-48 comprises the first 2152 amino acid residues of the N-terminal portion of B-100, there are six putative sites for N-linked oligosaccharides, of which 5 are likely to be glycosylated. (The first potential site at asparagine residue 7 has been shown not to be glycosylated in B-100 and B-48). Studies of hepatic B-100 from plasma LDL have revealed that 37% of the oligosaccharide structures consist of the high-mannose type with the rest being mainly bi-antennary complex oligosaccharides (Scott 1989 & 1990). A study of the oligosaccharide chains of human small intestinal apo B-48 by Sasak, Lown and Colburn (1991) showed that 16% of the oligosaccharides were of the high-mannose type, with 78% bi-antennary complex oligosaccharide structures and the remaining 6% consisting of higher-branched oligosaccharides. Of the bi-antennary complex sugars, half were sialylated, with a ratio of mono- to di-sialylation of 1:2. Knowing the mono-saccharide composition and the proportion of different types of N-linked oligosaccharides, it has

been calculated that human intestinal apo B-48 has, on average, one high-mannose type and 4 complex-type oligosaccharide chains.

There is thus a difference in the glycosylation pattern of B-100 and B-48. This may be organ-specific (liver versus small intestine) but the role of the carbohydrate modification is unknown. Some postulated functions have included protection against proteolysis (and hence increased molecular stability) and roles in affecting the insertion of the protein into membranes or the phospholipid layer of lipoproteins, intra-cellular migration and sorting as well as secretion. However, a study by Siuta-Mangano *et al.* in 1982 showed that N-linked glycosylation was not required for the secretion of apolipoprotein B from chicken or rat liver. In the condition of chylomicron retention disease, where malabsorption of fat occurs with low concentrations of plasma lipids and cholesterol and the absence of chylomicrons in the plasma, lipid droplets together with apolipoprotein B-48 accumulate in the small intestinal cells of these patients (Roy *et al.* 1987). When compared with normal subjects the incorporation of mannose into intracellular chylomicrons was reduced by 80% (Levy *et al.* 1987). Since apo B-48 is the only apolipoprotein present in chylomicrons that is known to contain N-linked oligosaccharides, it has been suggested that a specific apo B-48 glycolsylation deficiency occurs in this condition.

Tunicamycin is a compound which inhibits the enzyme catalyzing the addition of N-acetylglucosamine to dolichol phosphate, the first step in the biosynthesis of an oligosaccharide-pyrophosphoryl-dolichol complex (Murray 1988). Normally, following completion of the primary oligosaccharide chain, it is transferred en bloc from the dolichol pyrophosphoryl backbone to form an N-glycosidic bond with one or more specific asparagine residues on the protein during its synthesis on membrane-bound polyribosomes. The modification is therefore co-translational. Subsequent processing or trimming of the oligosaccharide chain on the protein to form either high-mannose or complex-type sugars takes place at first on the nascent peptide attached to endoplasmic

reticulum-bound polyribosomes, and later, on the cis, medial and trans cisternae of the Golgi apparatus.

5.2 AN EXPERIMENT USING TUNICAMYCIN

This experiment has previously been described in Chapter 3.10 (ii).

5.3 RESULTS AND DISCUSSION

The fluorogram (Fig. 5.1) showed a decrease from approximately 185 to 173 kilodaltons in the molecular weight of apolipoprotein B-48 in the tunicamycin-treated system after 60 minutes chase time compared with the system in which Tunicamycin was absent. This decrease was observed in both cell lysate immuno-precipitations and media samples. Furthermore, when quantified by densitometric scanning relative to the apo A-I or apo A-IV bonds of the same sample or lane (which were unaltered by tunicamycin treatment), the quantities of radio-labelled apolipoprotein B-48 were similar, with or without tunicamycin treatment. (The apparently denser B-48 bands in the tunicamycin-treated samples reflected differences in the total quantity of cell protein incubated in the 2 systems).

Thus, hamster small intestinal apo B-48 possesses N-linked oligosaccharide chains. This carbohydrate modification does not appear to affect its secretion or degradation in an *in vitro* pulse-chase incubation experiment of enterocyte sheets from a fat-fed animal. This finding is supported by work in rat and chicken hepatocytes which also showed no interference with apo B secretion by tunicamycin treatment (Siuta-Mangano *et al.* 1982; Forte & Nordhausen 1984).

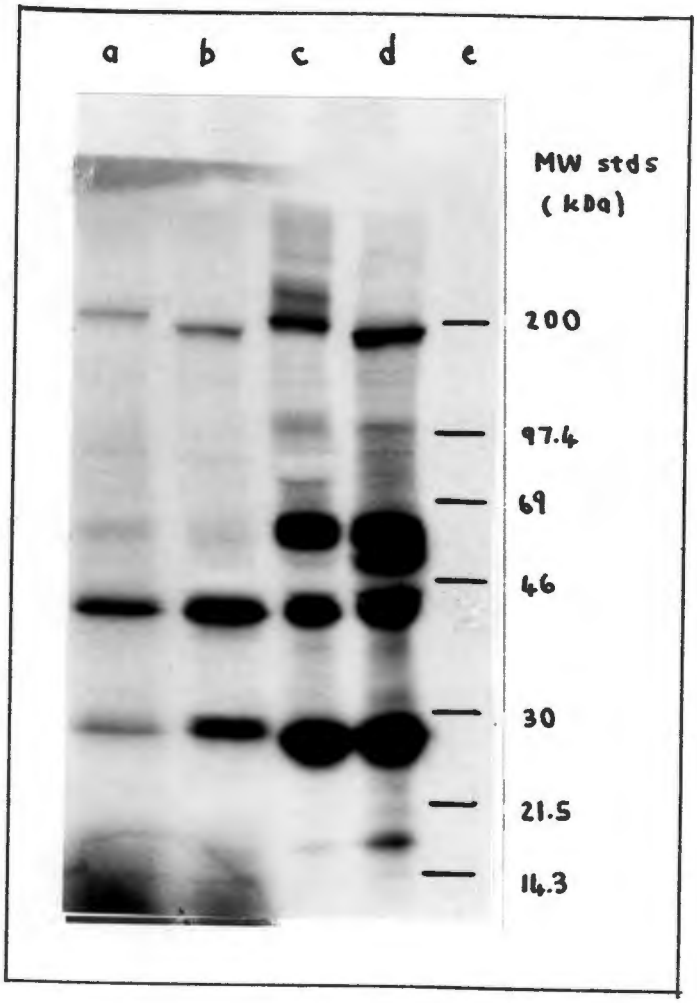


Fig. 5.1. Pulse-chase experiment in the presence or absence of tunicamycin. Enterocytes from a fat-fed hamster were incubated with ^{35}S-methionine in the presence or absence of tunicamycin (10 μg/ml final concentration). After a 20' pulse, cold methionine was added and a subsequent 60' "chase" period followed. The cell lysates and media samples were immuno-precipitated using "Rabbit 5" antiserum.

Key to lanes:

a = medium following chase period, without tunicamycin + R5 IP

b = medium following chase period in presence of tunicamycin

c = cell lysate, IP with R5 antiserum, without Tunicamycin

d = cell lysate, IP with R5 antiserum, in presence of tunicamycin.

5.4 PHOSPHORYLATION

The covalent modification of proteins and enzymes by reversible phosphorylation and dephosphorylation is an important mechanism by which intracellular events are regulated in mammalian tissue. A number of studies have demonstrated phosphorylation of apolipoprotein B (Davis *et al.* 1984 & 1986; Sparks *et al.* 1988 & Jackson *et al.* 1990).

Experiments were attempted to demonstrate phosphorylation of apo B-48 using [^{32}P] orthophosphate in the enterocyte sheet incubation system, but due to technical difficulties they were unsuccessful and inconclusive.

5.5 CONCLUSION

Hamster intestinal apolipoprotein B-48 undergoes N-linked glycosylation. Neither the secretion nor the degradation of the protein was affected when N-linked glycosylation was blocked in a pulse-chase experiment.

CHAPTER SIX

CONCLUSION:
APOLIPROTEIN BIOSYNTHESIS AND TURNOVER IN MAMMALIAN SMALL INTESTINAL EPITHELIUM

In order to study the dynamics of apolipoprotein biosynthesis and turnover in the mammalian small intestine, a system was established whereby intestinal epithelial cell (enterocyte) sheets could be freshly isolated from the hamster jejunum and incubated *in vitro* in a supportive medium. (The hamster was chosen as the best available small animal-model of human lipoprotein metabolism). The dietary treatments being tested were performed on the intact animals prior to killing and cell preparation on the assumption that metabolic activities would be maintained during the preparation and incubation of the cell sheets. The methods of cell preparation and incubation were based on a description by Towler *et al.* (1978) but were modified and optimized for the present purposes. The majority of the cells remained intact and viable during the incubation period and were capable of synthesizing and secreting apolipoproteins. The three principal intestinal apolipoproteins, B-48, A-IV and A-I, were identified by specific immuno-precipitation, and the method of pulse-chase labelling enabled measurements of apolipoprotein synthesis, secretion and degradation to be performed.

Previous systems devised to study the regulation of intestinal apolipoprotein synthesis (for example, the Glickman model of pulse labelling *in situ* in anaesthetized rats) precluded studies of protein degradation; there are, in fact, no reports in the literature to date on turnover studies of apolipoproteins in the intestinal epithelium. Studies have, however, been performed in rat hepatocytes in primary culture and in the hepatoma cell line, HepG2 (Borchardt & Davis 1987; Dixon *et al.* 1991; Dixon & Ginsberg 1993; White *et al.* 1992). A potential cell culture model for the human small intestinal epithelium, the human colonic carcinoma-derived CaCO-2 cell line, has been used to study lipid and

apolipoprotein synthesis and secretion (Hughes *et al.* 1987 and 1988) but many features of this cell line (e.g. the secretion of lipoproteins of unusual density, the predominance of apo B-100 secretion over apo B-48) have called into question the reliability of this model in reflecting normal small intestinal epithelium. In any event, no studies of apolipoprotein degradation have been reported in CaCO-2 cells.

In the past, the use of freshly isolated intestinal epithelial cells has generally been limited to studies of amino acid and sugar transport across the epithelial cell (Wilson & Vincent 1955; Alvarado, 1968; Bihler & Crane 1962; Kimmich (review), 1975). A paper by Yousef and Kuksis (1972) reported the *in vitro* release of chylomicrons from isolated cells of the rat intestine that had been "pre-loaded" *in vivo* by dietary-derived lipid. The group of Sviridov *et al.* (1986 and 1990) have used freshly isolated epithelial cells of the human small intestine to study their interactions with high density lipoproteins.

Isolated epithelial cells have a (theoretical) advantage over whole bowel or intact animal studies in allowing the study of the "genuine" newly secreted intestinal lipoproteins and apolipoproteins, free from the subsequent interactions and exchanges of material with other circulating lipoproteins that are thought to occur soon after the nascent particles are released from the cells. In order to produce sufficient quantities of material for subsequent compositional analysis, however, large preparations of cells are needed. The quantities available in this study for column chromatography and SDS-polyacrylamide gel analysis were unfortunately too small. (A study by Mahley *et al.* in 1971 analyzing the nascent VLDL particles associated with the Golgi apparatus of the rat small intestine required pooling of enterocyte preparations from 12 animals - this would probably be equivalent to 36 hamsters). Nevertheless, such "scaled-up" versions of the kinds of experiments reported in this project would provide additional information especially with regard to the protein and lipid composition of nascent intestinal lipoprotein particles.

An important concern in the use of isolated epithelial cells is the exposure of the basolateral surface of the cells to the potentially harmful effects of residual luminal bile acids. This could, in theory, result in dissolution of the basolateral membrane and cell lysis. In my experiments, however, the cells were washed twice prior to re-suspension in the incubation medium - this should have cleared away most or all the luminal contents that would have contaminated the initial cell preparation. Secondly, no significant lysis was ever demonstrated during a five hour incubation period (the pulse-chase labelling experiments involved a maximum incubation time of only two hours). Finally, morphological appearances of the released epithelial cells on light microscopy suggested that the cells were released in large groups or sheets from the villi tips, still attached to the basement membrane (which detached with the cells) and therefore still maintaining cell-to-cell contact (see fig. 4.2).

An important disadvantage of the use of epithelial cells or sheets of cells isolated from the bowel wall relates to the process of isolation itself which, however mild, must by its very definition result in cells that are somehow different from those in the intact bowel. This kind of alteration or damage is an escapable and inevitable consequence of the method - the real question is, by how much does the behaviour of these cells *in vitro* differ from that in the intact bowel? Do they synthesize and secrete proteins at rates similar to those achieved *in vivo*? Do the degradation rates measured *in vitro* bear any resemblance to those occurring *in vivo*? Do the isolated cells suffer from an artefactual secretion block? Are they deprived of hormones or nervous influences normally carried to the intestine from the systemic circulation that critically influence the processes of synthesis, secretion and degradation? Are they missing necessary local gut hormones? Are they adversely affected by a lack of normal cell-to-cell contact?

In one sense, the answer is simple - of course, by the very definition of being isolated, they must be lacking the normal systemic neural and endocrine influences. The isolated cell system cannot be *the* answer to all the questions of intestinal lipoprotein metabolism.

Nevertheless, when figures for intestinal apolipoprotein biosynthesis (expressed as a percentage of total radio-labelled protein synthesis) are compared between my system and the *in vivo* labelling system of Glickman *et al.*, the results are of a similar order of magnitude: Apo B-48, 2% in my system compared with 0.8% (Davidson *et al.* 1986), apo A-I (1.9% versus 1.8% (Davidson *et al.* 1985), and apo A-IV 3% versus 2% (non fat-fed, Apfelbaum *et al.* 1987). Some of the differences may be explained by the cross-species comparison (hamsters versus rats). When comparing degradation figures in hepatocytes with those in the isolated intestinal cell incubations, similar figures are seen. In my non fat-fed system, the average degradation rate for apolipoprotein B-48 was about 60% (of the previously pulse-labelled apolipoprotein) degraded in 1 hr; for the fat-fed system, the average figure was 15%. In Hep G2 cells in the absence of oleate in the medium, the degradation was 58% in 20 mins of :"chase" incubation; in the presence of oleate, it was 29% (Dixon *et al.*, 1991). Borchardt and Davis (1987) could only account for 36% of apo B-100 and 60% of apo B-48, previously synthesized intracellularly, appearing in the medium of rat hepatocytes in primary culture. The general principle seems to apply throughout: increased lipoprotein (including apolipoprotein B) secretion is associated with reduced rates of intracellular degradation and, conversely, degradation is high when the transmucosal lipid flux is low (or oleate is absent from the culture medium). The concordance with other systems of study adds credibility to the system itself.

There is a difficulty in the case of the results obtained for apolipoprotein A-IV secretion by the intestinal cells in this system (see discussion, chapt. 4.8). Perhaps lipoprotein assembly and secretion operated below normal and the normal pre-translationally controlled mechanism(s) for increasing the synthesis of apolipoprotein A-IV is lacking in the isolated epithelial cells. Or perhaps apo A-IV regulation in the hamster is controlled differently than it is in the more commonly studied laboratory rat? In any case, the isolated cells *do* secrete apolipoproteins into the medium, even if they do so at apparently lower rates than occur physiologically.

No attempts were made in my experiments to modify the lipid content of the incubation medium during an experiment. Such experiments may in the future provide useful insights into the precise mechanisms involved in regulating rates of apolipoprotein synthesis, secretion or degradation. For example, it would be useful to know whether the rate of apolipoprotein B-48 degradation can be reduced, *in vitro*, when oleate is added to an incubation of jejunal enterocytes from a low fat-fed hamster.

Measurements of apolipoprotein messenger RNA abundance and radio-immunoassays of the intracellular apolipoprotein pool sizes would also contribute potentially useful information in helping to formulate a model for the regulation of intestinal apolipoprotein fluxes but these were not possible in this study (except for a crude assay of the apo A-IV content - see chapt. 4.9).

Unanswered questions with respect to the regulation of apolipoprotein biosynthesis and turnover in the various intestinal segments could also, in the future, be addressed in the isolated enterocyte system. The proximal jejunum was used in all my experiments because it is known to be the most important site for the absorption of dietary lipid but other segments of the small bowel including the ileum may also be involved (Hoving & Valkema, 1969); preliminary work by Davidson *et al* (1985 and 1986) suggests that fundamental differences exist in the regulation of apo B-48 and apo A-I synthesis in ileal compared with jejunal enterocytes of the rat (for example, biliary lipid appears to be an important factor in regulating ileal but not jejunal apolipoprotein A-I synthesis).

Another unexplored avenue concerns variation along the villus-crypt axis. The cell preparation technique involved pooling the cell "effluents" from a series of "tapping" procedures designed to gently free the epithelial cells from the lamina propria. Initial cell collections would contain the terminally differentiated epithelial cells of the villus tip,

subsequent (more vigorous) tappings would be expected to free additional epithelial cells in the upper, middle and later, even lower, villus regions (see Weiser *et al.* 1973 and 1978). Thus, by collecting the sequential cell harvests separately, a differentiation of cells along the villus-crypt axis could be obtained and studied independently with regards apolipoprotein biosynthesis, etc. J.M. Dietschy and co-workers have previously demonstrated variations in cholesterol synthesis and LDL uptake along the villus-crypt axis (Stange & Dietschy review, 1985); it is likely that variations in apolipoprotein turnover also occur along this axis - the more differentiated cells of the upper villus are likely to be more active in apolipoprotein synthesis since they are most actively involved in the absorption of the luminal digestive products.

The question of intracellular processing and co- and post-translational modification of apolipoproteins has been briefly addressed (chapt. 5). A study using tunicamycin indicated that intestinal apolipoprotein B-48 was co- or post-translationally modified by N-linked glycosylation but this carbohydrate modification or lack of it did not appear to affect the secretion or susceptibility to degradation of the protein. With larger scale cell preparations, better subcellular fractionation techniques (see chapt. 3.8), and the use of a wider range of inhibitors of post-translational carbohydrate modifications, a more comprehensive picture of the intracellular processing of the apolipoproteins may emerge. Many other proteins thought to be synthesized by enterocytes with functions closely related to lipoprotein metabolism e.g. acylation stimulating protein (ASP), fatty acid binding proteins (FABP) g and h and the low density lipoprotein receptor (LDL-receptor) were not successfully identified in this project while the mystery of protein X (chapt. 3) remained unsolved. With improved antibodies and immuno-identification techniques these proteins may be identified and, like the apolipoproteins, may be subjected to similar studies of synthesis, secretion and degradation rates under various dietary treatments. Indeed, many other enterocyte proteins not necessarily related to lipoprotein metabolism e.g. the various digestive enzymes, intestinal transferrin, etc. may all be amenable to study following

dietary or experimental manipulations in the intact animals.

Finally, it should be remembered that the impetus for scientific inquiry into lipoprotein metabolism in general and intestinal lipoprotein metabolism in particular has been the association of dietary fat intake and hypercholesterolaemia with atherogenesis and cardiovascular disease. While this study has focused particularly on intestinal apolipoprotein dynamics, a fundamental long-term question remains - how do these changes induced by various diets affect overall lipid metabolism? Only by studying the subsequent metabolism of the intestinal lipoproteins and their component protein and lipid constituents can a more comprehensive picture emerge of the role or contribution of the small intestine to overall, whole-body lipid dynamics.

REFERENCES

Adams RLP. Cell Culture for Biochemists. 2nd revised Ed. Amsterdam: Elsevier, 1990: 64.

Akerstrom B, Brodin T, Reis K, Bjorck L. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J. Immunol.* 1985; 135: 2589-2592.

Alvarado F. Amino acid transport in hamster small intestine: site of inhibition by D-galactose. *Nature* 1968; 219: 276.

Apfelbaum TF, Davidson NO, Glickman RM. Apolipoprotein A-IV synthesis in rat intestine: regulation by dietary triglyceride. *Am. J. Physiol.* 1987; 252 (Gastrointest. Liver Physiol. 15): G662-G666.

Assmann G, Schmitz G, Brewer HB. Familial HDL deficiency: Tangier Disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic Basis of Inherited Disease. 6th Ed. New York: McGraw Hill Co., 1989: 1267-1282.

Bass NM, Manning JA, Ockner RK, Gordon JI, Seetharam S, Alpers DH. Regulation of the biosynthesis of two distinct fatty acid-binding proteins in rat liver and intestine. *J. Biol. Chem.* 1985; 260(3): 1432-1436.

Baum CL, Teng B-B, Davidson NO. Apolipoprotein B messenger RNA editing in the rat liver. Modulation by fasting and refeeding a high carbohydrate diet. *J. Biol. Chem.* 1990; 265(31): 19263-19270.

Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum. *J. Biol. Chem.* 1946; 164: 321-329.

Bihler J, Crane RK. Studies on the mechanism of intestinal absorption of sugars: the influence of several cations and anions on the active transport of sugars, in vitro, by various preparations of hamster small intestine. *Biochim. Biophys. Acta* 1962; 59: 78.

Bisgaier CL, Glickman RM. Intestinal synthesis, secretion and transport of lipoproteins. *Annu. Rev. Physiol.* 1983; 45: 625-636.

Bjorek L, Kronvall G. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J. Immunol.* 1984; 133: 969-974.

Blaufuss MC, Gordon JI, Schonfeld G, Strauss AW, Alpers DH. Biosynthesis of apolipoprotein CIII in rat liver and small intestinal mucosa. *J. Biol. Chem.* 1984; 259: 2452-2456.

Boguski MS, Elshourbagy N, Taylor JM, Gordon JI. Rat apolipoprotein AIV contains 13 tandem repetitions of a 22 amino acid segment with amphipathic helical potential. *Proc. Natl. Acad. Sci. USA.* 1984; 81: 5021-5025.

Bollman JL, Cain JC, Grindlay JH. J. Lab. Clin. Med. 1949; 33: 1349. Cited in: Tso P, Simmonds WJ. The absorption of lipid and lipoprotein synthesis. In: Lipid Research Methodology. Laboratory and Research Methods in Biology and Medicine. New York: Alan P. Liss Inc., 1984; 10: 191-216.

Borchardt RA, Davis RA. Intrahepatic assembly of very low density lipoproteins. *J. Biol. Chem.* 1987; 262(34): 16394-16402.

Borer K. Regulation of energy balance in the golden hamster. In: Siegel HI, editor. The Hamster. Reproduction and Behaviour. New York: Plenum Press, 1985: 363-408.

Burton PM, Chiou YM. Isolation, characterisation and quantification of apolipoproteins A-I and B of the Golden Syrian Hamster (Mesocricetus auratus) and modification of their levels by dietary cholesterol. *Comp. Biochem. Physiol.* 1989; 92B(4): 667-673.

Buschmann RJ, Manke DJ. Morphometric analysis of the membranes and organelles of small intestinal enterocytes. I. Fasted hamster. II. Lipid-fed hamster. *J. Ultrastructure Res.* 1981; 76: 1-14, 15-26.

Cameron-Clarke A, Manchester KL. Effect of dietary carbohydrate and lipid on very low density lipoprotein secretion by rat hepatocytes. *Nutr. Reports International* 1985; 32: 1451-1460.

Chan SDH, Atkins D. Distribution and properties of Ca^{2+}-ATPase, phytase, and alkaline phosphatase in isolated enterocytes from normal and vitamin D-deficient rats. *Gut* 1983; 24: 886-892.

Cianflone K, Vu H, Walsh M, Baldo A, Sniderman A. Metabolic response of Acylation Stimulating Protein to an oral fat load. *J. Lipid Res.* 1989; 30: 1727-1733.

Cori CF. The fate of sugar in the animal body. I. The rate of absorption of hexoses and pentose from the intestinal tract. *J. Biol. Chem.* 1925; 66: 691 cited in: Tso P, Simmonds WJ. The absorption of lipid and lipoprotein synthesis. In: Lipid Research Methodology. Laboratory and Research Methods in Biology and Medicine. New York: Alan P. Liss Inc., 1984; 10: 191-216.

Crane RK, Mandelstam P. The active transport of sugars by various preparations of hamster intestine. *Biochim. Biophys. Acta* 1960; 45: 460.

Davidson NO, Drewek MJ, Gordon JI, Elovson J. Rat intestinal apolipoprotein gene expression. *J. Clin. Invest.* 1988a; 82: 300-308.

Davidson NO, Carlos RC, Drewek MJ, Parmer TG. Apolipoprotein gene expression in the rat is regulated in a tissue-specific manner by thyroid hormone. *J. Lipid Res.* 1988b; 29: 1511-1522.

Davidson NO, Powell LM, Wallis SC, Scott J. Thyroid hormone modulates the introduction of a stop codon in rat liver apolipoprotein B messenger RNA. *J. Biol. Chem.* 1988c; 263(27): 13482-13485.

Davidson NO, Glickman RM. Apolipoprotein A-I synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. *J. Lipid Res.* 1985; 25: 368-379.

Davidson NO, Kollmer ME, Glickman RM. Apolipoprotein B synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. *J. Lipid Res.* 1986; 27: 30-39.

Davidson NO, Magun AM, Brasitus TA, Glickman RM. Intestinal apolipoprotein A-I and B-48 metabolism: effects of sustained alterations in dietary triglyceride and mucosal cholesterol flux. *J. Lipid Res.* 1987; 28: 388-402.

Davis RA, Borchardt RA. Phosphorylation-dephosphorylation of apolipoprotein B. In: Albers JJ, Segrest JP, editors. Plasma Lipoproteins. Methods in Enzymology 1986; 129: 536-542.

Davis RA, Clinton GM, Borchardt RA, Malone-McNeal M, Tan T, Lattier GR. Intra-hepatic assembly of very low density lipoproteins. Phosphorylation of small molecular weight apolipoprotein B. *J. Biol. Chem.* 1984; 259: 3383-3386.

Dietschy JM. Regulation of cholesterol metabolism in man and in other species. *Klin. Wochenschrift* 1984; 62: 338-345.

Dixon JL, Furukawa S, Ginsberg HN. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* 1991; 266(8): 5080-5086.

Dixon JL, Ginsberg HN. Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J. Lipid Res.* 1993; 34: 167-179.

Drayna D, Jarnagin AS, Mclean J, Henzel W, Kohr W, Fielding C, Lawn R. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature* 1987; 327: 632-634.

Driscoll DM, Casanova E. Characterization of the apolipoprotein B mRNA editing activity in enterocyte extracts. *J. Biol. Chem.* 1990; 265(35): 21401-21403.

Eder HA, Gidez LI. The clinical significance of the plasma high density lipoproteins. *Med. Clin. North Am.* 1982; 66: 431-440.

Elshourbagy NA, Walker DW, Biguski MS, Gordon JI, Taylor JM. The nucleotide and derived amino acid sequence of human apolipoprotein A-IV mRNA and the close linkage of its gene to the genes of apolipoproteins A-I and CIII. *J. Biol. Chem.* 1986; 261: 1998-2002.

Faggiotto A, Ross R, Harker L. Studies of hypercholesterolaemia in the non-human primate I. *Arteriosclerosis* 1984; 4: 323-340.

Faggiotto A, Ross R. Studies of hypercholesterolaemia in the non-human primate II. *Arteriosclerosis* 1984; 4: 341-356.

Faust RA, Albers JJ. Regulated vectorial secretion of cholesteryl ester transfer protein (LTP-1) by the CaCO-2 model of human enterocyte epithelium. *J. Biol. Chem.* 1988; 263(18): 8786-8789.

Feingold KR, Wiley MH, MacRae G, Moser AH, Lear SR, Siperstein MD. The effect of diabetes mellitus on sterol synthesis in the diabetic rat. *Diabetes* 1982; 31: 388-395.

Field FJ, Albright E, Mathur SN. Regulation of cholesterol esterification by micellar cholesterol in CaCO-2 cells. *J. Lipid Res.* 1987; 28: 1057-1066.

Field FJ, Albright E, Mathur SN. Regulation of triglyceride-rich lipoprotein secretion by fatty acids in CaCO-2 cells. *J. Lipid Res.* 1988; 29: 1427-1437.

Fong LG, Bonney E, Kosek JC, Cooper AD. Immunohistochemical localisation of low density lipoprotein receptors in adrenal gland, liver and intestine. *J. Clin. Invest.* 1989; 84: 847-856.

Forester GP, Tall AR, Bisgaier CL, Glickman RM. Rat intestine secretes spherical high density lipoproteins. *J. Biol. Chem.* 1983; 258: 5938-5943.

Forte TM. Primary hepatocytes in monolayer culture: a model for studies on lipoprotein metabolism. *Annu. Rev. Physiol.* 1984; 46: 403-415.

Forte T, Nordhausen RW. Electron microscopy of negatively stained lipoproteins. In: Albers JJ, Segrest JP, editors. Plasma Lipoproteins. *Methods in Enzymology* 1986; 128: 442-456.

Forte T, Norum KR, Glomset JA, Nichols AV. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: structure of low and high density lipoproteins as revealed by electron microscopy. *J. Clin. Invest.* 1971; 50: 1141-1148.

Fraser R. Size and lipid composition of chylomicrons of different Svedberg units of flotation. *J. Lipid Res.* 1970; 11: 60-65.

Fujiki Y, Hubbard AL, Fowler S, Lazarow PB. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 1982; 93: 97-102.

Fujimoto K, Fukagawa K, Sakata T, Tso P. Suppression of food intake by apolipoprotein A-IV is mediated through the central nervous system in rats. *J. Clin. Invest.* 1993; 91: 1830-1833.

Garvey JS, Cremer NE, Sussdorf DH. Methods in Immunology. 3rd ed. Reading: WA Benjamin Inc., 1977: 7-38.

Geigy Scientific Tables, Vol. One (8th Ed.), 1981. Ciba-Geigy Ltd., Basle, Switzerland: 251 & 254.

Ghiselli G, Crump III WL, Gotto AM. Binding of apo A-IV-phospholipid complexes to plasma membranes of rat liver. *Biochem. Biophys. Res. Commun.* 1986; 139: 122-128.

Glickman RM, Green PHR, Lees RS, Lux SE, Kilgore A. Immunofluorescence studies of apolipoprotein B in intestinal mucosa. Absence in abetalipoproteinaemia. *Gastroenterology* 1979; 76: 288-292.

Glickman RM, Green PHR, Lees RS, Tall A. Apoprotein A-1 synthesis in normal intestinal mucosa and in Tangier Disease. *New Engl. J. Med.* 1978b; 299: 1424-1427.

Glickman RM, Green PHR. The intestine as a source of apo A-1. *Proc. Natl. Acad. Sci. USA* 1977; 74: 2569-2573.

Glickman RM, Kilgore A, Khorana J. Chylomicron apoprotein localization within rat intestinal epithelium: studies of normal and impaired lipid absorption. *J. Lipid Res.* 1978a; 19: 260-268.

Glickman RM, Kirsch K, Isselbacher KJ. Fat absorption during inhibition of protein synthesis: studies of lymph chylomicrons. *J. Clin. Invest.* 1972; 51: 356-363.

Glickman RM, Kirsch K. Lymph chylomicron formation during the inhibition of protein synthesis. Studies of chylomicron apoproteins. *J. Clin. Invest.* 1973; 52: 2910-2920.

Glickman RM, Magun AM. High density lipoprotein formation by the intestine. In: Albers JJ, Segrest JP, editors. Plasma Lipoproteins. Methods in Enzymology 1986a; 129: 519-536.

Glickman RM, Rogers M, Glickman JN. Apolipoprotein B synthesis by human liver and intestine *in vitro*. *Proc. Natl. Acad. Sci. USA* 1986b; 83: 5296-5300.

Go MF, Schonfeld G, Pfleger B, Cole TG, Sussman NL, Alpers DH. Regulation of intestinal and hepatic apolipoprotein synthesis after chronic fat and cholesterol feeding. *J. Clin. Invest.* 1988; 81: 1615-1620.

Goldstein JL, Brown MS. Familial hypercholesterolaemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D. The Metabolic Basis of Inherited Disease. 6th Ed. New York: McGraw-Hill Information Services Company, 1989: 1215-1250.

Goodman DS, Blomstrand R, Werner B, Huang HS, Shiratori T. The intestinal absorption and metabolism of vitamin A and beta-carotene in man. *J. Clin. Invest.* 1966; 45: 1615-1623.

Gordon JI, Bisgaier CL, Sims HF, Sachdev DP, Glickman RM, Strauss AW. Biosynthesis of human pre-apolipoprotein A-IV. *J. Biol. Chem.* 1984; 259: 468-474.

Gordon JI, Smith DP, Alpers DH, Strauss AW. Proteolytic processing of the primary translation product of rat intestinal apolipoprotein A-IV mRNA. Comparison with pre-pro-apolipoprotein A-I processing. *J. Biol. Chem.* 1982b; 257: 8418-8423.

Gordon JI, Smith DP, Andy R, Alpers DH, Schonfeld G, Strauss AW. The primary translation product of rat intestinal apolipoprotein AI mRNA is an unusual pre-protein. *J. Biol. Chem.* 1982a; 257: 971-978.

Gordon T, Castelli WP, Hjortland MC, *et al.* High density lipoprotein as a protective factor against coronary heart disease: the Framingham study. *Amer. J. Med.* 1977; 62: 707-717.

Green PHR, Glickman RM, Riley JW, Quinet E. Human apolipoprotein A-IV: intestinal origin and distribution in plasma. *J. Clin. Invest.* 1980; 65: 911-919.

Green PHR, Glickman RM, Saudek CD, Blum CB, Tall AR. Human intestinal lipoproteins: studies in chyluric subjects. *J. Clin. Invest.* 1979; 64: 233-242.

Green PHR, Glickman RM. Intestinal lipoprotein metabolism. *J. Lipid Res.* 1981; 22: 1153-1173.

Green PHR, Lefkowitz JH, Glickman RM, Riley JW, Quinet E, Blum CB. Apolipoprotein localization and quantification in the human intestine. *Gastroenterology* 1982; 83: 1223-1230.

Green PHR, Tall AR, Glickman RM. Rat intestine secretes discoid high density lipoprotein. *J. Clin. Invest.* 1978; 61: 528-534.

Griffiths A. Centrifugation Techniques. In: Williams BL, Wilson K, editors. A Biologist's Guide to Principles and Techniques of Practical Biochemistry. 2nd Ed. London: Edward Arnold, 1981: 48.

Hamilton RL, Williams MC, Fielding CJ, Havel RJ. Discoidal bilayer structure of nascent HDL from perfused rat liver. *J. Clin. Invest.* 1976; 58: 667-680.

Hamosh M. Fat digestion in the newborn: Role of lingual lipase and pre-duodenal digestion. *Paediatric Res.* 1979; 13: 615-622.

Harrer DS, Stern BK, Reilly RW. Removal and dissociation of epithelial cells from the rodent gastro-intestinal tract. *Nature* 1964; 203: 319.

Havel RJ, Kane JP. Structure and metabolism of plasma lipoproteins. In: Scriver CR, Beaudet AL, Sly WS, Valle D. The Metabolic Basis of Inherited Disease. 6th Ed. New York: McGraw-Hill Information Services Company, 1989: 1129-1138.

Hayashi H, Fujimoto K, Cardelli JA, Nutting DF, Bergstedt S, Tso P. Fat feeding increases size, but not number, of chylomicrons produced by small intestine. *Amer. J. Physiol.* 1990; 259 (Gastrointest. Liver Physiol. 22): G709-G719.

Hayashi H, Nutting DF, Fujimoto K, Cardelli JA, Black D, Tso P. Transport of lipid and apolipoproteins A-I and A-IV in intestinal lymph of the rat. *J. Lipid Res.* 1990; 31: 1613-1625.

Hernell O, Blackberg L. Digestion of human milk lipids: physiological significance of sn-2 monoacylglycerol hydrolysis by bile salt-stimulated lipase. *Paediatric Res.* 1982; 16: 882-885.

Hodges P, Scott J. Apolipoprotein B mRNA editing: a new tier for the control of gene expression. *Trends Biochem. Sci.* 1992; 17: 77-81.

Hoeg JM, Meng MS, Ronan R, Demosky SJ, Fairwell T, Brewer B. Apolipoprotein B synthesized by Hep G2 cells undergoes fatty acid acylation. *J. Lipid Res.* 1988; 29: 1215-1220.

Hoving J, Valkema AJ. Effect of dietary fat content on the site of fat absorption in hamster small intestine *in vitro*. *Biochim. Biophys. Acta* 1969; 187: 53-58.

Howell KE, Palade GE. Hepatic Golgi fractions resolved into membrane and content subfractions. *J. Cell Biol.* 1982; 92: 822-832.

Huang KC. Uptake of L-tyrosine and 3-O-methylglucose by isolated intestinal epithelial cells. *Life Sci.* 1965; 4: 1201. Cited in: Kimmich GA. Preparation and characterisation of isolated intestinal epithelial cells and their use in studying intestinal transport. In:

Korn ED, editor. Methods in Membrane Biology. New York: Plenum Press, 1975; 5: 51-115.

Hughes TE, Ordovas JM, Schaefer EJ. Regulation of intestinal apolipoprotein B synthesis and secretion by CaCO-2 cells. *J. Biol. Chem.* 1988; 263(7): 3425-3431.

Hughes TE, Sasak WV, Ordovas JM, Forte TM, Lamon-Fava S, Schaefer EJ. A novel cell line (CaCO-2) for the study of intestinal lipoprotein synthesis. *J. Biol. Chem.* 1987; 262(8): 3762-3767.

Imaizumi K, Havel RJ, Fainaru M, Vigne JL. Origin and transport of the AI and arginine-rich apolipoproteins in mesenteric lymph of rats. *J. Lipid Res.* 1978; 19: 1038-1046.

Jackson TK, Salhanick AI, Elovson J, Deichman ML, Amatruda JM. Insulin regulates apolipoprotein B turnover and phosphorylation in rat hepatocytes. *J. Clin. Invest.* 1990; 86: 1746-1751.

James RW, Hochstrasser D, Tissot J-D, Funk M, Appel R, Barja F, Pellegrini C, Muller AF, Pometta D. Protein heterogeneity of lipoprotein particles containing apolipoprotein A-I without apolipoprotein A-II and apolipoprotein A-I with apolipoprotein A-II isolated from human plasma. *J. Lipid Res.* 1988; 29: 1557-1571.

Jersild AR. Time-sequence study of fat absorption in the rat jejunum. *Amer. J. Anat.* 1966; 118: 135-141.

Jiang XC, Moulin P, Quinet E, Goldberg IJ, Yacoub LK, Agellon LB, Compton D, Schnitzer-Polokoff R, Tall AR. Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. *J. Biol. Chem.* 1991; 266(7): 4631-4639.

Johnston JM. Esterification reactions in the intestinal mucosa and lipid absorption. In: Dietschy JM, Gotto AM, Ontko JA, editors. Disturbances in Lipid and Lipoprotein Metabolism. Baltimore: American Physiological Society, 1978: 57-68.

Kane JP, Havel RJ. Disorders of the biogenesis and secretion of lipoproteins containing the B apolipoproteins. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic Basis of Inherited Disease. 6th Ed. New York: McGraw Hill Co., 1989: 1139-1164.

Kayden HJ, Karmen A, Dumont A. Alterations in the fatty acid composition of human lymph and serum lipoproteins by single feedings. *J. Clin. Invest.* 1963; 42: 1373-1381.

Kern R, Borgstrom B. Quantitative study of the pathways of triglyceride synthesis by hamster intestinal mucosa. *Biochim. Biophys. Acta* 1965; 98: 520-531.

Kessler SW. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* 1975; 115: 1617-1624.

Kimmich GA. Preparation and characterisation of isolated intestinal epithelial cells and their use in studying intestinal transport. In: Korn ED, editor. Methods in Membrane Biology. New York: Plenum Press, 1975; 5: 51-115.

Kinter WB, Wilson TH, Mullen DA. Autoradiographic study of sugar and amino acid absorption by everted sacs of hamster intestine. *J. Cell Biol.* 1965; 25: 19.

Knott TJ, Pease RJ, Powell LM, Wallis SC, Rall SC, Innerarity TL *et al.* Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature* 1986; 323: 734-738.

Kraft HG, Menzel HJ, Hoppichler F, Vogel W, Utermann G. Changes of genetic apolipoprotein phenotypes caused by liver transplantation. *J. Clin. Invest.* 1989; 83: 137-142.

Kreisberg RA. Lipids, lipoproteins, apolipoproteins, and atherosclerosis. *Ann. Intern. Med.* 1983; 99: 713-715.

Kruse and Patterson (eds.). Tissue Culture: Methods and Applications. Academic Press. 1406-1408.

Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.

Langone JJ. Protein A of Staphylococcus aureus and related immunoglobulin receptors produced by Streptococci and Pneumococci. *Adv. Immunol.* 1982; 32: 157-252.

Lawn RM. Lipoprotein (a) in heart disease. *Sci. Am.* 1992; 266(6): 26-32.

Lentner C (ed.). Geigy Scientific Tables. 8th Ed. Basle: Ciba-Geigy Ltd, 1986; 4: 26.

Levy E, Marcel Y, Deckelbaum RJ, Milne R, Lepage G, Seidman E, Bendayan M, Roy CC. Intestinal apo B synthesis, lipids and lipoproteins in chylomicron retention disease. *J. Lipid Res.* 1987; 28: 1263-1274.

Levy E, Marcel Y, Deckelbaum RJ, Milne R, Lepage G, Seidman E, Bendayan M, Roy CC. Intestinal apoB synthesis, lipids, and lipoproteins in chylomicron retention disease. *J. Lipid Res.* 1987; 28: 1263-1274.

Lossow WJ, Lindgen FT, Murchio KC. Stevens GR. Jensen LC. Particle size and protein content of six fractions of the Sf > 20 plasma lipoproteins isolated by density gradient centrifugation. *J. Lipid Res.* 1969; 10: 68-76.

Lowry OM, Rosebrough NJ, Farr AK, Randall RJ. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 1951; 193: 265-275.

Magun AM, Brasitus TA, Glickman RM. Isolation of high density lipoproteins from rat intestinal epithelial cells. *J. Clin. Invest.* 1985; 75: 209-218.

Magun AM, Mish B, Glickman RM. Intracellular apo A-I and apoB distribution in rat intestine is altered by lipid feeding. *J. Lipid Res.* 1988; 29: 1107-1116.

Mahley RW, Bennett BD, Morre DJ, Gray ME, Thistlewaite W, LeQuire VS. Lipoproteins associated with the Golgi apparatus isolated from epithelial cells of rat small intestine. *Lab. Invest.* 1971; 25: 435-444.

Malloy MJ, Kane JP, Hardman DA, Hamilton RL, Dalal KB. Normotriglyceridaemic abetalipoproteinaemia. Absence of the B-100 apolipoprotein. *J. Clin. Invest.* 1981; 67: 1441-1450.

Mans RJ, Novelli D. Measurement of the incorporation of radioactive amino acids into protein by a filter disk method. *Arch. Biochem. Biophys.* 1961; 94: 48-53.

Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 1978; 87: 206-210.

Matoba N, Kuroki S, Cohen BI, Mosbach EH, McSherry CK. Effect of 7-methylated bile acids and bile alcohols on cholesterol metabolism in hamsters. *Lipids* 1988; 23(5): 465-468.

Mayes PA. Lipid transport and storage. In: Murray RK, Granner DK, Mayes PA, Rodwell VW. Harper's Biochemistry. 21st Ed. Norwalk: Appleton and Lange, 1988: 226-240.

Montgomery R, Dryer RL, Conway TW, Spector AA. Biochemistry: A Case-Orientated Approach. 2nd Ed. St. Louis: CV Mosby Co., 1977: 363-417.

Murray RK. Glycoproteins and proteoglycans. In: Murray RK, Granner DK, Mayes PA, Rodwell VW. Harper's Biochemistry. 21st Ed. Norwalk: Appleton and Lange, 1988: 589-598.

Nistor AN, Bulla A, Filip DA, Radu A. The hyperlipidaemic hamster as a model of experimental atherosclerosis. *Atherosclerosis* 1987; 68: 159-173.

Ockner RK, Hughes FB, Isselbacher KJ. VLDL in intestinal lymph: origin, composition, and role in lipid transport in the fasting state. *J. Clin. Invest.* 1969; 48: 2079-2088.

Ockner RK, Jones AL. An electron microscopic and functioning study of VLDL in intestinal lymph. *J. Lipid Res.* 1970; 11: 284-292.

Ontko JA, Cheng Q, Yamamoto M. Metabolic factors underlying high serum triglycerides in the normal hamster. *J. Lipid Res.* 1990; 31: 1983-1992.

Patsch JR. Postprandial lipaemia. In: Shepherd J, editor. Lipoprotein Metabolism. Baillière's Clinical Endocrinology and Metabolism. London: Baillière Tindall, 1987; 1(3): 551-580.

Pearlman BJ, Bonorris GG, Phillips MJ, Chung A, Vimadalal S, Marks JW, Schoenfield LG. Cholesterol gallstone formation and prevention by chenodeoxycholic and ursodeoxycholic acids. A new hamster model. *Gastroenterology* 1979; 77: 634-641.

Persson R, Ahlström E, Fries E. Differential arrest of secretory protein transport in cultured rat hepatocytes by azide treatment. *J. Cell Biol.* 1988; 107(6) (Pt 2): 2503-2510.

Phares CK. Streptozotocin-induced diabetes in Syrian hamsters: New model of diabetes mellitus. *Experimentia* 1980; 36: 681-682.

Popper PD, Shiau YF, Reed M. Role of the small intestine in the pathogenesis of hyperlipidaemia in diabetic rats. *Amer. J. Physiol.* 1985; 249; G 161-167.

Powell LM, Wallis SC, Pease RJ, Edwards YH, Knott TJ, Scott J. A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. *Cell* 1987; 50: 831-840.

Pullinger CR, North JD, Teng B-B, Rifici VA, de Brito AER, Scott J. The apolipoprotein B gene is constitutively expressed in Hep G2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurements of the mRNA half-life. *J. Lipid Res.* 1989; 30: 1065-1077.

Rachmilewitz D, Albers JJ, Saunders DR, Apoprotein B in fasting and post-prandial human biopsies. *J. Clin. Invest.* 1976; 57: 530-533.

Rachmilewitz D, Albers JJ, Saunders DR. Apoprotein synthesis by human duodeno-jejunal mucosa. *Gastroenterology* 1978; 75: 667-682.

Redgrave TG. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* 1970; 49: 465-471.

Reeves WJ, Fimognari GM. An improved procedure for the preparation of crystalline lactic dehydrogenase from hog heart. *J. Biol. Chem.* 1983; 238 (12): 3853-3858.

Renner F, Samuelson A, Rogers M, Glickman RM. Effect of saturated and unsaturated lipid on the composition of mesenteric triglyceride-rich lipoproteins in the rat. *J. Lipid Res.* 1986; 27: 72-81.

Rifai N. Lipoproteins and apolipoproteins. *Arch. Pathol. Lab. Med.* 1986b; 110: 694-701.

Riley JW, Glickman RM, Green PHR, Tall AR. The effect of chronic cholesterol feeding on intestinal lipoproteins in the rat. *J. Lipid Res.* 1980; 21: 942-952.

Risser TR, Reaven GM, Reaven EP. Intestinal contribution to secretion of very low density lipoproteins into plasma. *Amer. J. Physiol.* 1978; 234: E277-281.

Rosenberg L, Duguid WP, Brown RA, Vinik AI. Induction of Nesidioblastosis will reverse diabetes in Syrian Golden Hamster. *Diabetes* 1988; 37: 334-341.

Roy CC, Levy E, Green PHR, Sniderman A, Letarte J, Buts J-P *et al.* Malabsorption, hypocholesterolaemia and fat-filled enterocytes with increased intestinal apoprotein B: chylomicron retention disease. *Gastroenterology* 1987; 92: 390-399.

Rubin EC, Perkins WD, Surawicz CM, McDonald GB, Albers JJ. Ultrastructure apoprotein B localization within human jejunal absorptive cells during fat absorption. *Gastroenterology* 1980; 78: 1248.

Rudel LL, Marzetta CA, Johnson FL. Separation and analysis of lipoproteins by gel filtration. In: Albers JJ, Segrest JP, editors. Plasma Lipoproteins. Methods in Enzymology. Academic Press, Inc., 1986; 129(Pt B): 45-56.

Sabesin SM, Frase S. Electron microscopic studies of the assembly, intracellular transport, and secretion of chylomicrons by rat intestine. *J. Lipid Res.* 1977; 18: 496-511.

Sakata N, Wu X, Dixon JL, Ginsberg HN. Proteolysis and lipid-facilitated translocation are distinct but competitive processes that regulate secretion of apolipoprotein B in Hep G2 cells. *J. Biol. Chem.* 1993; 268 : 22967-22970.

Sasak WV, Lown JS, Colburn KA. Human small-intestinal apolipoprotein B-48 oligosaccharide chains. *Biochem. J.* 1991; 274: 159-165.

Schonfeld G, Bell E, Alpers DH. Intestinal lipoproteins during fat absorption. *J. Clin. Invest.* 1978; 61: 1539-1550.

Scott J. Regulation of the biosynthesis of apolipoprotein B100 and apolipoprotein B48. *Current Opinions in Lipidology* 1990; 1: 96-103.

Scott J. The molecular and cell biology of apolipoprotein-B. *Mol. Biol. Med.* 1989; 6: 65-80.

Segrest JP, Albers JJ, editors. Plasma Lipoproteins. Part A: Preparation, Structure and Molecular Biology. Section II. Preparation of Plasma Lipoproteins. *Methods in Enzymology* 1986; 128: 151-212.

Shepherd P, Simmonds WJ. Aust. J. Exp. Biol. Med. 1959; 37: 1. Cited in: Tso P, Simmonds WJ. The absorption of lipid and lipoprotein synthesis. In: Lipid Research Methodology. Laboratory and Research Methods in Biology and Medicine. New York: Alan P. Liss Inc., 1984; 10: 191-216.

Shiau Y-F. Lipid digestion and absorption. In: Johnson LR, editor. Physiology of the Gastrointestinal Tract. New York: Raven Press, 1987: 1527-1556.

Siuta-Mangano P, Janero DR, Lane MD. Association and assembly of triglyceride and phospholipid with glycosylated and unglycosylated apoproteins of very low density lipoprotein in the intact liver cell. *J. Biol. Chem.* 1982; 257: 11463-11467.

Sjostrand FS. A simple and rapid method to prepare dispersions of columnar epithelial cells from the rat intestine. *J. Ultrastruct. Res.* 1968; 22: 424.

Sognen E. A method for the preparation of suspensions of intestinal mucosal cells by means of calcium chelation. *Acta Vet. Scand.* 8: 76. Cited in: Kimmich GA. Preparation and characterization of isolated intestinal epithelial cells and their use in studying intestinal transport. In: Korn ED, editor. Methods in Membrane Biology, New York: Plenum Press, 1975; 5: 51-115.

Soutar AR, Knight BL, Myant NB. The characterization of lipoproteins in the high density fraction obtained from patients with familial lecithin:cholesterol acyltransferase deficiency and their interaction with cultured human fibroblasts. *J. Lipid Res.* 1982; 23: 380-390.

Spady DK, Bilheimer DW, Dietschy JM. Rates of receptor-dependent and independent low density lipoprotein uptake in the hamster. *Proc. Natl. Acad. Sci. USA* 1983a; 80: 3499-3503.

Spady DK, Dietschy JM. Sterol synthesis *in vivo* in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster and rat. *J. Lipid Res.* 1983b; 24: 303-315.

Sparks JD, Sparks CE, Roncone AM, Amatruda JM. Secretion of high and low molecular weight phosphorylated apolipoprotein B by hepatocytes from control and diabetic rats. J. Biol. Chem. 1988; 263: 5001-5004.

Stange EF, Dietschy JM. Cholesterol absorption and metabolism by the intestinal epithelium. In: Danielsson H, Sjövall J, editors. Sterols and Bile Acids. Elsevier Science Publishers, 1985: 121-147.

Stange EF, Dietschy JM. Cholesterol synthesis and LDL uptake are regulated independently in rat small intestinal epithelium. *Proc. Natl. Acad. Sci. (USA)* 1983; 80: 5739-5743.

Stein Y, Dabach Y, Hollander G, Stein O. Cholesteryl ester transfer activity in hamster plasma: Increase by fat and cholesterol-rich diets. *Biochim. Biophys. Acta* 1990; 1042: 138-141.

Steinberg D. Lipoproteins and Atherosclerosis. Duff Memorial Lecture. *Arteriosclerosis* 1983; 3: 283-301.

Strauss EW. Electron microscopic study of intestinal fat absorption *in vitro* from mixed micelles containing linolenic acid, monolein and bile salt. *J. Lipid Res.* 1976; 7: 307-323.

Strauss EW, Jacob JS. Some factors affecting the lipid secretory phase of fat absorption by intestine *in vitro* from golden hamster. *J. Lipid Res.* 1981; 22: 147-156.

Suissa M (1983). Spectrophotometric quantitation of silver granules eluted from autoradiograms. *Anal. Biochem.* 133: 511-514.

Suzuki N, Fidge N, Nestel P, Yin J. Interaction of serum lipoproteins with the intestine: evidence for specific high density lipoprotein-binding sites on isolated rat intestinal mucosal cells. *J. Lipid Res.* 1983; 24: 253-264.

Sviridov DD, Pavlov MY, Safonova IG, Repin VS, Smirnov VN. Inhibition of cholesterol synthesis and esterification regulates high density lipoprotein interaction with isolated epithelial cells of human small intestine. *J. Lipid Res.* 1990; 31: 1821-1830.

Sviridov DD, Safonova IG, Gusev VA, Talalaev AG, Tsibulsky VP, Ivanov VO, Preobrazensky SN, Repin VS, Smirnov VN. Specific high affinity binding and degradation of high density lipoproteins by isolated epithelial cells of human small intestine. *Metabolism* 1986; 35(7): 588-595.

Swaney JB, Reese H, Eder HA. Polypeptide composition of rat HDL: characterization by SDS-gel electrophoresis. *Biochem. Biophys. Res. Commun.* 1974; 59: 513-518.

Tall AR. Metabolism of postprandial lipoproteins. In: Albers JJ, Segrest JP, (editors). Methods in Enzymol. 1986; 129: 469-482.

Tall AR, Green PHR, Glickman RM, Riley JW. Metabolic fate of chylomicron phospholipids and apoproteins in the rat. *J. Clin. Invest.* 1979; 64: 977-989.

Thomson AR. Absorption of fat-soluble vitamins and sterols. *J. Clin. Path.* 1971; 5: 85-89.

Thomson ABR, Dietschy JM. Intestinal lipid absorption: major extracellular and intracellular events. In: Johnson LR, editor. Physiology of the Gastrointestinal Tract. New York: Raven Press, 1981: 1147-1220.

Thurnhofer, H, Hauser H. Uptake of cholesterol by small intestinal brush border membrane is protein-mediated. *Biochemistry* 1990; 29: 2142-2148.

Tollefson JH, Albers JJ. Plasma lipid transfer proteins. In: Albers JJ, Segrest JP, editors. Plasma Lipoproteins. *Methods in Enzymology* 1986; 129: 797-816.

Towler CM, Pugh-Humphreys GP, Porteous JW. Characterisation of columnar absorptive epithelial cells isolated from rat jejunum. *J. Cell Sci.* 1978; 29: 53-75.

Traber MG, Kayden HJ, Rindler MJ. Polarised secretion of newly synthesized lipoproteins by the CaCO-2 human intestinal cell line. *J. Lipid Res.* 1987; 28: 1350-1363.

Trotter PJ, Storch J. Fatty acid esterification during differentiation of the human intestinal cell line CaCO-2. *J. Biol. Chem.* 1993; 268(14): 10017-10023.

Tso P, Drake DS, Black DD, Sabesin SM. Evidence for separate pathways of chylomicron and very low density lipoprotein assembly and transport by small intestine. *Amer. J. Physiol.* 1984b; 247: G599-G610.

Tso P, Kendrick H, Balint JA, Simmonds WJ. Gastro-enterology 1981; 80: 60. Cited in: Tso P, Simmonds WJ. The absorption of lipid and lipoprotein synthesis. In: Laboratory and Research Methods in Biology and Medicine. New York: Alan P. Liss Inc., 1984; 10: 191-216.

Tso P, Lindström MB, Borgström B. Factors regulating the formation of chylomicrons and very-low-density lipoproteins by the rat small intestine. *Biochim. Biophys. Acta* 1987; 922: 304-313.

Tso P, Simmonds WJ. The absorption of lipid and lipoprotein synthesis. In: Lipid Research Methodology. Laboratory and Research Methods in Biology and Medicine. New YorK: Alan P. Liss Inc., 1984a; 10: 191-216.

Wang S-Y, Williams DL. Biosynthesis of the vitellogenins. *J. Biol. Chem.* 1982; 257(7): 3837-3846.

Weinberg RB, Spector MS. Structural properties and lipid binding of human apolipoprotein A-IV. *J. Biol. Chem.* 1985; 260: 4914-4921.

Weinberg RB. Differences in the hydrophobic properties of discrete alpha-helical domains of rat and human apolipoprotein A-IV. *Biochim. Biophys. Acta* 1987; 918: 299-303.

Weingand KW, Daggy BP. Effects of dietary cholesterol and fasting on hamster plasma lipoprotein lipids. *Eur. J. Clin. Chem. Clin. Biochem.* 1991; 29: 425-428.

Weiser M, Newmeier MM, Quaroni A, Kirsch K. Synthesis of plasmalemmal glycoproteins in intestinal epithelial cells. *J. Cell Biol.* 1978; 77: 722-734.

Weiser MM, Intestinal epithelial cell surface membrane glycoprotein synthesis. *J. Biol. Chem.* 1973; 247(7): 2536-2541.

Westergaard H, Dietschy JM. The uptake of lipids into the intestinal mucosa. In: Andreoli TE, Hoffman JF, Fanestil DD, Schultz SG, editors. Physiology of Membrane Disorders. New York: Plenum Publishing Corp., 1986: 597-608.

White AL, Graham DL, LeGros J, Pease RJ, Scott J. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. *J. Biol. Chem.* 1992; 267(22): 15657-15664.

Williams SC, Grant SG, Reue K, Carrasquillo B, Lusis AJ, Kinniburgh AJ. cis-Acting determinants of basal and lipid-regulated apolipoprotein A-IV expression in mice. *J. Biol. Chem.* 1989; 264: 19009-19016.

Wilson TH, Vincent TN. Absorption of sugars *in vitro* by the intestine of the golden hamster. *J. Biol. Chem.* 1955; 216: 851.

Wilson TH, Wiseman G. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J. Physiol. (London)* 1954; 123: 116.

Windmueller HG, Spaeth AE. Respiratory fuels and nitrogen metabolism *in vivo* in small intestine of fed rats. *J. Biol. Chem.* 1980; 255: 107-122.

Windmueller HG, Wu A-L. Biosynthesis of plasma apolipoproteins of rat small intestine without dietary or biliary fat. *J. Biol. Chem.* 1981; 256: 3012-3016.

Winkler KE, Marsh JB. Characterization of nascent high density lipoprotein subfractions from perfusates of rat liver. *J. Lipid Res.* 1989; 30: 979-987.

Wong L, Pino R. Biosynthesis of very-low-density lipoproteins in rat liver. Intracellular distribution of apolipoprotein B. *Eur. J. Biochem.* 1987; 164: 357-367.

Woollet LA, Spady DK, Dietschy JM. Mechanisms by which saturated triacylglycerols elevate the plasma low density lipoprotein-cholesterol concentration in hamsters. *J. Clin. Invest.* 1989; 84: 119-128.

Wu A-L, Windmueller HG. Identification of circulating apolipoproteins synthesized by rat small intestine *in vivo*. *J. Biol. Chem.* 1978; 253: 2525-2528.

Wu A-L, Windmueller HG. Relative contribution by liver and intestine to individual plasma apolipoproteins in the rat. *J. Biol. Chem.* 1979; 254: 7316-7322.

Yang C-Y, Chen S-H, Gianturco SH, Bradley WA, Sparrow JT, Tanimura M *et al.* Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature* 1986; 323: 738-742.

Yousef IM, Kuksis A. Release of chylomicrons by isolated cells of rat intestinal mucosa. *Lipids* 1972; 7(6): 380-386.