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Post Prandial Lipoprotein Metabolism

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Submitted towards the degree of PhD.
University of Glasgow
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Declaration

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I hereby declare that the work described herein was conducted entirely by myself except where indicated and acknowledged.

21/6/1990

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Abbreviations

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ACAT	acyl: cholesterol acyl transferase
apo	apoprotein
ARAT	acyl: retinol acyl transferase
CAD	coronary artery disease
CCK-PZ	cholecystokinin-pancreozymin
CE	cholesteryl ester
CHD	coronary heart disease
CoA	coenzyme A
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immuno sorbent assay
ER	endoplasmic reticulum
FABP	fatty acid binding protein
FC	free cholesterol
FFA	free fatty acid
HDL_2	high density lipoprotein subfraction 2
HDL_3	high density lipoprotein subfraction 3
HL	hepatic lipase
HMG CoA	hydroxymethylglutaryl coenzyme A
HPLC	high pressure liquid chromatography
IDL	intermediate density lipoprotein
IHD	ischæmic heart disease
LCAT	lecithin: cholesterol acyl transferase
LDL	low density lipoprotein
LpL	lipoprotein lipase
LTP	lipid transfer proteins
MI	myocardial infarction
PL	phospholipid
PUS	poly-unsaturated
ρ	density (g/ml)
RER	rough endoplasmic reticulum
RP	retinyl palmitate
rpm	revolutions per minute
SD	standard deviation
SER	smooth endoplasmic reticulum
Sf	flotation rate in Svedbergs
sn-	stereospecific numbering

TG ..... triglyceride

TLC ..... thin layer chromatography

UWL ..... unstirred water layer

VLDL ..... very low density lipoprotein

Summary

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An oral fat tolerance test was administered to groups of normal male and female volunteers, and to a group of patients undergoing coronary angiography. The changes in plasma lipoprotein fractions were monitored during the post prandial phase. Vitamin A (retinyl palmitate) was added to the fat load to mark lipoproteins of intestinal derivation.

In healthy male subjects the post prandial rise in plasma triglyceride was associated with an increase in the size of lipoproteins of density less than 1.006kg/l, and with characteristic changes in the composition of the other lipoprotein classes. Triglyceride rose in LDL and HDL in parallel with the rise in $\rho<1.006$ triglyceride. Phospholipid in LDL and HDL continued to rise beyond the peak of triglyceridæmia. HDL cholesteryl esters fell at the peak, and the free cholesterol content of LDL and HDL rose in the late phase of lipæmia. Retinyl palmitate was present in significant amounts 24 hours after the initial fat meal.

The pattern of changes was interpreted in terms of lipoprotein surface and core lipid compositions. There was a decline in the ratio of free cholesterol to phospholipid in the surface regions of LDL, and a fall in the ratio of cholesteryl ester to triglyceride in the core of both LDL and HDL.

Post prandial triglyceridæmia correlated positively with fasting levels of the apo B containing lipoproteins, and negatively with HDL. There was an inverse correlation with post heparin lipoprotein and hepatic lipase activities. Triglyceridæmia was positively related to the actual free cholesterol content of LDL, but negatively to the post prandial changes in LDL free cholesterol. The fall in HDL cholesteryl ester and cholesteryl ester/triglyceride ratio were negatively related to triglyceridæmia.

Females had lower fasting VLDL and higher HDL than males. Post prandial triglycerides were less and post heparin lipoprotein lipase activity was greater. There was a greater increase in plasma free cholesterol during lipæmia and a fall as opposed to a rise in plasma cholesteryl ester. Retinyl palmitate levels were less at all times, but most significantly at 24 hours after the fat meal.

In subjects undergoing coronary angiography fasting LDL cholesterol was positively related and HDL cholesterol negatively related to angiogram score. Post prandial triglyceridæmia was positively related to angiogram score, but fasting triglycerides were not. The best single predictor of angiogram score was retinyl palmitate levels 24 hours after the fat load. Retinyl ester levels were also strongly related to LDL cholesterol.

Increasing the cholesterol content of the fat load had no effect on post prandial lipids.

Decreasing the fatty acid saturation of the test meal decreased triglyceridæmia only slightly. Chylomicrons obtained after saturated or unsaturated fat loads differed in the composition of their triglyceride content, but not in the composition of phospholipid fatty acids. These chylomicrons were catabolised at similar rates during *in vitro* lipolysis.

Three weeks treatment with fenofibrate reduced fasting VLDL and LDL, and increased HDL_3. Post prandial triglycerides were decreased, and the post prandial fall in HDL cholesteryl esters was eliminated. Retinyl palmitate was apparently increased during lipæmia but levels were unchanged at 24 hours.

Three weeks treatment with nicotinic acid reduced fasting VLDL and LDL and increased HDL_2. Post prandial triglycerides were reduced but retinyl esters were unchanged. There were no significant changes in other aspects of post prandial lipid flux.

Possible mechanisms explaining these findings are discussed.

Chapter 1

Introduction

Less than an hour after consuming a meal containing fat the blood serum takes on a white, cloudy appearance, sometimes with white streaks floating on the surface. The subject matter of this paper concerns the nature of this post-prandial or alimentary lipæmia in terms of the alterations in plasma fat transport which it represents.

1.1 Of the White Serum

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Among the earliest accounts of this phenomenon, Robert Boyle in 1665 reported an incident which had come to his attention in which *"they had, in the house of a physician, newly opened a man's vein, wherein they found milk instead of blood".*[1] In his book 'De Corde' published in 1669 Dr Richard Lower, a colleague of Boyle's extends these observations, stating that *"vessels appeared fuller of milk than of blood, if a vein was incised after a good breakfast or luncheon".*[2] He goes on to describe a series of experiments in which he illustrates the route whereby food is digested and assimilated into the blood. It is clear from these writings that he was well aware of the nature of the white serum. *"Although this phenomenon had been observed by the doctors of earlier days"*, he grants, *"they were quite unaware of its cause".*

Over one hundred years later, in 1772 William Hewson described several cases of people with white serum, perhaps the first documented cases of hyperlipidæmia.[3]

"Mary Rider, about twenty five years of age, of a fresh complexion, and lusty, has not had her menses for these seven months. She discharges blood sometimes by vomiting and sometimes by stool; complains of a pain in her left side and in her stomach; she has an inclination to eat, but when she tries, she soon loathes her food. She complains of great lassitude and sleepiness; her pulse is ninety-five in a minute. She has been bled twelve times within these six months, and every time the serum was as white as milk".

Hewson examined the serum from these cases of whiteness under a microscope and found it to contain a number of small globules. These globules he observed were spherical and agreed more with the globules of milk than the red particles of blood, which were smaller and were flat. Hewson considered the particles to be oily in nature. *"Recollecting that butter had been got from such human serum I tried, by agitating some of it, a little diluted, to separate its oil or churn it without success"*. He eventually allowed the serum to dry on a piece of paper and found the residue to be greasy.

In a footnote to these studies in the collected works of Hewson,[4] George Gulliver confirms his estimate of the size of these particles *"to be scarcely* $\frac{1}{30000}$ *th of an inch and agreeing in all respects with the molecular base of the chyle"*, a term adopted by Gulliver to signify the particulate nature of the chyle whilst discussing the subject at length in his appendix to Gerber's Anatomy of 1842.[5]

The advent of the dark field microscope provided a basis for future quantitative studies of lipæmia, and inspired those researchers of a lyrical disposition. In the first description of the appearance of these particles in the dark field Edmunds[6] noted that blood *"... seems like a wholly new substance, with multitudes of glancing particles which look like motes in a sunbeam"*. Simon Gage[7] imagined the blood to be *"literally alive with these dancing particles"* which, he continued, *"were shown as clearly as the stars in the milky way on a clear dark night"*. Other authors have variously described these bright particles as 'fat dust', and 'hæmokonia' -blood dust.[8] The term chylomicron was proposed by Gage in 1920,[7] to designate their origin (in the chyle) and their dimensions (diameter 1.0 μm).

In 1924 the same author, in collaboration with Pierre Fish published a review of their work[8] in which they laid a foundation for the quantitative assessment of post prandial chylomicronæmia, and investigated some of its physiological and environmental determinants. Using the techniques of dark field microscopy they established that it was the fat component alone of newly absorbed food that gave rise to chylomicrons, and that it took from $\frac{1}{2}$ to $1\frac{1}{2}$ hours after eating fatty food for them to appear in serum. They examined the effects of different kinds of fat, inter-individual differences in man and animals, the effects of age and gender, and the influence of mental conditions on alimentary lipæmia.

Quantitation of lipæmia by dark field microscopy[9,10] involved visualisation of a capillary blood sample (obtained by thumb prick) at set times after eating, followed by a direct count of the number of chylomicrons in a specified grid area of the field __ a technical exercise likened by some authors to counting snowflakes in a storm.[8] The rapid Brownian motion of the particles necessitated several counts of each area by different individuals in order to improve the accuracy of the mean count. This number was then plotted against time to produce a 'chylomicrograph', the area of which was determined by planimetry and used as a measure of the magnitude of lipæmia. This technique was used by several groups of workers at this time,[8,11] and continued in use as late as 1950.[12,13]

Alternative techniques for the quantitation of lipæmia included measurement of the thickness of the 'cream line' after spinning serum in a centrifuge at 15000 revolutions per minute (rpm) for a set time,[14] or the use of the Tyndall Light Box which measured the scattering of incident light by lipæmic sera, the electrical signal generated being proportional to the size as well as the number of particles.[14]

Other groups used chemical methods based on the estimation of neutral fats to examine the effects of a fatty meal on blood lipids,[15] and it was established that the chylomicrograph and blood fat curves rose and fell in parallel.[11] Evidence for the composition of chylomicrons remained largely circumstantial however, till the advent of the ultracentrifuge allowed their preparation in purified form.

Early workers were hindered by the inconvenience of having to halt spins every fifteen minutes to allow the equipment to cool down.[16] Nevertheless they were able to confirm that the main component of the largest particles at least was triglyceride, and it was soon confirmed that they also carried cholesterol.[14] Although investigations into the nature of chylomicrons continued, the arrival of the ultracentrifuge signaled a decline in the use of the chylomicrograph as attention was diverted to the newly discovered lipoproteins, and only recently has its use been revived.[17]

Much of the early interest in chylomicronæmia was stimulated by the recognition that blood lipids were somehow involved in the development of atherosclerosis. Similarly the discovery that plasma lipids were transported as lipoproteins led to an explosion of interest, which continues today, in the study of lipoproteins and atherosclerosis.

1.2 The lipoproteins

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In several laboratories the ultracentrifuge soon became an essential tool in the study of serum lipids, and techniques quickly achieved a high degree of sophistication.[18,19] Lipoproteins were identified as large molecular structures in which polar and non-polar lipids are complexed with specific amphiphilic proteins (the apolipoproteins or apoproteins) for purposes of trans-portation through the plasma.

The general structure of these macro-molecules places neutral lipids (cholesteryl esters and triglycerides) in the core of a spherical particle, separated from the external aqueous environment by a surface monolayer consisting of apoproteins and the more polar lipids, mostly phospholipids, but including free cholesterol.[20] The mutual solubilities of these lipids may allow appreciable quantities of cholesterol to be distributed into the core, and small but significant amounts of cholesteryl ester and triglyceride to enter the surface monolayer.[21] In this model relative amounts of the more dense surface components increase with the square of the lipoprotein radius, and amounts of the less dense core components increase with the cube of the particle radius.

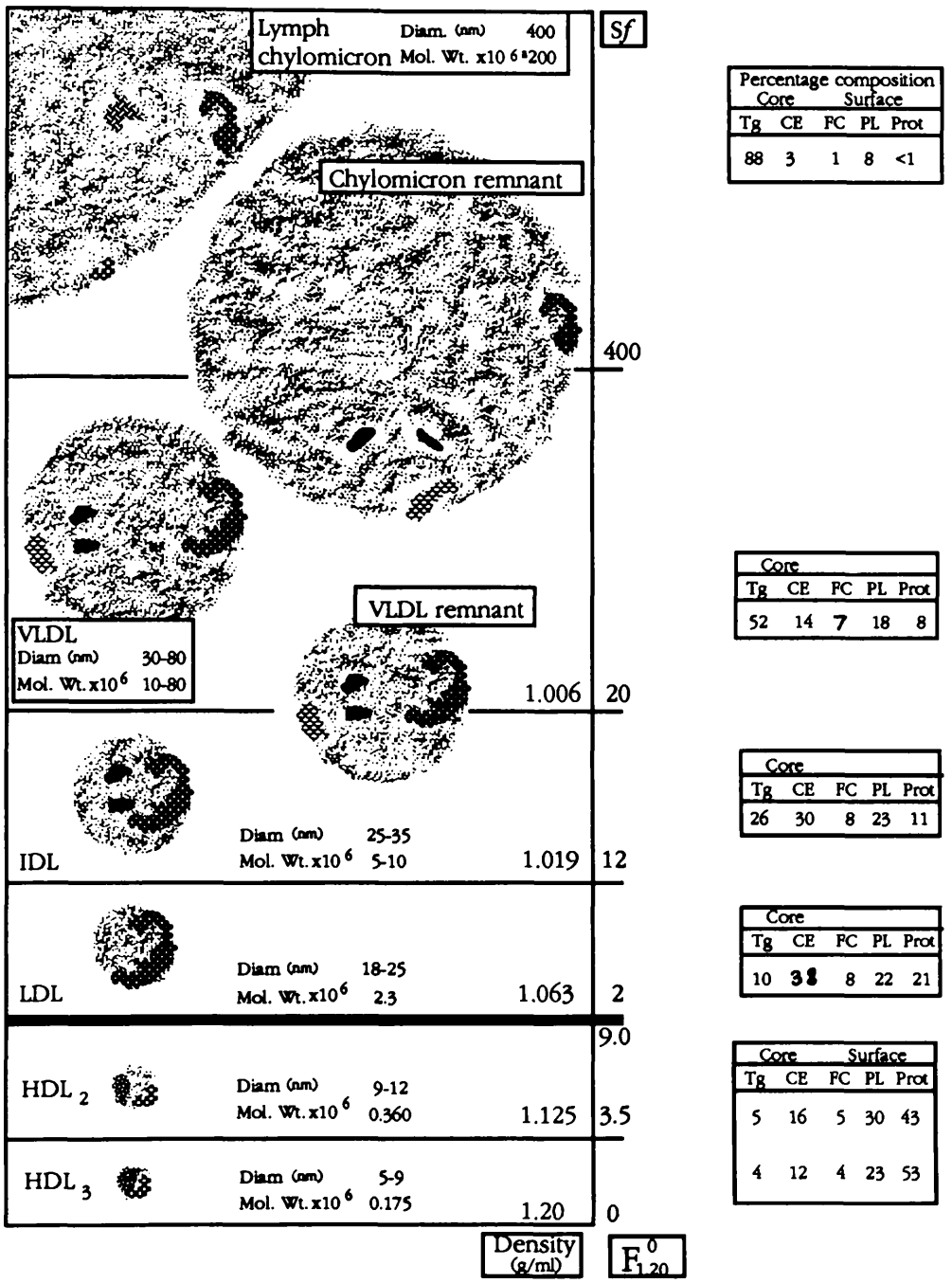

Figure 1.1 The lipoproteins occupy a continuum of size and density determined by the relative contributions of lipid and protein to their total mass. This continuum can be divided by empirical means into several classes with apparent physiological significance, and within which the majority of plasma lipoproteins lie. Nonetheless such operational definitions are to some extent arbitrary, and obscure the existence of particles with similar physical properties but different physiological background.

In the ultracentrifuge five major lipoprotein classes and several subclasses have been identified, and have gained widespread recognition and use in clinical as well as research laboratories (Figure 1.1). They are defined either in terms of their hydrated density (g/ml), or their rate of flotation through a salt solution of specific gravity 1.063 g/ml. The latter is expressed in terms of Svedberg units of flotation (S*f*)*[22]. The following section provides only a brief description of the main characteristics of these lipoprotein classes.

Chylomicrons

These are the largest and least dense of the plasma lipoproteins. They are synthesized by the intestine and transport newly absorbed dietary fat through the bloodstream. Under normal physiological conditions they are not present in fasting blood.

Very low density lipoproteins (VLDL)

These particles share similarities with chylomicrons in that their main component is triglyceride. They differ however in that they are synthesized from endogenous materials in the liver, and are present in fasting as well as post prandial blood.

The triglyceride component of both chylomicrons and VLDL are hydrolysed in the plasma by the enzyme lipoprotein lipase (LpL) present on the endothelial surfaces of blood vessels. Both chylomicrons and VLDL may give rise to 'remnants' __ catabolic products which seem less susceptible to further lipolysis. These remnants are relatively enriched in cholesterol and are efficiently cleared from the plasma by hepatocytes.

Intermediate density lipoproteins (IDL)

Present only in low concentration in normal plasma these lipoproteins represent an intermediate stage in the formation of LDL from VLDL. They contain relatively less triglyceride and more cholesteryl ester than VLDL.

Low density lipoproteins (LDL)

These are the major carriers of cholesterol in plasma. They are formed in the circulation by catabolism of hepatic VLDL and IDL, and are removed by hepatic and extra-hepatic tissues in a specific receptor mediated process which allows regulation of cellular cholesterol uptake.

High density lipoproteins (HDL)

These are the smallest and most dense lipoproteins. They are formed in the plasma either from precursors synthesised in the gut and liver, or from catabolic products generated at the surface of triglyceride rich lipoproteins during lipolysis.

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*One S*f* unit is equal to 10^{-13} cm/s/dyne/g at 26°C.

HDL exhibit considerable structural and metabolic heterogeneity. The two major subfractions of physiological significance are termed HDL_2 and HDL_3, and are separated on the basis of size or density. Other species of HDL, notably those with and without apo E, can be separated by affinity techniques and also appear to be physiologically important.

Table 1.1 *Key proteins involved in lipid metabolism*

Protein	Function
a). Apoproteins	
AI	Structural protein in HDL; LCAT activator; Receptor ligand
AII	Structural protein in HDL
B_{48}	Structural protein in chylomicrons
B_{100}	VLDL-LDL structural protein; Receptor ligand
CI	?LCAT activator
CII	LpL cofactor
CIII	LpL modulator
E	Receptor ligand
b). Receptors	
apo B/E	Cellular uptake and degradation of VLDL an LDL
apo E	Hepatic uptake of chylomicron remnants; ? uptake of apo E rich HDL
c). Enzymes	
LCAT*	Cholesterol sequestration and esterification in lipoproteins
LpL¶	Hydrolysis of triglycerides in chylomicrons and VLDL
HL§	Hydrolysis of triglycerides and phospholipids in IDL, LDL, and HDL.
LTP#	Several species facilitate the movements of phospholipid, cholesteryl esters, and triglycerides between lipoprotein classes in plasma.

*Lecithin: cholesterol acyl transferase, ¶Lipoprotein lipase, §Hepatic lipase, #Lipid transfer protein.
Adapted from reference 23.

The metabolism of these lipoprotein classes is directed by a number of protein components; __ receptors on cell membranes, enzymes in plasma, and apoproteins distributed on the lipoprotein surface (Table 1.1).As well as a structural role apoproteins regulate the metabolism of lipoproteins by modulating the activity of the plasma enzymes or by mediating their uptake by specific lipoprotein receptors. Their distribution in the various lipoprotein classes is given in Table 1.2.

Apo AI is a cofactor for the enzyme lecithin: cholesterol acyl transferase (LCAT) which transfers fatty acids from phospholipids to cholesterol forming cholesteryl esters. The activity of LCAT is mostly associated with HDL, the major repository of apo AI in plasma, and

leads to esterification of free cholesterol present in the surface monolayer of these lipoproteins.

Apo CII is an essential activator of LpL whereas the presence of apo CIII on triglyceride rich lipoproteins may suppress LpL activity and also modify the ability of receptors to bind with apoE.

Apo B_{100} is present on VLDL, IDL, and LDL of hepatic origin, and is the ligand for the LDL receptor. This receptor is responsible for the uptake of LDL by cells and plays an important role in the regulation of cellular and plasma cholesterol levels. Apo B_{48} is present only on triglyceride rich lipoproteins of intestinal origin and is not thought to be important in receptor mediated processes.

Apo E is also a ligand for the LDL receptor. It exists on chylomicrons and VLDL, and on a subgroup of HDL. The presence of apo B_{100} or E on a lipoprotein does not guarantee uptake by a particular receptor however, and it seems likely that the lipid content and physical properties of a lipoprotein particle may modify receptor interactions.

The presence of apo E on chylomicrons seems to be important in the clearance of remnant particles by the liver. The receptor responsible for uptake of these remnants appears to be distinct from that responsible for uptake of LDL, and has apo E alone as its ligand. A number of other receptors have been proposed to mediate the uptake of abnormal lipoproteins from the circulation.

Table 1.2 *Concentration of apoproteins in serum lipoproteins*

	Chylomicrons	VLDL	LDL	HDL_2	HDL_3
AI	33	trace	trace	65	62
AII	trace	trace	trace	10	23
AIV	14	-	-	?	trace
B	5	25	95	3	-
C	32	55	2	13	5
D	?	?	?	2	4
E	10	15	3	3	1

Figures are grams per 100g of protein. Adapted from reference 24.

1.3 Lipoproteins and atherosclerosis

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"Atherosclerosis is generally considered to be the major disease of this era. Its consequences in the coronary, cerebral, and peripheral arteries, in the form of occlusive phenomena, are responsible for more death and disability than any other disease".[19]

Thus wrote John Gofman in 1950, in the introduction to an article in which he and his collaborators at the Donner Laboratory in Berkely presented new evidence on the role of the serum lipoproteins in atherosclerosis. In 1988 Nobel laureates Michael Brown and Joseph Goldstein, introducing a symposium on the same subject stated that__*"In recent years the case against plasma lipoproteins as inciting agents in atherosclerosis has been established beyond reasonable doubt".*[25]

The intervening years have witnessed a massive investment by the scientific and medical communities in their investigations into the nature of this major disease, and the involvement of the lipoprotein system in its initiation and progression.

1.3.1 Pathological and epidemiological aspects of atherosclerosis

Atherosclerosis consists anatomically of a combination of changes in the intima of arteries involving an accumulation of lipids and complex carbohydrates with blood and its constituents, accompanied by a hardening and thickening of the arterial wall. The initiating factors are not precisely known but the condition seems to develop slowly over a period of decades. So called 'fatty streaks', visible in the walls of arteries from puberty, contain cells filled with many lipid droplets giving them a characteristic appearance which has prompted the descriptive term 'foam cell'. However it is not known what causes these streaks, or even whether they progress to become *bona fide* atherosclerotic plaque.

Progression of the disease involves the accumulation of cell debris, cholesterol crystals & esters, and calcium in a necrotic centre, overlying which is a fibrous cap of proliferated smooth muscle cells, extra- and intra-cellular lipid, and collagen. As it develops this plaque occupies progressively more space within the vascular lumen, eventually interfering with normal blood flow. When the lumen is reduced to about a third of its normal diameter ischæmia of the tissues distal to the narrowing ensues, leading to clinical conditions such as coronary heart disease (CHD), cerebrovascular insufficiency, and peripheral arterial occlusive disease. In a further stage of development the plaque may rupture or fissure, leading to hæmorrhage, ulceration and thrombosis.

Coronary heart disease (eg. angina pectoris, myocardial infarction, sudden cardiac death) is a widespread manifestation of atherosclerotic disease, and is the major cause of premature death and disability in many industrialised countries. For these nations it represents a major financial drain in terms of lost working hours and medical resources. The United Kingdom (UK) is prominent in the world ranking of CHD mortality rates, and within the UK Scotland

fares worse than England or Wales.[26] Even within Scotland there are large regional variations in mortality, with the Greater Glasgow area demonstrating the highest rate.[27]

Lacking precise knowledge of the causative factors in atherosclerosis, and noting these large population differences, epidemiologists have been prompted to introduce the concept of risk factors __ factors within populations which have been found to be associated with an increased likelihood of developing the disease. Many such risk factors have been identified[28] and new ones are frequently suggested. Some of these are genetic in origin, and some relate to dietary or social habits. Very often however, risk is associated with a combination of genetic disposition and unhealthy lifestyle, thus the origins of the disease are generally referred to as ***multifactorial***, with several possible factors, perhaps individually but more probably interacting in unhappy concert, to produce the symptoms.

Foremost and most consistent among established risk factors have been cigarette smoking, hypertension, and raised blood lipids.[28,29] Of these the most relevant to this study is the role of lipids in atherosclerosis.

1.3.2 Cholesterol and atherosclerosis

The ubiquity of cholesterol deposition in the atherosclerotic lesion,[30] the strong positive correlations between serum cholesterol and the incidence of the disease,[31,32] and the recent finding that therapy aimed at reducing serum cholesterol levels in hypercholesterolæmic subjects is accompanied by a reduction in the incidence of the disease,[33-35] leaves little doubt that cholesterol metabolism is somehow involved in atherosclerosis. It has even been suggested that some minimum serum cholesterol may be *necessary* for the development of atherosclerosis.[36]

The relationship of cholesterol levels to CHD extends in a continuum down through the normal cholesterol levels found in Western society,[37] and has led to the advocation of nationwide cholesterol lowering, with the introduction of action limits encompassing a substantial proportion of the population.[38]

1.3.3 Triglycerides and atherosclerosis

Despite the fact that populations at risk from CHD consume large amounts of triglyceride daily, and that the quantity and quality of this fat is intimately related to the development of the disease,[31] the concentration of triglyceride in plasma has never received the unanimous recognition as a risk factor that cholesterol has. There are probably several reasons for this.

Firstly, triglyceride is not directly associated with the atherosclerotic lesion in the incriminating fashion that cholesterol is.[30] Secondly, people suffering from conditions such as Type I hyperlipidæmia, in which serum triglyceride can reach levels in excess of 10 mmol/l, are not particularly susceptible to developing atherosclerosis.[39] Lastly, the results of epidemiological studies have not demonstrated a consistent *independent* relationship

between plasma triglycerides and CHD, and so have failed to convince the medical profession of their importance.[40]

Nevertheless concerns over the possible role of triglycerides in heart disease remain.[41] Serum triglycerides are frequently either moderately or markedly elevated in individuals suffering coronary infarction. In a widely quoted review of the subject Hulley *et al* noted 26 out of 27 studies examined in which there was a positive association between CHD and serum triglyceride.[40] It was discovered however, that this ***univariate*** relationship disappeared when other risk factors, with which serum triglycerides are correlated, were included in multiple logistic regression analyses. They concluded that there is little reason to believe that triglycerides are causally related to the disease, since the associations observed derive from a mutual association with other risk factors, namely serum and HDL cholesterol, and body mass.

However, by introducing an ***interaction term*** into their multiple regression analyses a recent report suggests that triglycerides may be an 'independent' predictor of CHD in subjects with relatively low serum cholesterol (<5.7mmol/l)[42] and in the ongoing Framingham study[43] there is a suggestion that high triglycerides are a risk factor in those who have a ratio of serum to HDL cholesterol which is greater than 3.5.

These examples illustrate the fundamental problem presented by the multifactorial nature of the disease. Static measurement of serum cholesterol provides useful information regarding the likelihood of developing CHD. Similarly, measurement of HDL cholesterol, blood pressure, body mass, smoking habits, the unsaturated fat content of the diet, or plasma triglyceride bring additional information. Taken together these factors may be integrated to provide an overall picture of an ***at risk*** person, or population.

However these factors are frequently physiologically related, statistically correlated, and quite possibly interactive rather than merely additive in their effects.[44] It may be futile therefore to attempt to extract the relative risk attached to any one factor in searching for the causes of the disease.

Morphologically, triglyceride and cholesterol are united in the lipoproteins for transport through the plasma. Physiologically, the metabolism of triglycerides is intimately related to that of all the lipoprotein classes,[45,46] and in hypertriglyceridæmic subjects there are distinctive abnormalities in lipoprotein structure and metabolism, which can be corrected by correction of the hypertriglyceridæmia.[47]

It was not a conclusion of Hulley *et al* that triglycerides were uninvolved in atherosclerosis, only that there was no evidence to support screening for, or treatment of, *isolated* hypertriglyceridæmia.[40] In their paper these authors complained that ***"Triglyceride is often lumped together with cholesterol in this fashion, which tends to discourage physicians from making separate judgements for the two hyperlipidæmias"***.

They concede however that it is difficult to test separate intervention in triglyceride or cholesterol levels by dietary or pharmacologic means due to their close relationship in a common transport system. It may be therefore that serum cholesterol and triglyceride *cannot* be considered separately, and that elevations in one or the other reflect alterations in specific lipoprotein fractions which, although perhaps showing a disproportionate effect on the levels of one lipid, ultimately affect both.

In the recently conducted Helsinki Heart Study[35] the drug Gemfibrozil was found to reduce the incidence of CHD in dyslipidæmic men. This drug lowered serum cholesterol by 10%. It also lowered triglyceride by 43%, non HDL cholesterol by 14%, and raised HDL cholesterol levels by 10%. In other words it corrected the *overall pattern* of lipoprotein transport in a manner favourable to the reduction of heart disease.

1.3.4 Chylomicronæmia and atherosclerosis

Post prandial chylomicronæmia was among the first of the lipid disorders suspected in the genesis of atherosclerosis. It was a consistent finding of early studies that lipæmia was more severe in people suffering from ischæmic heart disease.[13,48-51] As an hypothesis it was attractive since its transient nature could explain the common occurrence of the disease in otherwise normo-cholesterolæmic subjects.

The atherogenicity of lipæmia was initially considered to reside in the large size and particulate nature of the chylomicrons, and that post prandial triglyceridæmia was merely an indication of the number of chylomicrons present. Moreton[49] for example, considered intimal deposition *"to be directly related to the physical state of the lipids"*, and deemed it therefore *"meaningless and unnecessary to measure triglycerides during alimentary lipæmia"*.

He theorised that intimal macrophages of the reticulo-endothelial system identified these large particles as foreign bodies and engulfed them, and that while the triglyceride could be easily catabolised and absorbed by the cells of the intima, the residual cholesterol could not, and gradually accumulated in the cytosol leading to the formation of cholesterol laden foam cells.

Denborough[51] considered the relationship to CHD to be derived from an association of lipæmia with fasting triglycerides, with exogenous and endogenous triglyceride rich particles competing for a common removal mechanism. Lipæmia in itself was not thought to be causally related to the disease.

In more recent times the theory has been revived in slightly different form by Zilversmit,[52] who suggested that the interaction with the endothelium during lipolysis may provide an environment conducive to cholesterol deposition. The finger of blame had now shifted however from the chylomicron itself to the chylomicron remnant, a cholesteryl ester enriched particle, resistant to further lipolysis and removed directly from the circulation by receptor uptake.

The appeal of this theory was again that it might constitute a 'latent dyslipidæmia' which could explain the development of the disease in normolipidæmic individuals.[53] It was lent further support by the finding that cholesteryl ester enriched chylomicron remnants closely resembled ß-VLDL, an abnormal lipoprotein found in circumstances of high risk for the development of atherosclerosis.[54]

More recently still the growing appreciation of the inter-related and dynamic nature of lipoprotein metabolism[55] (see later) suggests another possibility, not exclusive of the above, that lipæmia may influence atherosclerosis indirectly by altering lipid flux through the lipoproteins, shifting cholesterol into less well regulated and potentially atherogenic pathways. Such a concept is founded in the belief that certain lipoprotein classes possess particularly atherogenic characteristics.

1.3.5 Atherogenic lipoproteins

Cholesterol in foam cells is generally thought to be derived from circulating lipoproteins. When there is a positive cholesterol balance in which uptake of lipoprotein cholesterol exceeds catabolism or efflux, cholesterol accumulation ensues.[56] The nature of the lipoprotein particle that gives rise to this situation and induces foam cell formation has been the focus of much research activity. Candidates include native LDL,[57] large LDL,[58] chemically or physically modified LDL,[59] oxidised LDL,[36] ß-VLDL,[60] and the remnants of triglyceride rich lipoproteins.[61] Central to the arguments regarding foam cell formation by these lipoproteins is the regulation of cellular cholesterol content by specific cell surface receptors.

The LDL receptor, because of its ability to recognize specific domains on apo B and E, binds lipoproteins carrying these peptides and leads to internalization of the whole lipoprotein by endocytosis. Hydrolysis of internalized lipoproteins releases cholesterol into the cytoplasm where it initiates a process of auto-regulation, suppressing further receptor synthesis and reducing cholesterol production by inhibiting the enzyme hydroxymethylglutaryl coenzyme A reductase (HMG CoA reductase). Cholesterol itself may be put to use by the cell, exported as bile acids or in lipoproteins, or esterified by ACAT and stored as cholesteryl esters.

Since the LDL receptor also recognises apo E on certain lipoproteins it is thought that a species of HDL carrying apo E may return cholesterol from the periphery to the liver,[54] although the quantitative significance of this pathway in man remains to be established.

Whereas cholesterol balance in most cells is controlled by the LDL receptor, macrophages and certain other cells of reticulo-endothelial origin seem to possess only a few of these.[59] They are furnished however with other distinct receptors thought to play a role in the uptake of certain abnormal lipoproteins. These receptors differ from the LDL receptor in that their expression is only poorly regulated by the cholesterol load of the cell. Therefore even in the presence of large amounts of intra-cellular cholesterol they continue to be

expressed and to remove their ligands from the plasma, resulting in a critical accumulation of cholesteryl ester, and leading eventually to foam cell formation.

Among these the acetyl LDL receptor seems to specifically identify LDL which has been chemically modified in certain ways, for example by acetylation, aceto-acetylation, or treatment with malondialdehyde.[59] The native ligand for this receptor remains unknown.

ß-VLDL is a peculiar lipoprotein which has the flotation properties of VLDL, and the electrophoretic mobility of LDL. These particles are present in Type III hyperlipoproteinæmia, and can be induced by feeding diets high in cholesterol and fat __ both conditions associated with a greatly increased risk of developing CHD. They are thought to represent exaggerated forms of the remnants of triglyceride rich lipoproteins which have become particularly enriched in cholesteryl esters, and they are capable of provoking massive cholesterol accumulation in macrophages, and foam cell formation.[54,62]

A ß-VLDL receptor, like the acetyl LDL receptor is found only on cells of reticuloendothelial origin, and in keeping with the general function of these cells has been suggested to play a backup or cleanup role in conditions where normal routes are overloaded.[59] Chylomicrons, hypertriglyceridæmic (but not normal) VLDL, and ß-VLDL are all sequestered by this receptor.[63]

Chylomicron remnants enriched in cholesteryl ester are among the most potent naturally occurring initiators of foam cell formation.[62] and a specific chylomicron remnant receptor thought to exist on hepatocytes has been postulated to be present on macrophages also. It has never been isolated however, and its identity has recently been questioned.[64] By virtue of their size, cholesteryl ester and apo E content, chylomicron remnants bear some resemblance to the ß-VLDL mentioned above, and there has been evidence to suggest that they may enter macrophages by the same route.[63] The activity of this receptor seems to be constitutive and not suppressed by cholesterol feeding, or drug therapy. It is therefore probably not functional in regulating cellular cholesterol levels.

If plasma cholesterol were to remain within lipoproteins which are taken into cells by the LDL receptor then cellular cholesterol uptake would be afforded some degree of regulation. (As long as LDL receptor capacity does not become overloaded) However factors which tend to divert cholesterol transport from this path into abnormal lipoprotein species may lead to increased amounts being removed from the circulation by less well regulated pathways, and may favour the deposition and accumulation of cholesterol in cells. Such factors may arise in a variety of situations, including, as will be discussed later, periods of high triglyceride flux.

1.4 Exogenous fat transport

§

"The relative rate at which the fat is being poured into the blood is indicated by the steepness of the upward limb of the (chylomicrograph) curve between any two intervals.

The relative rate at which the fat is being removed from the blood between any two intervals is indicated by the steepness of the downward limb of the curve.

The ascending and descending limbs of the curve are a composite of the opposing factors, viz, if there is an excess of addition of fat over the subtraction at any given time, the curve will go up, and its steepness will indicate the relative excess addition. On the other hand, if the elimination of the fat is greater than the addition at any given time, then there will be a descent of the curve, and its steepness will indicate the relative excess of the reduction "

SH Gage and PA Fish. (1924) *American Journal of Anatomy*, 34 p17.

The magnitude of alimentary lipæmia is determined by the composite effects of environmental and physiological factors affecting rates of chylomicron entry into the bloodstream, and factors affecting their clearance. The mechanisms involved comprise a system of 'exogenous fat transport' whereby dietary fats are assimilated by the body. These processes may conveniently be set into three stages:-

a) the digestion and absorption of fat in the gut,
b) the formation of chylomicrons from newly absorbed or synthesized materials in the endothelial cells of the intestine,
c) the transport and catabolism of chylomicrons in the plasma.

During any of these phases events may influence the development of lipæmia in the plasma. Details of the digestion and absorption of fats have largely been established over the past fifty years, and have been reviewed extensively in the literature.[65-67] Only a brief summary of present understanding is included here, followed by a description of the principal stages in chylomicron formation and catabolism including their interactions with other lipoprotein classes during lipæmia

1.4.1 The fat component of the diet

The term 'fat' properly refers to triglycerides which, along with phospholipids and cholesterol, are the major classes of lipid in the diet. The fat soluble vitamins are biologically important but are quantitatively a minor component. It has been estimated[68] that the average Western adult consumes approximately 100g of fat per day, providing some 40% of total calories. Dietary intake of phospholipid, mainly lecithin, is approximately 2.2g[69] and cholesterol 1.0g per day,[70] to which a further 12g of phospholipid and 1.0g of cholesterol derived from bile and gut endothelial cell debris are added in the gut [69].

The structure and physico-chemical properties of triglycerides, phospholipids and cholesterol are to be found in many chemistry and biochemistry texts. In addition to the conventional chemical definitions biologically active lipids have been classified by Patton[71] on the basis of their occurrence in foods, and by Small[72] on the basis of their behaviour in aqueous systems.

Table 1.3 *Classification of biologically active lipids*

a). Composition of dietary lipid

Physical form	eg	Chemical Composition
Surface lipids	external seed coat	Hydrocarbons, wax esters, ketones, triglycerides, fatty acids, alcohols, aldehydes, sterols, alkane diols, terpenoids
Fat droplets	adipose tissue	Triglycerides (terrestrial plants and animals) Wax esters and triglycerides (marine animals).
Membranes	internal cell membranes	Phospholipids, sterols, sphingolipids, galactolipids, carotenoids.

b). Lipids in aqueous systems

Non polar
Insoluble in water and do not interact with water eg. *cholesterol esters, carotene.*

Polar
Insoluble non-swelling amphiphiles:-
Low solubility in water, form an immiscible liquid or solid phase eg. *long chain fatty acids, diglycerides, triglycerides, sterols.*

Insoluble swelling amphiphiles:-
Insoluble in water, but interact to form a liquid crystalline phase in which water is sandwiched between polar groups (known as swelling), eg. *phospholipids, monoglycerides, ionised fatty acids.*

Soluble amphiphiles:-
Soluble in water at low concentration. Form micelles in which polar group faces out and non-polar group faces inward. eg. *bile acids, ionic detergents, soaps.*

The scheme of Patton (Table 1.3a) recognises that the digestive system has evolved to deal with foodstuffs in their natural environment - their 'molecular ecology' as he calls it - where they occur not only in molecular mixture with other lipids, but also with carbohydrates, proteins, minerals, and indigestible fibres. Quantitatively lipid digestion is virtually synonymous with the digestion of triglyceride stored in fat droplets, although minor components of the diet, the surface and membrane lipids of this classification, are nonetheless essential to the physiological processes of digestion, absorption, and transport of fat.

The bio-availability of lipids is heavily dependent on their ability to interact with water, and the rationale underlying the evolution of physiological fat transport systems has been to increase this ability. In this respect the classification of Small (Table 1.3b) is of interest.

Although this scheme is based on the physical behaviour of purified lipids when mixed with water, it has been useful in predicting their behaviour in physiological systems where polar and non-polar lipids occur in the various combinations already mentioned. Thus the amphipathic nature of certain lipids is employed to solubilize those which are more hydrophobic.

1.4.2 The digestion of fat

The basic reaction of digestion is the conversion of triglyceride, a non-swelling insoluble lipid, into a form which can better interact with water. This function is performed by a series of lipases—enzymes which catalyse the hydrolysis of fatty acid ester bonds. The intermediate product is a diglyceride which is also insoluble, and the end products are monoglycerides, fatty acids, and glycerol. In order to facilitate this reaction fats are first emulsified.

Mechanical disruption of bulk fat takes place in the mouth, by the action of chewing, and in the stomach by its peristaltic activity, progressively forming a more finely divided emulsion. In the acid (pH 1.0) environment of the stomach peptic digests of protein, complex polysaccharides, and phospholipids derived from the membranous materials of ingested food provide a potential source of the amphiphiles necessary for the formation of emulsions.[73] By orientation at the surfaces of lipid droplets these amphiphiles reduce lipid/water interfacial tension, stabilize the emulsion and significantly increase the surface area of fatty substrate available for hydrolysis by surface active lipases.[74]

Triglyceride remains in the stomach for 2 to 4 hours[75] during which time 10-30% may be hydrolysed.[68] Lingual lipase, secreted by glands on the dorsal surface of the tongue, is particularly suited to the gastric environment and is probably responsible for this initial hydrolytic activity.[76] It has an acid pH optimum, is resistant to peptic digestion, and does not require bile salts for its action. It also shows much higher activity with short or medium chain than with long chain fatty acids, and is specific for primary esters of triglycerides, attacking the sn-3 position twice as fast as the sn-1 position.[76] The main products of hydrolysis by this enzyme are long chain sn-1,2 diglycerides and fatty acids.

Shorter chain fatty acids liberated at this stage are soluble in water and are absorbed directly through the stomach wall and transported bound to albumin to the liver via the portal vein, while longer chain fatty acids tend to partition within the hydrophobic core of emulsion particles.[73] The acidic emulsion (at this stage referred to as chyme), is dispensed intermittently into the upper small intestine (duodenum) through the pyloric sphincter. The squirting of chyme through this valve produces shear forces which further assist in fine emulsification. Lipid droplets entering the duodenum are less than 0.5 μm in diameter and highly stable.[65]

The low pH of the chyme and the presence of fatty acids causes the release of the hormone cholecystokinin pancreozymin (CCK-PZ) from duodenal mucosal cells into the

bloodstream, and induces gall bladder contraction and pancreatic enzyme secretion. The combined action of bile and pancreatic juice, which enter the duodenum close to the pyloric sphincter, bring about a marked change in the chemical and physical form of the ingested lipid emulsion.

Although in themselves poor emulsifying agents, bile salts aid emulsification when in dilute combination with lipolytic products from the stomach (lecithin, monoglyceride, fatty acids) and biliary lipids. By lowering the bulk pH at which fatty acids are 50% ionised from >7.0 to 6.5 the bile acids allow the formation of acid soaps.[73] Bile salts intercalate with these and other hydrolytic products and amphiphiles in the surface monolayer thus increasing ionisation at the interface and maximising emulsion stability.

Pancreatic secretions have a pH of 7.5 - 8.0, and contain Na^+, K^+, HCO^-_3, Cl^- and other salts, in addition to many digestive enzymes. The major enzymes in lipid digestion are cholesterol esterase, phospholipase A2 and pancreatic lipase.

• *Cholesteryl ester hydrolase* has a broad specificity catalysing the hydrolysis of water soluble carboxyl esters and insoluble esters (cholesterol and vitamins A,D,and E) in bile salt micelles. The monomeric form is dimerised to the active form in the presence of 3α and 7α hydroxylated primary bile salts. The end products are fatty acids and free cholesterol.[77]

• *Pancreatic phospholipase A2* is activated by tryptic hydrolysis of a pro-enzyme in the small intestine. It catalyses the hydrolysis of fatty acids at the sn-2 position of phosphoglycerides (but not sphingolipids). There is an absolute requirement for Ca^{++} ions and for aggregation of the substrate, an arrangement which appears to facilitate the non-equivalent interaction of sn-2 fatty acid ester with the catalytic site of the enzyme. The product is a lysophospholipid. No further hydrolysis of lysolecithin appears to take place in the intestinal lumen.

• *Pancreatic triglyceride lipase* can bind to a triglyceride/water interface where it is highly active. Bile salts, phospholipids, cholesterol and other physiological emulsifiers however appear to inhibit binding.[66] The polar surface layer is penetrated by a co-lipase, a factor secreted by the pancreas which attaches to ester bonds and provides an anchor for the lipase which is then in suitable conformation for hydrolysis of the substrate.[71]

A number of factors promote the binding of co-lipase to emulsion particles. Small amounts of partially ionised fatty acids formed during lingual lipase hydrolysis increase binding.[73] Bile salts may also help by forming aggregates with co-lipase and enhance the binding of phospholipase A2. Calcium ions required for the activity of phospholipase A2 may facilitate the desorption of lipolytic products from the catalytic interface. Pancreatic lipase is virtually specific for sn-1 and sn-3 fatty acids, and probably hydrolyses sn-2 acids only after isomerisation. Since isomerisation generally occurs very slowly the main products of

pancreatic lipase activity are sn-2 monoglycerides and fatty acids, with less than a quarter of ingested triglyceride completely broken down to glycerol and fatty acid.

The final products of this digestive phase are glycerol, sn-2 monoglycerides, fatty acids, cholesterol, and phospholipids (mainly lysolecithin) in dilute suspension at pH6.5. Due to their limited solubility in aqueous solution, these materials are not readily available for absorption.

As their concentration approaches between 5 -15 μmol/ml however, (the critical micellar concentration), bile salts spontaneously form micelles in which the polar hydroxyl and amino groups face the surrounding aqueous phase, and the non-polar steroid nuclear portions form a hydrophobic core.[67] Interaction of these negatively charged micellar particles with lipolytic products leads to the formation of ***mixed*** micelles. At the pH of the upper intestine fatty acids are partially ionised. The non-polar chains of fatty acids, monoglycerides and phospholipids, along with cholesterol, enter the micelles and are thus solubilized. Successful micelle formation permits diffusion of lipid products to the surface of the intestinal epithelium, there to make contact with the microvillus membrane.

1.4.3 The absorption of fat

The luminal surface area of the small intestine is greatly expanded by the presence of millions of finger-shaped villi approximately 1 mm long and occurring at a density of some hundreds per mm^2.[78] Each of these villi have access to the lymphatic and blood systems via the central lamina propria which is supplied with blood capillary and lymphatic channels. The epithelial cells of the villus (enterocytes) are specialised both for the uptake of digestive products and for their re-packaging for transport to the bloodstream. At their luminal (apical) surface the membrane is thrown up into multiple projections (microvilli) to form the so-called 'brush-border', further expanding the area available for absorption. Lipid digestion products are thought to enter these cells by passive diffusion down a concentration gradient.[67]

Adjacent to the brush border is a layer of water which is not in equilibrium with the bulk intestinal fluid. This unstirred water layer (UWL) as it is known, represents an effective barrier to the passage of hydrophobic materials, and diffusion through this area is thought to govern their overall rate of absorption.[67] Micellar solubilization of lipids enhances their passage through this region.

Micelles cannot permeate the cell membrane as intact structures however, since it is lipid in nature and therefore does not readily admit polar materials. The products of lipid digestion are in dynamic equilibrium with the mixed micelle phase, thus maintaining a saturated aqueous solution of lipids within the UWL. Lipid moieties also partition into the lipid environment of the membrane, and diffuse from there into the cell. Rapid removal from the membrane and esterification within the cell ensures a favourable concentration gradient from the intestinal lumen into the cells.

A low molecular weight cytosolic protein has been found to bind fatty acids avidly,[79] and it has been suggested that this protein, named fatty acid binding protein (FABP), facilitates removal of fatty acid from the membrane and aids its transport through the cytosol to sites of complex lipid synthesis. Fatty acids of chain length less than 10-12 carbon atoms pass without re-esterification directly from mucosal cells into the portal circulation, and free glycerol may also leave by this route. Longer chain fatty acids are re-esterified to form triglyceride.

Cholesteryl ester must be hydrolysed prior to absorption, the free cholesterol then entering micellar solution. Even in the presence of bile salt micelles the solubility of cholesterol is still poor and depends on the local concentration of monoglyceride and fatty acid since these expand the hydrophobic core in which the cholesterol is dissolved.[80] Free cholesterol passes through the cell membrane as a monomer and must ultimately dissociate from the micelle, possible aided by the micro-environment created by similarly dissociated fatty acids.

Lysolecithin is the main form of absorbed phospholipid, and partitions into the micellar phase. Sphingolipids are thought to be absorbed intact.

1.4.4 The formation of chylomicrons

Topologically the lumen of the gastrointestinal tract is still outside the body. Having crossed the barrier of the intestinal microvillus membrane the lipid digestive products can be considered as inside. The intestine however does not merely passively admit absorbed dietary materials to the circulation, but plays an active part in plasma lipoprotein formation and metabolism. Once inside, absorbed lipid materials are quickly re-esterified to form triglycerides, phospholipids and cholesteryl esters. In addition to the enzymes required for lipid resynthesis the enterocyte is a major source of newly synthesized apoproteins, and possesses the complex and highly organised machinery for the assembly and export of several classes of lipoprotein, the most important of which are the chylomicrons

Lipid components

• *Triglyceride* is synthesized from fatty acid, sn-2 monoglyceride and/or glycerol. The necessary enzymes are present on the inner membrane of the endoplasmic reticulum (ER) and are increasingly abundant in enterocytes near the villus tip. There are two enzymatic pathways by which triglyceride may be synthesised, both of which have a common first step - the activation of fatty acid to its coenzyme-A (CoA) derivative. This is accomplished by the enzyme fatty acid:CoA ligase, also called acyl CoA synthetase, which specifically utilises long chain fatty acid as substrate.

In the *monoglyceride pathway* fatty acyl CoA is transferred to sn-2 monoglyceride to form a diglyceride which is then further acylated to form triglyceride. The three enzymes

necessary for this reaction - acyl CoA synthetase, monoglyceride transacylase and diglyceride transacylase are present as a complex called triglyceride synthetase.

In the *phosphatidic acid pathway* glycerol is first phosphorylated by glycerol kinase and then converted to phosphatidic acid by glycerolphosphate and acyl transferase. Fatty acyl CoA is then transferred to phosphatidic acid to form diglyceride (phosphatidate phosphohydrolase) and then triglyceride (diglyceride transacylase).

These two pathways function independently__the enzyme system for the monoglyceride pathway is present on the membranes of the smooth endoplasmic reticulum (SER) and those of the phosphatidic acid pathway on the rough endoplasmic reticulum (RER).[81] In the enterocyte the monoglyceride pathway is quantitatively more important, although it depends to some extent on the lipid mixture available. During absorption, when monoglyceride is in abundance the monoglyceride pathway will be favoured. During fasting however, the intestine still produces triglyceride, contributing some 20-40% of plasma VLDL,[82] at which time the phosphatidic acid pathway may predominate. Since the glycerol for this pathway can be derived from glucose metabolism it provides a link between carbohydrate and lipid metabolism.

• *Phospholipids*, mainly lecithin, are formed by the acylation of absorbed lysophospholipids due to the enzyme lysophosphatidyl-acyltransferase present in the ER. Alternatively they can be synthesized *de novo* from diglyceride formed by the phosphatidic acid pathway, and cytidine diphosphocholine (synthesised from absorbed choline). Between 20-30% of phospholipids may be formed in this way.[83] Lysolecithin may can be hydrolysed within the enterocyte by lysolecithinase to form glycerolphosphorylcholine and fatty acid. The fatty acyl composition of phospholipids synthesized in the enterocyte does not reflect the composition of dietary fatty acids as accurately as does that of newly synthesized triglyceride.[84]

The pathway of phospholipid synthesis may be determined by the availability of the respective substrates, or it may be suited to the needs of the cell. Phospholipids are required for the surface coating of chylomicrons and depletion of phospholipid leads to the formation of larger lipoproteins,[85] whereas excess lecithin leads to the formation of smaller intestinal VLDL.[86] In addition phospholipids are required for the maintenance of enterocyte membrane function.[87] During protein biosynthesis and intracellular chylomicron transport the membranes of the ER and Golgi are turning over rapidly[88] and their continued synthetic activity requires constant replenishment with phospholipid.

Sphingomyelin is absorbed intact but is broken down in the mucosal cell to ceramide and phosphotidylcholine[69]

• *Cholesterol* within the enterocyte may be derived from a variety of sources: from absorbed material, both from the diet and from biliary and cellular breakdown, from *de novo* synthesis, or from infiltration of plasma LDL and HDL.[66] Esterification of cholesterol

probably takes place just prior to incorporation into chylomicrons since most cellular cholesterol remains free, whereas 80-85% of that exported is in the form of esters. Oleic acid is the principal fatty acid found in cholesteryl esters of intestinal origin.[89]

It seems that the rate of cholesterol absorption may be determined to some extent by its rate of esterification.[90] Cholesteryl ester hydrolase of pancreatic origin hydrolyses cholesteryl esters in the duodenum where pH is neutral to basic. At pH 5-6 however the enzyme catalyses the reverse reaction.[91] This enzyme has been found in the enterocyte and has been suggested to play a role in cholesterol esterification there.[92] Acyl CoA: cholesterol acyl transferase (ACAT) is another enzyme capable of esterifying cholesterol, and is also present in microsomal fractions of mucosal cells, mostly in the villus tips of jejunum and proximal ileum.[93] It shows a preference for oleoyl CoA, thus agreeing with the main species of cholesterol ester exported from the intestine. It has been shown however that in the presence of antibodies against ACAT cholesterol absorption and esterification can continue.[94]

Apoprotein components

Approximately 10-20% of the surface of a chylomicron is occupied by proteins similar to those found in the plasma lipoproteins. They are crucial in directing the pattern of export, catabolism and clearance of chylomicrons. Comprising only 1-2% of total chylomicron mass, the large daily flux of lipid through the enterocyte nonetheless ensures a significant contribution to the plasma apoprotein pool. Some of these apoproteins are actively synthesized by the enterocyte and incorporated during chylomicron assembly, others are acquired by transfer from other lipoproteins during transport through lymph and plasma.

• *ApoAI* is synthesized in the enterocyte[95] and is present there in appreciable amounts even in the post absorptive state.[96] A high rate of synthesis and export is suggested by the fact that daily output may be 20-50 times the total mucosal content of AI[97] It has been calculated that the intestine may contribute up to 56% of AI in rat plasma.[98]

Much of the AI in enterocytes and in lamina propria is present in free form, only 10% being associated with lipoprotein. In mesenteric lymph almost all is associated with lipid.[99] Increasing amounts of enterocyte AI becomes associated with lipid during absorption, and secretion into the lymph may increase several fold.[97] Rates of synthesis however seem to be little altered by triglyceride flux,[100] therefore the level at which AI synthesis is regulated and the point of action of fat feeding is unknown.

• *ApoAII* is also synthesized in small amounts by enterocytes.[101]

• *ApoB* found in chylomicrons shows immunological similarities with that found in hepatic VLDL and LDL but has a molecular weight which is only 48% that of the LDL form. This variety of apoB has therefore been called B_{48} and is apparently specific to intestinal lipoproteins.[102]

Synthesis seems to occur in the RER from whence apoB is transferred to the SER and incorporated into the nascent chylomicron at or near the junction of the two.[103] These then move to the Golgi cisternæ where the apoB is glycosylated and chylomicrons prepared for export.[104]

Lipid absorption in rats was associated with an increase in apoB concentration within 10-15 minutes of exposure to fat,[97] though in humans there was a decrease in apoB following fat feeding.[105] This finding was taken to indicate depletion of a preformed pool of apoB at a rate exceeding that of *de novo* synthesis. More recently it has been suggested that B_{48} synthesis is bile salt dependent and not subject to regulation by acute trans-epithelial triglyceride flux.[106]

The importance of apoB in lipid absorption is clearly exemplified by the inherited condition of abetalipoproteinæmia in which a total lack of apoB synthesis is associated with a complete absence of chylomicrons, VLDL, and LDL from the circulation.[107] Although lipid droplets accumulate in the SER of these people they seem unable to enter the Golgi apparatus and chylomicrons are not exported.

- *ApoAIV*, at a concentration of 12μg/mg protein in the fasting state rising to 46μg/mg after lipid feeding, is present in human intestinal mucosa in substantially greater amounts than other apoproteins.[96] Its role in chylomicron formation and transport remains unknown.
- *ApoC* constitutes 25-50% of post prandial chylomicron proteins.[108] Most of these are acquired by transfer from other lipoproteins however, since less than 10% of plasma apoC are thought to be synthesised in the intestine.[109]
- *ApoE* is probably not produced by the intestine[109] but acquired by transfer from other lipoproteins during transport through lymph and plasma.[108] ApoE plays a crucial role in the catabolism of chylomicrons.
- *Apo[a]* was found in a fraction of postprandial lipoproteins which were also enriched in apoE and $apoB_{48}$.[110] The origin or significance of the apo[a] component in terms of chylomicron formation or catabolism is unknown.

The assembly of chylomicrons

Chylomicrons are not synthesized in the form in which they eventually appear in the plasma, but are continuously modified as they pass through the enterocyte, the lymphatics, and the blood. Triglyceride re-synthesis is localised in the SER and lipid droplets subsequently accumulate in the Golgi apparatus of the supra nuclear portion of the cell. Apo B is localised initially in the RER and on triglyceride droplets in the profiles of the SER, suggesting an addition early in chylomicron formation. Glycosylation of lipid and protein components takes place in the endoplasmic reticulum and in the Golgi.

There are two proposals, not mutually exclusive, regarding the involvement of the Golgi apparatus.[81] Firstly vesicles containing maturing pre-chylomicrons may bud off from the tubules of the SER and merge with the Golgi apparatus. Secondly the SER are connected by a

group of tubules called the 'Boulevard Peripherique' which may transport lipid droplets between the two organelles. Vesicles containing nascent chylomicrons pinch off from the Golgi and migrate towards the baso lateral plasma membrane with which they fuse and release their contents into the inter-cellular space.[111]

Redgrave[12] found lymph chylomicrons to differ in their composition from the intra-cellular lipid droplets found during absorption, and called these droplets 'pre-chylomicrons'. They contained more free fatty acids, free cholesterol, more protein and differed in their phospholipid composition, indicating a possible change in the surface coat during transit through the Golgi apparatus to the plasma membrane.

Intestinal VLDL It is apparent that triglyceride rich lipoproteins of S*f* 20-400 and diameter 30-80nm are formed and exported from the intestine during fasting[82] and fat absorption.[113] They have also been observed in human enterocytes[114] and resemble chylomicrons in their lipid and apoprotein content. Although qualifying as VLDL the triglyceride composition of these particles clearly differs from that of plasma VLDL.

Incubation of these intestinal VLDL with the $\rho<1.006$ plasma fraction leads to an increase in apoE at the expense of apoAI and AIV, so that they then resemble plasma VLDL. They become more prominent after duodenal infusion of lecithin[86] probably due to an increased ratio of surface to core materials. It has been suggested that they represent two separate populations of triglyceride rich lipoprotein,[81] although it is also possible that they represent one end of a spectrum of particles varying in size according to the relative amounts of core and surface materials available.

1.4.5 Rate limiting steps in chylomicron production

In the complex series of events described above there are several potentially rate limiting steps. The rate at which food leaves the stomach is influenced by many factors, in particular the volume and composition of the meal.[75,115] Upon entering the intestine lipases are secreted in excess,[71] and so it seems unlikely that their activity would be rate limiting. Diffusion through the unstirred water layer has been suggested as limiting where large micellar structures are concerned,[67] and if competition between excess lipolytic products for micellar solubilization occurs this step may indeed affect rates of lipid absorption.

Rates of re-esterification, as has already been mentioned, may influence the overall rate of absorption.[90] In this respect fatty acid binding proteins (FABP) must be produced at an adequate rate to ensure a steady supply of fatty acids to the synthetic machinery of the enterocyte. In addition sufficient surface materials such as apoproteins or phospholipids are required not only for incorporation into lipoproteins, but for all membrane bound activities. If their supply is restricted then absorption of triglyceride may be compromised.[87]

Lipid absorption and chylomicron formation occur mainly in the jejunum, and less efficiently in the ileum.[111] In the proximal jejunum the limiting step seems to be uptake into the enterocyte, whereas in the ileum it is the removal of lipid which is most important.[109,117]

Having left the enterocyte chylomicrons must cross approximately 50μm of interstitium before reaching the central lacteal. Rates of passage through this gel like material depends on the degree of hydration[118] and the flow rate of lymph.[119]

1.4.6 The digestion and absorption of retinol (Vitamin A)

The unique features of vitamin A absorption have been exploited in studies of exogenous fat metabolism, and are therefore included here in some detail. Dietary retinyl esters, retinol and provitamin A carotenoids are dispersed and emulsified during digestion in a manner analogous to that of cholesteryl esters. A relatively non-specific carboxylic ester hydrolase, thought to be the same enzyme responsible for cholesterol ester hydrolysis, hydrolyses long chain retinyl esters. Only free retinol may cross the mucosal cell membrane, and to do so is solubilized in mixed micelles.

Within the enterocyte retinol is re-esterified with long chain fatty acids. The fatty acid composition of retinyl esters is largely independent of the fatty acid composition of meals with which they are consumed, palmitate predominating and saturated retinyl esters (palmitate and stearate approx 2:1) comprising three fourths. Small amounts of retinyl oleate and linoleate may also be present.[120] It has been proposed that retinol reacts with a fatty acyl CoA in a reaction catalysed by an acyl CoA: retinol acyl transferase (ARAT), again in analogous fashion to the ACAT reaction forming cholesteryl esters. ARAT shows a preference for palmitoyl over oleoyl CoA.[121]

Retinyl esters are thereafter packaged in the hydrophobic core of chylomicrons and transported to the liver where they are taken up by hepatocytes along with chylomicron remnants. Once within hepatocytes they are either stored as retinyl ester or are hydrolysed to retinol and exported to the general circulation bound to a high density plasma retinol binding protein. Retinol is not exported from the liver in the form of long chain fatty acid esters.

1.4.7 Chylomicron catabolism

Within an hour of fat consumption chylomicrons begin to traverse the thoracic duct and enter the general circulation through the subclavian vein. In the plasma they are fitted for catabolism by an immediate and rapid series of interactions with other lipoproteins. ApoAI and AIV, synthesised by the intestine, leave the chylomicron surface and enter the HDL and lipoprotein free density ranges.[122] $ApoB_{48}$, the other intestinally derived apoprotein remains an integral part of the chylomicron throughout its career. Apoproteins CI, CII, and CIII transfer in the opposite direction, from HDL to chylomicrons,[123] as does apoE.[124] These

transfers occur rapidly and spontaneously upon entry of chylomicrons to the plasma, leaving a particle whose apoprotein composition is quite different from its lymphatic precursor.

The catabolic processes which rapidly ensue may be divided into two phases. Firstly a large percentage of triglyceride is removed from the core of the particle (lipolysis) reducing its size considerably, and liberating redundant surface materials which enter the HDL density range. Secondly the remainder of the particle - the chylomicron remnant.- is removed *'en masse'* by a receptor mediated process into hepatocytes. Both of these processes are apparently closely regulated by the fluctuating affinities of the apoC and E components for the surface of the diminishing chylomicron.

Lipolysis

Hydrolysis of the bulk of chylomicron triglyceride is accomplished by the actions of lipoprotein lipase (LpL). This enzyme is found attached to the endothelium of capillaries by cell surface glycosaminoglycans such as heparin sulphate,[125] and may be released into the bloodstream by the administration of heparin.[126] The products of hydrolysis are free fatty acids and monoglycerides, which eventually enter cells or are bound to circulating albumin. LpL shows some stereospecific preference for the sn-1 over the sn-3 position of triglyceride but has a much lower affinity for the sn-2 position.[125] There is debate over its fatty acid specificities (see later). The enzyme also has phospholipase activity.[125]

Lipoprotein lipase activity has an absolute requirement for apoCII,[127] as is evident from an inherited condition in which its complete absence leads to massive chylomicronæmia.[128] Activity of the enzyme requires the formation of a complex comprising apoCII, dimeric lipoprotein lipase, and a triglyceride rich lipoprotein substrate.[129] It seems likely that lipoprotein lipase, an enzyme which catalyses a reaction ultimately requiring water, encounters its triglyceride substrate at the surface of the chylomicron.

Triglyceride in the core equilibrates rapidly with the small amount of triglyceride solubilized in the surface monolayer,[130] thus factors affecting the partitioning of triglyceride between core and surface (eg cholesteryl ester content, or increasing saturation of fatty acid chains) may influence the rate of hydrolysis. The affinity of lipoprotein lipase and apoCII for the lipid surface of the lipoprotein may also be important. A build up of lysolecithin, a product of phospholipid hydrolysis, decreases fatty acid release by approximately 10 fold,[131] and fatty acids not efficiently removed from the active site may lead to product inhibition.[132] An accumulation of free cholesterol in surface layers has also been associated with a decrease in lipase activity.[21]

As triglyceride is removed from the particle the core volume decreases leaving an excess of surface material. It is thought that this material, mainly phospholipid, apoAI and AII, with some free cholesterol, forms bilammellar outfoldings which eventually dissociate to form

discoidal leaflets.[133] These then enter the HDL density range where they interact with the enzyme lecithin : cholesterol acyl transferase (LCAT) to give rise to HDL.

It is not clear from the literature whether the triglyceride content of the chylomicron is hydrolysed 'at one sitting' or whether a dynamic equilibrium exists between bound and circulating chylomicrons at various stages of catabolism, an equilibrium which gradually moves from bound to unbound. In any case, at a stage when approximately 75% of triglyceride has been removed, the chylomicron becomes a relatively poor substrate for LpL and continues to circulate as a chylomicron remnant until it is removed by hepatocytes.

There is some evidence that the enzyme hepatic triglyceride lipase may be involved in chylomicron catabolism in its later stages[134] although the role of this enzyme remains unclear.

The signal marking the end of LpL hydrolysis (remnant production) and the beginning of the remnant clearance phase of lipæmia remains to be established. Presumably factors which influence the affinity of the particle for either LpL or its apoCII activator are important in reducing its susceptibility to further hydrolysis.

The composition of the chylomicron remnant differs from its precursor in a number of important respects. The loss of triglyceride from the core of the chylomicron represents in effect a loss of solvent for core located free cholesterol, resulting in a net movement of unesterified cholesterol into surface regions.[21] In addition there is a movement of unesterified cholesterol down a concentration gradient from red and white blood cells and other lipoproteins. Enrichment of surface areas with free cholesterol can reduce the solubility of triglyceride (from 3-4% to 0.15% at 33% surface cholesterol)[21] in the surface phase and also reduce protein binding.[130] An increase in the lysophosphatidyl choline content of the surface has also been associated with a reduced affinity for C apoproteins.[135]

These changes in surface composition may be attenuated in part by the loss of most of this material to HDL (along with apoAI and AII, and apoAIV which enters the lipoprotein free fraction)[136] as the chylomicron core dwindles, leading to redundancy of the surface coat. This process may in itself be important since the greatly reduced surface area may provoke competition for space between $apoB_{48}$, apoE and the apoC peptides, with the attendant effects on LpL and receptor interactions.

$ApoB_{48}$ and apoE remain with the particle until it is eventually removed by hepatocytes. ApoC's however are gradually lost and re-enter the HDL pool. It has been suggested that differing affinities of the shrinking chylomicron for apoCII, CIII, and E regulate not only the cessation of lipolysis but the uptake of the remnant by the hepatic apoE receptor.[137] In this scenario the presence of apoC, particularly apoCIII, on chylomicrons prevents their uptake by the apoE receptor. The affinity of the C peptides declines however, as lipolysis nears completion and they depart the particles surface. ApoCII is postulated to leave slightly before apoCIII, being more influenced by the accumulation of lysophospholipids in the surface, at which stage lipolysis is halted. The departure of apoCIII frees apoE to interact with

its receptor.[138] Modulation of lipase action in this way may ensure a slower, stepwise and more complete delivery of lipolytic products to the tissues.

Remnant clearance

Chylomicron remnants are removed from the circulation by liver parenchymal cells in a high affinity receptor mediated process. ApoE seems to be the ligand responsible for receptor interaction[139] and apoCIII seems to inhibit uptake.[138]

Although its identity has not been clearly resolved, the chylomicron remnant receptor appears to be distinct from the LDL receptor[59] which recognises both apoB and apoE. Chylomicron clearance occurs normally in homozygous familial hypercholesterolæmic humans and in Watanabe Heritable Hyper-lipidæmic (WHHL) rabbits,[140] both of which have little or no production of LDL receptors. In addition the chylomicron remnant receptor is not affected by factors which regulate LDL receptor activity[141-143] and so may lack the ability to control cholesterol homeostasis.

Beyond a lack of functional identity with the LDL receptor however, the state of knowledge concerning the character of the chylomicron remnant receptor is at present unsettled. The validity of liver membrane binding studies which led to the proposal that a specific apoE receptor was the chylomicron remnant receptor[144] have recently been cast into doubt by the discovery that protein components binding apoE now seem unrelated to lipoprotein transport.[145]

Van lenten *et al* [63] demonstrated that chylomicron remnants can enter human monocyte macrophages by the ß-VLDL receptor pathway. Recent work has shown however that the receptor responsible for ß-VLDL uptake in these cells is in fact the 'classic' LDL receptor,[64] and that chylomicron remnants can be internalised by human monocyte derived macrophages through the LDL receptor. The relevance of ß-VLDL and chylomicron remnant uptake by macrophages in the development of atherosclerosis was discussed earlier.

In the absence of apoE on chylomicrons remnant clearance was reduced to about one third that of native particles.[146] Since $apoB_{48}$ is not thought to play a significant part in receptor binding[147,148] some other mechanism must operate. It is possible that some non specific uptake may occur, in which respect macrophages may again be important. Phenotypic expression of different apoE isomorphs may also affect clearance by remnant receptors.[149,150] The rate of clearance of remnants from the plasma seems to be independent of the rate of triglyceride hydrolysis since the stimulation of lipolysis by heparin injection did not affect remnant decay.[151]

Once within hepatocytes chylomicron remnants are subject to lysosomal degradation which releases their components for degradation, storage or resynthesis, thus completing the corporeal assimilation of dietary materials.

1.4.8 Post-prandial lipæmia and lipoprotein metabolism

Although often discussed as discrete physiological entities it is now apparent that plasma lipoproteins exist throughout their intravascular metabolic transformations in a state of dynamic equilibrium with each other and with tissues.[55] The entry of a large number of chylomicrons into the system introduces a transient dys-equilibrium which has repercussions for lipid transport in all lipoprotein classes. The effects of post-prandial lipæmia on lipoproteins involve both surface and core materials.

Interactions of surface areas

The intestine is a major source of apoproteins AI, AII, and C. During intravascular lipolysis these apoproteins, in association with phospholipid and free cholesterol, are shed from the chylomicron surface as bilammellar leaflets. This material is thought to associate with existing HDL_2 and HDL_3 in the plasma,[152] and by so doing decrease its density and increase the amount of material in the HDL_2 density range.[153] Alternatively, or additionally, these bilammellar leaflets may form vesicles or discoidal structures which are precursors of mature spherical HDL.[154]

Formation of genuine HDL_2 from these discs requires the activity of LCAT which esterifies free cholesterol with fatty acid moieties derived from the sn-2 position of phospholipid. The cholesteryl esters so formed partition into the more hydrophobic regions of these structures, eventually forming an oily core and leaving the surface relatively depleted of cholesterol. A concentration gradient is thus formed down which free cholesterol may travel. HDL_3 are rich in phospholipids and are avid acceptors of free cholesterol from the plasma membrane. The movement of free cholesterol to HDL under the impetus of LCAT activity forms the basis of the so called 'reverse cholesterol transport' pathway.[155]

Movement into HDL may not be the only route by which lipæmia affects cellular cholesterol content. Nascent chylomicrons contain little cholesterol (<1% of their total weight), whereas red blood cells contain a reservoir roughly equivalent to the circulating pool of free cholesterol. Miller[21] has suggested that free cholesterol may enter the chylomicron pool from this source during lipæmia. In this respect it is of interest to note that LCAT may also act on lipoproteins other than HDL,[156] and perhaps may be able to act on chylomicron surface material prior to or during its transformation to HDL precursor.

Interactions of core materials

Incubation of fasting human plasma results in a net transfer of triglyceride from VLDL to LDL and HDL, and a reciprocal transfer of cholesteryl esters from LDL and HDL to VLDL down their concentration gradients.[157] Although these transfers may occur spontaneously they are facilitated *in vivo* by the existence of specific transfer proteins.[158]

A scheme has been described by Patsch[159]whereby postprandial lipæmia, through the combined actions of lipid transfer proteins and hepatic lipase, affects the distribution of HDL subfractions. During lipæmia chylomicron triglyceride enters the core of HDL particles with a concomitant loss of cholesteryl esters from HDL to chylomicrons, both catalysed by the action of lipid transfer proteins. Enrichment of HDL_2 with triglyceride at the expense of cholesterol esters makes it a substrate for hepatic lipase, which hydrolyses the triglyceride, depleting the core, and converting it to HDL_3. He suggests that if lipæmia is regularly elevated this sequence of reactions may lead to lowered levels of HDL_2 in the steady state .

Studies of hypertriglyceridæmic subjects carried out by Eisenberg and colleagues[160] indicate the consequences in LDL and HDL classes of increased circulating triglyceride-rich lipoproteins. The prolonged lifetime of VLDL in the plasma of these subjects led to an excessive modification of all lipoprotein classes due to the activity of lipid transfer proteins. Hypertriglyceridæmic VLDL contained less protein and triglyceride than normal VLDL and was abnormally enriched in free and esterified cholesterol. The converse was true of LDL and HDL. These abnormalities were related to the degree of hyper-triglyceridæmia and reverted to normal when drug therapy alleviated the problem.

It should be noted that these studies refer specifically to hepatic VLDL elevation in which condition the synthesis of LDL may be also be affected,[161] thus com-pounding the influence of transfer activity. No similar studies of post-prandial triglyceridæmia have been documented.

Chapter 2

Materials and Methods

2.1 Outline of the study

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Post prandial lipæmia was examined following the administration of an oral fat tolerance test, conducted under standardised conditions, to several groups of volunteers. The test used was based on that of Patsch,[162] and consisted of a liquid format, cream based meal consumed in the morning following a 12 hour fast.[163]

The absorption and plasma transport of orally ingested vitamin A parallels to some extent that of cholesterol, but differs in that once incorporated into hepatocytes retinol does not re-enter the bloodstream in esterified form. Thus plasma retinyl esters represent newly absorbed material in transit with chylomicrons from the gut to the liver. This feature of vitamin A absorption has been utilized for the *in vivo* marking of chylomicrons and their remnants as they pass through the plasma.[164] Since the differentiation of exogenously (intestinal) and endogenously (hepatic) derived lipoproteins has been a notorious technical problem in this type of research, the fat tolerance test employed in this study was modified to include retinyl esters.

After consumption of the fat load serum lipoproteins were prepared from samples obtained at regular intervals during the post prandial phase. The fluctuations in lipid and apoprotein components were quantified and related to an individuals fasting lipoprotein profile.

The influence of gender, the effects of lipid lowering drugs, and of varying the composition of the fat load, were examined in sub-groups drawn from a pool of healthy volunteers. The relationship of lipæmia to coronary artery disease (CAD) was studied in a group of men whose coronary arteries were assessed angiographically for evidence of atherosclerotic stenoses.

2.2 Materials

§

Aldritch Chemical Company, Gillingham, Dorset, England

CH_3ONa

Amersham International plc, Little Chalfont Buckinghamshire, England
^3H triolein

Baker Instruments Ltd, Windsor, Berkshire, England
Encore centrifugal analyser

BDH (Chemicals) Ltd, Poole, Dorset, England
NH_4SO_4, NaCl, NaBr, NaOH, Na_2EDTA, KBr, K_2HPO_4, K_2CO_3, K_2BO_3, $MnCl_2$ Cocktail T

Beckman Instruments, Spinco Division, Palo Alto CA, USA
L8-80 Ultracentrifuge, centrifuge tubes

Boehringer Corporation Ltd, Lewes, East Sussex, England
Enzymatic kits for cholesterol, free cholesterol, and phospholipid.

Brownlea Laboratories, Santa Clara, CA USA
RP-300 Reverse phase HPLC columns

Cockburn Farm Dairy, Balerno, Midlothian
Unpasteurised milk

Laboratoire Fournier, France
Fenofibrate

FSA Laboratory Supplies, Loughborough, England
Isopropanol, chloroform, ethanol, methanol, glacial acetic acid, formic acid

Gilson Medical Electronics, Middleton WI 53562, USA
Twin pump HPLC system with variable UV monitor

Leo Laboratories Ltd, Aylesbury, England
Sodium heparin for injection

Merck, Darmstatd, West Germany
TLC plates, Enzymatic triglyceride kits

Minitab Inc., PA 16801, USA
Statistical software, release 5

Napp laboratories, Cambridge, England
Nicotinic acid

Orion Diagnostica, Espoo, Finland
Immuno turbidimetric kits for apoAI, and apoB

Phillips Analytical, Cambridge, England
Pye Unicam 204 Gas Chromatograph

Rathburn Chemicals Ltd, Walkerburn, Scotland
Acetonitrile (HPLC grade), diethyl ether, hexane, heptane

Roche Products Ltd, Welwyn Garden City, Herefordshire, England
Ro-A-Vit vitamin A supplement

Shimadzu Corporation Ltd, Tokyo 163, Japan
Integrator (HPLC)

Sigma London Chemical Co, Poole, Dorset, England
Retinyl palmitate, retinyl acetate, triolein, glycerol, cholesterol, heparin agarose gel, gum arabic, trisodium citrate dihydrate, sodium heparin (reagent grade)

SPSS Inc., Chicago, Ill 60611 USA
Statistical software

Supelco Chromatography, Sawbridgeworth, Herefordshire, England
SP2330 liquid phase and Chromasorb WAW gas chromatography column packings

Trivector Systems International, Sandy, Bedfordshire, England
Trilab II integrator (gas chromatograph)

2.3 Fat tolerance tests

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2.3.1 Fat load test

The following protocol applied to all participants. Those who fulfilled the selection criteria, and who agreed to take part were asked to refrain from alcohol for 24 hours prior to the fat tolerance test, and to fast for 16 hours.

Blood sampling: On the morning of the test a forearm intravenous catheter was inserted and a fasting blood sample collected prior to consumption of the fat load. All blood samples were collected into plain tubes. Serum was separated immediately by low speed centrifugation and stored at 4°C until lipoprotein preparations were commenced. Samples (20ml) were withdrawn at two hourly intervals till eight hours in normal subjects, and twelve hours in CAD patients, and then again on the following morning (24 hours).

Fat load: The fat load was a liquid format, cream based meal and was made up in the following manner. 280ml double cream, 19g sucrose, 12.5g dried skimmed milk and 40ml of flavoured syrup were made up to 500ml with water and mixed thoroughly but not blended. The total energy content was 1371 kilocalories, derived from 11.4g protein, 137g fat, and 50.3g carbohydrate, and contained 400mg of cholesterol. 30,000 units (165mg) of retinyl palmitate (Roche Products Ltd) was added as an oil before homogenisation. Subjects drank the mixture within 15 minutes, and timing of blood sampling commenced at the beginning of that period.

A light, low fat meal, free of vitamin A or provitamin A carotenoids, was permitted at 8 hours in normals and 10 hours in CAD subjects, who were then fasted till the next morning. Otherwise only calorie free drinks were permitted, and participants were asked to avoid any unnecessary exertion throughout the study period.

2.3.2 Definitions and Calculations

No consistent definitions or terminology exist to describe quantitatively the events of post prandial lipæmia following an oral fat load. It is appropriate at this stage therefore, to define certain parameters and to make clear some of the terms used in this study.

VLDL: In this study VLDL refers specifically to *fasting* lipoproteins of density less than 1.006 g/ml, whether derived from intestine or liver, and is used to distinguish fasting from post prandial lipoproteins in this density range.

ρ<1.006 lipoproteins: This term embraces all lipoproteins isolated at a density of less than 1.006g/ml. Because of the frequent use made of this term the units 'g/ml' are omitted when used in the sense of a class of lipoproteins rather than as a specific reference to density.

Therefore $\rho<1.006$ lipoproteins will be equivalent to VLDL while fasting (in normolipidæmics), and during lipæmia would include fasting and post prandial lipoproteins (VLDL & chylomicrons).

Lipæmia: This term refers to the period of sampling subsequent to a fat load, and to the condition of the serum during this period.

Lipæmic response: This term refers to the difference from fasting levels of any parameter either in terms of its magnitude at a particular time, or its magnitude sustained over the post prandial sampling period. Lipæmic responses were quantified in two ways. These are described here for $\rho<1.006$ triglyceride, although they could be applied to any variable altered during lipæmia (Figure 2.1).

a). The area under the curve described by $\rho<1.006$ triglyceride was calculated after normalisation to fasting values using the formula:-

$$\text{Area} = [2(n_2 + n_4 ..... + n_{x-1}) + n_x] - (2x-1)n_0$$

where n = the measured value at a particular time in hours, and x = the last time point on the curve. This variable is referred to as the '$\rho<1.006$ triglyceride area'.[162]

b). The difference between fasting value and the amount of $\rho<1.006$ triglyceride at any given time is referred to as 'Δ $\rho<1.006$ triglyceride' at that particular time.

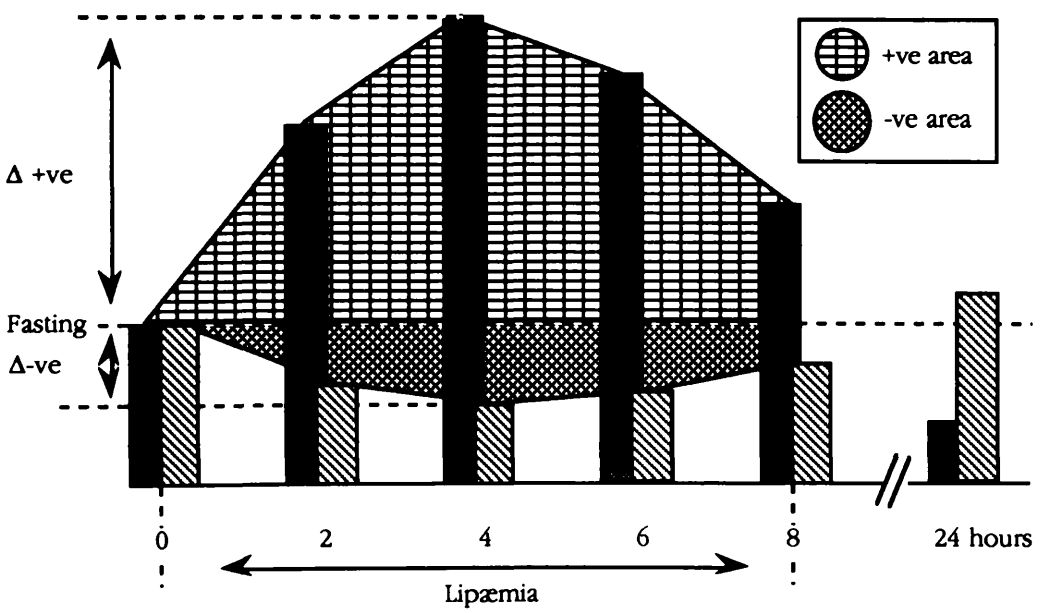

Figure 2.1 The chylomicrograph. Post prandial lipæmia was quantified as a response rather than in absolute terms. Responses were expressed as Δ values representing change from fasting level at a particular time, or as an area giving a cumulative figure for change experienced over the entire period of measurement.

2.4 Biochemical analyses

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2.4.1 Lipoprotein preparations

Lipoprotein classes while fasting and during lipæmia were prepared by ultra-centrifugation followed by analysis of lipid and protein composition. Two different ultracentrifugal techniques were employed at various stages in the study. Solutions of precise density are required for these procedures, and were made up as detailed below. Densities were checked by refractometry prior to use.[165]

Density solutions (g/ml):

ρ 1.006 ..... 11.4 g of NaCl and 0.1 g of EDTA Na_2 were dissolved in 500 ml of distilled water and 1.0 ml of 1.0 M NaOH added. This was made up to 1 litre in a volumetric flask and 13.0 ml of distilled water added.

ρ1.182 ..... 24.98 g of NaBr was added gradually to 100.0 ml of ρ1.006 solution

ρ1.019 ..... 270.0 ml of ρ1.006 and 30.0 ml of ρ1.182 were mixed.

ρ1.063 ..... 75.0 ml of ρ1.006 and 36.9 ml of ρ1.182 were mixed.

ρ1.20 ..... 11.06 g NaCl + 284.6 g NaBr + 100mg Na_2EDTA were made up to 1.00 litre with distilled water.

ρ1.31 ..... 11.82 g NaCl + 473.2 g NaBr + 100mg Na_2EDTA were made up to 1.00 litre with distilled water.

ß quantification: Lipoprotein classes were prepared by a combination of ultracentrifugation and selective precipitation, using standard methodologies.[166] VLDL (and chylomicrons if present) were separated as a floating fraction. The infranatant was then treated with heparin/Mn^{++} (at a final concentration of 1.3mg/ml heparin and 0.092M Mn^{++}) to precipitate LDL and leave HDL in solution.[167] The cholesterol content of whole serum, of the top (VLDL), and bottom (LDL & HDL) fractions, and of the heparin/Mn^{++} supernatant (HDL) were measured as described below.

• *Ultracentrifugation:* 5ml of serum was placed in a Beckman Ultra-clear centrifuge tube (13x64 mm) and overlayered with 2ml of ρ1.006g/ml solution. Tubes were capped and centrifuged overnight at 110,000g[#], 4°C in a Beckman 40.3 rotor, then sliced 20mm from the top and the supernatant collected volumetrically into a 3ml flask. The contents of the bottom fraction were transferred to a 5.0ml volumetric flask, the tube washed with saline, the wash added to the flask, and the volume adjusted to 5.0ml with saline.

[#] g is the standard acceleration of gravity (9807m/s^2)

• *Precipitation of LDL:* 1.0ml of the bottom fraction was placed in a Beckman centrifuge tube and 50µl of precipitating reagent (10.39g $MnCl_2 \cdot 4H_2O$ + 1.05g (approximately $5x10^5$ units) heparin sodium salt (Sigma London Chemical Co.) in 25ml saline were added and mixed. The mixture was kept at 4°C for 30 minutes then centrifuged at 10,000 rpm for 30 minutes, and the supernatant separated immediately for cholesterol analysis.

Sequential flotation: The lipoprotein classes VLDL, IDL, LDL were isolated at limit densities of 1.006, 1.019, and 1.063g/ml respectively by repeated ultracentrifugations after progressively raising the solvent density. HDL were collected from the infranatant after removal of the lighter density classes.[22,168] All centrifugations took place in Beckman Ultraclear tubes (13x64 mm) for 22 hours at 110,000g and 4°C, in a Beckman 40.3 rotor.

• *ρ<1.006 lipoproteins:* 4ml of serum were overlayered with 2 ml of ρ1.006g/ml solution. After centrifugation tubes were uncapped and ρ<1.006 lipoproteins removed volumetrically in 2.0ml by careful aspiration at the surface of the supernatant.

• *IDL:* The density of the infranatant was increased to 1.019g/ml by mixing 0.32ml of ρ1.182g/ml solution with the remaining 4 ml infranatant. This was overlayered with 1.68ml of ρ1.019g/ml solution, the tubes capped and centrifugation recommenced. IDL was carefully aspirated from the surface into a 1.0ml volumetric flask and a further 1.0ml removed and discarded.

• *LDL:* The infranatant was then adjusted to ρ1.063g/ml by the addition of 1.47ml of ρ1.182g/ml solution, and the mixture overlayered with 0.53ml ρ1.063g/ml solution. After further centrifugation LDL were removed volumetrically from the surface in 1.0ml.

• *HDL:* At this stage 1.0ml of the infranatant was discarded and the remainder (4.0ml) used for the analysis of HDL lipids.

2.4.2 Analytical ultracentrifugation

HDL subfraction masses were estimated by analytical ultracentrifugation in a Beckman Model L8 ultracentrifuge equipped with an ultraviolet scanning attachment, (Beckman Instruments) using an AnF rotor with double sector centrepiece cells.[169]

Sample preparation: A total lipoprotein fraction was prepared from 2.0ml of serum after adjusting the density to 1.216g/ml by the addition of 4.0ml of ρ1.31g/ml solution. This was mixed and centrifuged in a Beckman 40.3 rotor for 30 hours at 110,000g and 18°C. After centrifugation the tube was uncapped and the top 1.0ml removed quantitatively by gentle

aspiration from the surface. This preparation was then diluted 1:8 with ρ1.20g/ml solution to adjust its optical density at 280nm to between 0.8 and 0.9, a range found to be optimal for operation of the ultra violet scanner.

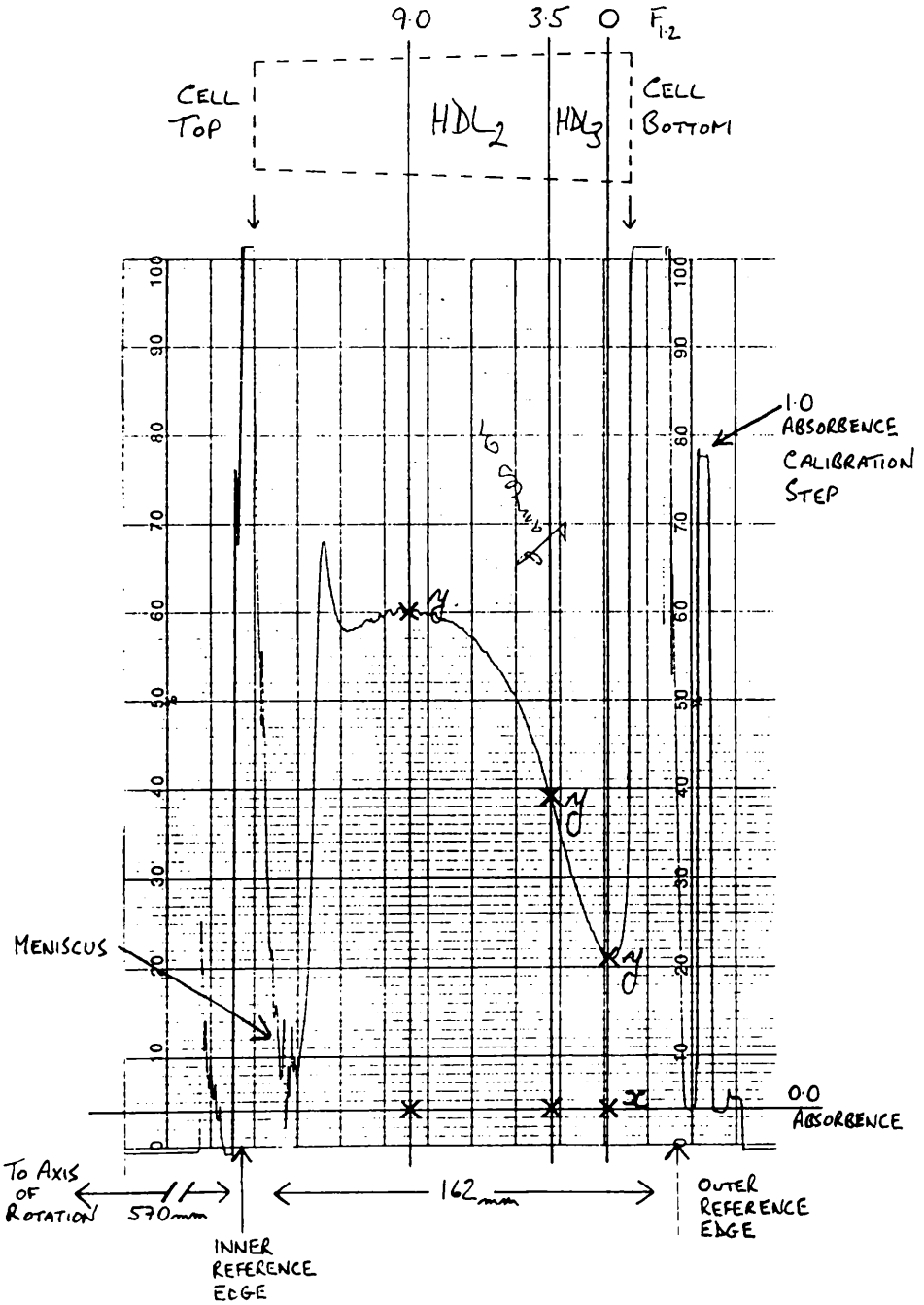

Figure 2.2 HDL subfractions by analytical centrifugation. A typical recorder trace. Knowing the distance between inner and outer reference images and the distance from the inner image to the axis of rotation allows radial distances to be measured on the trace. The distances x,y at the *Sf* boundaries 0, 3.5, and 9.0 are measured in mm and the differences multiplied by a factor (43.74 for HDL_3 and 36.58 for HDL_2), which incorporates specific molar absorptivities and the conversion of millimeters to units of absorbence, giving HDL subfraction concentrations in mg/dl.

Ultracentrifugation: An aliquot of the above material was placed in the sample side of a double sector cell of an AnF rotor, and a similar aliquot of ρ1.20g/ml solution placed in the other side as a blank. Centrifugation was conducted at 42,000rpm at 26°C for 2 hours and 11 minutes. Under these conditions HDL is buoyant and so floats through the cell under the influence of the gravitational field at a rate which is a function of its density. As it does so absorbence at 280nm may be monitored periodically by the UV scanning attachment, revealing a decrease in absorbence at the periphery of the cell.

After a defined ultracentrifugal energy has been applied ($\omega^2 t = 1.52 \times 10^{11}$)* the cell is scanned and the change in absorbence within the flotation ($F_{1.2}$)* intervals 3.5 - 9.0 and 0 - 3.5 are recorded. A typical recorder trace is shown in Figure 2.2. A template, marked with perpendiculars at previously determined boundaries for $F_{1.2}$ of 0, 3.5 and 9.0 is placed over the trace and the height at which they cross the absorption curve measured in millimeters. The differences between 3.5 and 9.0, and 0 and 3.5 are calculated and this data converted, using specific molar absorptivities, to give concentrations of HDL_2 and HDL_3 respectively.

2.4.3 Compositional analyses

Cholesterol, triglyceride, and phospholipid in serum and lipoprotein fractions were assayed by enzymatic procedures on a Centrifichem Encore centrifugal analyzer. (Baker Instruments)

Cholesterol was assayed using Boehringer kit (Nº. 692905, Boehringer Mannheim)
The enzymatic reactions are summarised below:-

$$\text{Cholesterol ester} + H_2O \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \Delta 4 \text{ - cholestenone} + H_2O_2$$

$$2H_2O_2 + \text{4 - aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{4 - (}p\text{ - benzoquinone - monoimino) - phenazone} + 4H_2O$$

The resultant colour is measured at 500nm. The centrifugal analyser was programmed for kinetic (fixed time) measurement.

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* ω is the angular velocity in radians per second (2π rpm/60) and t is the time in seconds.
*$F_{1.2}$ is the negative sedimentation co-efficient (in Svedbergs) of the lipoprotein at solvent density 1.2g/ml and 26°C.

Free (unesterified) cholesterol was estimated with a Boehringer kit. (Nº 310328) in which the cholesterol esterase above is omitted from the enzyme reagents. Esterified cholesterol was calculated from the formula

Esterified cholesterol = (total - free cholesterol)x1.68

where 1.68 is equal to the molecular weight of cholesteryl linoleate divided by the molecular weight of cholesterol, linoleic acid being the most common fatty acid found in the cholesteryl esters of lipoproteins. Because of the small amounts of cholesterol found in VLDL and IDL it was not possible to estimate free and esterified forms separately in these fractions by these methods.

Triglyceride was assayed using Merckotest kit (Nº 1431, E. Merck). By this method triglyceride is hydrolysed enzymatically to glycerol and free fatty acids by a combination of lipases. Glycerol reacts further to give formazone which is monitored at 520nm.

Phospholipid was assayed with Boehringer kit Nº 691844. This method employs an enzymatic, colorometric assay dependent on the liberation of choline by phospholipase D, therefore only phospholipids containing this moiety are measured. Contrary to the manufacturers claims it was found that ethylenediamine tetra acetic acid (EDTA), commonly used as an anti-coagulant in the collection of plasma for lipoprotein analyses, inhibited this reaction, probably by inhibition of phospholipase D.[170] For this reason serum was used for all blood collections.

Total protein in lipoproteins was estimated by the method of Lowry[171] modified to reduce interference from turbidity in $\rho<1.006$ preparations by the addition of sodium dodecyl sulphate (SDS) to the Biuret reagent.[172]

Apoproteins AI and B were measured by immuno turbidimetric methods[173] in the centrifugal analyser using kits Nº D10051 and Nº D10019 (Orion Diagnostica) respectively. Polyclonal antibodies specific to these apoproteins are mixed with sample in the optical cells of the centrifugal analyser. The antibody-antigen complex so formed absorbs light at 340nm. Standard materials were supplied with these kits, and reference material 1883 from the Centre for Disease Control Atlanta, Georgia, USA was used for quality control.

Apoproteins AII, CIII, and E were estimated by enzyme linked immunosorbent assay (ELISA) by Dr Pascal Puchois of the Pasteur Institute in Lille, France. The principles of this technique are as follows.[174]

A polystyrene microtitre plate is coated with a purified polyclonal antiserum raised against the apoprotein of interest. Standards and unknowns are added to the wells in this plate and incubated at room temperature for a number of hours, during which time the antigen becomes bound to the antibody. After washing the plate a second antibody preparation, to which the enzyme alkaline phosphatase has been conjugated, is added to the wells and the plate incubated further. Following another wash a solution of *p*-nitrophenyl phosphate is added to the wells for a short time before stopping enzyme activity and reading the optical density at 410nm with a purpose built plate reader.

2.4.4 Calculation of particle diameters

Using compositional data obtained by the above procedures, and the partial specific volumes listed below, it is possible to estimate the particle diameters of VLDL, IDL, and LDL.[175] To perform this calculation it is assumed that lipoprotein particles are spherical, that the non-polar lipids cholesteryl ester and triglyceride are in the core of the particle, and that the polar lipids free cholesterol & phospholipid, and protein are distributed at the lipoprotein surface. From a knowledge of the total amounts and the space occupied by these materials it is possible to calculate the ratio of available surface to core material, and from that to calculate particle diameter.

	Partial specific volume (ml/g)
Triglyceride	1.093
Cholesteryl ester	1.044
Free cholesterol	0.968
Phospholipid	0.970
Protein (apo B)	0.777

The total volume of lipoprotein present (V_1) is calculated as follows (lipid weights are g/ml):-

$$V_1 = (\text{cholesteryl ester} \times 1.044) + (\text{triglyceride} \times 1.093) + (\text{free cholesterol} \times 0.968) + (\text{phospholipid} \times 0.970) + (\text{protein} \times 0.777)$$

The apolar constituents, triglyceride and cholesteryl ester, lie within the core of these lipoproteins. The volume of the core materials (V_2) was calculated:-

$$V_2 = (\text{cholesteryl ester} \times 1.044) + (\text{triglyceride} \times 1.093)$$

The units of V_1 and V_2 are ml of lipid per ml of solvent.

In the case of $\rho<1.006$ fractions, where free and esterified cholesterol were not separately assessed, an estimate was made based on published values of the percentage of these lipids found in a fasting preparation of human $\rho<1.006$ lipoproteins.[21]
The radius (r_1) of the lipoprotein and the radius of the core lipid/surface boundary (r_2) were then calculated assuming a spherical particle.

If $C = \frac{r_2}{r_1}$ then $V = \frac{4\pi r^3}{3}$

$$r = \left(\frac{3V}{4\pi}\right)^{1/3}$$

$$C = \left(\frac{V_2}{V_1}\right)^{1/3}$$

The more polar components, phospholipid, protein, and free cholesterol, occupy a surface layer whose thickness seems to be fairly constant for all VLDL particles at about 2.15nm [176]__ the approximate length of extended phospholipid fatty acyl side chains. It follows therefore:-

$$r_1 - r_2 = 2.15$$

since $C = \frac{r_2}{r_1}$

$$r_1 - (r_1 \times C) = 2.15$$

$$r_1 = \frac{2.15}{(1 - C)}$$

Calculation of the radius by this method has been found to correlate well with direct measurement in the electron microscope.[175] An estimate of particle numbers can be obtained by dividing the total volume of material present (V_1) by the volume of one particle of radius r.

2.4.5 Retinyl esters

Retinyl esters in the $\rho<1.006$ fraction were estimated by reversed phase High Pressure Liquid Chromatography (HPLC) using retinyl acetate as an internal standard.

Standards: Stock standards of retinyl acetate and retinyl palmitate were made by dissolving commercially available materials (Sigma products R3000 and R3375 respectively), in isopropanol at a concentration of 1 mg/ml. A working standard was prepared as a 1:100 dilution of the stock in isopropanol. Standards contained 0.009% butylated hydroxyanisole,

and 0.009% butylated hydroxytoluene (w/w ester) to retard oxidation. Standards and samples were stored in the dark at 4°C.

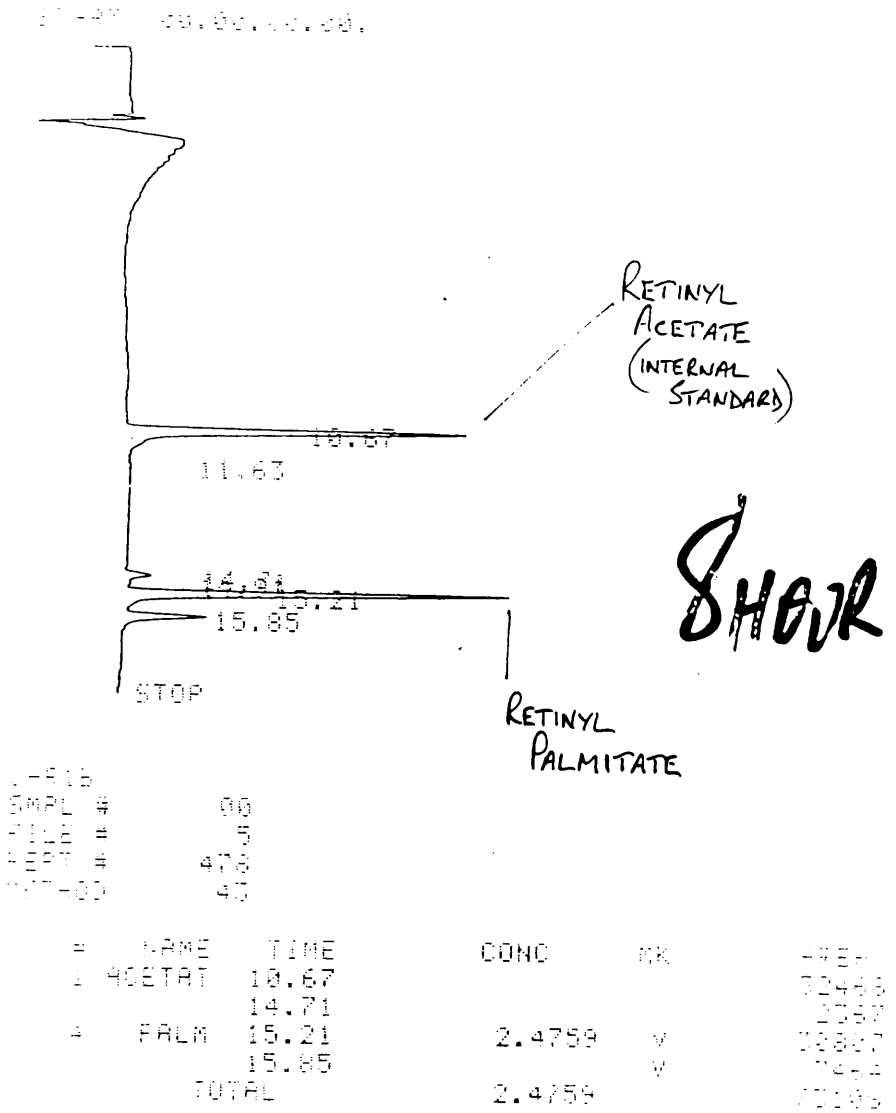

Figure 2.3 Retinyl esters by HPLC. 0.02µg of retinyl acetate injected in 100µl of isopropanol onto a reverse phase HPLC column (250x4.6mm) containing a lipophilic silica gel of 10µm spheres, 300Å pore size. A linear gradient of 50-100% acetonitrile was pumped for 15 minutes at 2.0ml/min. Effluent was monitored by absorption at 326nm.

Extraction: 0.05μg (50μl) of retinyl acetate internal standard, 500μl of saline, and 50μl of 10% acetic acid were added to 500μl of sample (ρ<1.006 lipoproteins). The sample was then extracted 3 times with 1.0ml aliquots of diethyl ether and the combined extracts dried under nitrogen. The residue was re-dissolved in 250μl of isopropanol and injected onto the HPLC column immediately.

Analysis: 100μl of the extract in isopropanol was injected onto a Brownlee Aquapore RP300 reversed phase column (Brownlee Laboratories), mounted on a Gilson HPLC system (Gilson Medical Electronics) fitted with dual pumps.

Quantitation: Retention times and response factors for retinyl acetate and retinyl palmitate were determined after multiple injections of known amounts of standard materials, and integration of the absorption peaks on a Shimadzu Chromatopac C-R1B (Shimadzu Corporation). Subsequent injections of unknowns provided absolute concentrations of retinyl palmitate in samples. A typical trace is shown in Figure 2.3.

Performance: Linearity of extraction and analytical procedures was checked by analysing serial dilutions of a particularly lipæmic sample from a patient who had consumed retinyl palmitate along with a fat load. The assay was linear up to 5.00μmol/l (Figure 2.4).

Coefficients of variation were measured by repeated assay of stored (-50°C) aliquots of lipæmic ρ<1.006 preparations. Within batch variation for this material was 10.1% (n=6, mean = 0.88±0.01μmol/l), and between batch was 8.6% (n=15, mean = 0.93±0.01μmol/l).

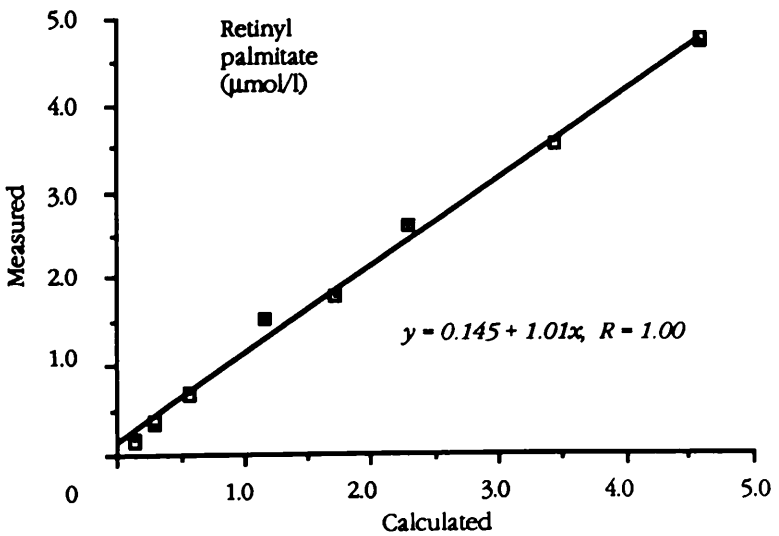

Figure 2.4 Linearity of the retinyl palmitate assay.

2.4.6 Fatty acid compositions

Total lipids in fat loads and in lipoprotein fractions were extracted into organic solvent and separated by thin layer chromatography (TLC) into the different lipid classes. The fatty acid components of these lipids were converted to their methyl esters and analysed by gas liquid chromatography (GLC).

Extractions: [177] 1.0ml of sample was shaken for 5 minutes with 3.0ml of methanol in a 15ml glass tube with a ground glass top. Two consecutive aliquots of chloroform (3.0ml) were then added, each time shaking for 5 minutes. 2.5ml of aqueous KCl (0.88% w/v) is then added and the mixture shaken for another 5 minutes.

Tubes were allowed to stand at room temperature for two hours, then spun at 750g, 23°C for 5minutes. The upper aqueous phase was removed and discarded, and the remaining organic phase evaporated to dryness under vacuum on a rotary evaporator and the residue re-dissolved in 100µl of chloroform.

Thin Layer Chromatography: The total lipid extract was spotted as a band 2cm from the bottom of a 0.25mm thick silica gel TLC plate (Merck plate 5721), and the plate placed in a covered glass tank containing 100ml of hexane: diethyl ether: formic acid, 80:20:2 (v:v:v). The solvent was allowed to rise across the plate for 60 minutes, during which time the lipid classes move at a rate determined by their differing solubilities in the tank solvent. The plate was dryed and sprayed with POPOP/PPO and the lipid bands identified under ultra violet light. These were then scraped with the silica gel into separate glass tubes.

Transmethylation: The fatty acid moieties of these lipids were prepared for gas chromatography by base catalysed esterification with methyl groups.[178] 1.0ml of toluene and 2.0ml of sodium methoxide (0.5M in dry methanol, Aldrich Chemical Co) were added to each tube and heated to 50°C for ten minutes. To extract the methyl esters 100µl of glacial acetic acid, 5.0ml of distilled water, and 5.0ml of hexane were added to the tubes which were then shaken for 5 minutes and centrifuged at 750g, 23°C, for a further 5 minutes

5.0ml of the top layer was removed and evaporated to dryness in a separate tube and the extraction repeated with a second 5.0ml of hexane. The combined residues were re-dissolved in 25µl of chloroform and applied to the gas chromatograph.

Gas Liquid Chromatography: 5µl of the above sample was injected into a glass column (4mm x 1.5m) packed with Chromasorb WAW (Supelco) coated with a 10% SP2330 liquid phase (Supelco). This was mounted in a Pye Unicam 204 gas chromatograph (Phillips Analytical). Carrier gas was nitrogen (50ml/min). Injection temperature was 220°C, and the temperature of the analytical oven was programmed to 180°C for 3minutes, rising at 3°/min to

a top temperature of 250°C, at which it remained for 5minutes. The flame ionisation detector was set at 300°C, and peaks were integrated on a Trilab II (Trivector Systems International). Data were presented as percentages of total peak areas.

2.4.7 Lipase activity

Lipoprotein and hepatic lipase activities were estimated in fasting and post heparin plasma after selective inhibition of each by specific antibodies.[179] Samples were collected in Glasgow as described below, and activities were assayed by Dr Thomas Olivecrona of the University of Umeå, Sweden.

Sample preparation: After withdrawal of a 10ml fasting blood sample sodium heparin, 100units/kg body weight, (Leo Laboratories Ltd) was administered intravenously. A further 10ml sample was collected 15minutes after injection. Blood was collected in EDTA K_2 and plasma separated immediately and placed in a -50°C freezer, at which temperature it was stored until transported to Sweden in dry ice.

Assay: The principles of the method are briefly as follows. A synthetic emulsion of triolein (containing trace amounts of ^{14}C in the fatty acid moiety) stabilised by gum arabic is prepared by sonication of an oil/aqueous buffer mixture. An aliquot of post heparin plasma is added to this mixture and incubated at 25°C for a short time. Lipolytic activity of plasma is proportional to the amount of radio-labelled free fatty acid liberated. This is obtained by extraction of the free fatty acid into an organic solvent followed by liquid scintillation counting.

2.5 *In vitro* lipolysis

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ρ<1.006 lipoproteins were isolated 5 hours after consumption of an oral fat load. Radioactively labelled triglyceride was incorporated into these lipoproteins, and after standardising their triglyceride content they were subjected to lipolysis *in vitro* by a purified bovine milk lipoprotein lipase. The extent of lipolysis was estimated as the amount of radioactive free fatty acid liberated during the incubation, and this was compared with the fatty acid compositions of fatty meals and lipid substrates.

2.5.1 Preparation of substrate

5 hours after an oral fat tolerance test 100ml of blood was collected and ρ<1.006 lipoproteins isolated by ultracentrifugation. 20ml of plasma was placed in Beckman tubes (25 x 89mm), and overlayered with 6.0ml of ρ1.006g/ml solution. Tubes were capped and placed in a Beckman Ti50.2 rotor and centrifugation conducted at 140,000g, 4°C for 18 hours in a

Beckman L8-80 ultracentrifuge. The supernatant was aspirated gently from the surface of each tube with a fine tipped glass Pasteur pipette, and collected into 3ml volumetric flasks. Individual preparations were then dialysed against a Tris buffer (0.01M Tris HCl, 0.15M NaCl, 0.001M EDTA, pH 7.4). The concentration of triglyceride in the dialysate was measured by enzymatic procedures and adjusted with Tris buffer to 30mmol/l.

2.5.2 Preparation of total lipoprotein and ρ<1.006 lipoprotein deficient plasmas

Plasma deficient in ρ<1.006 lipoproteins was required for incubations of substrate with lipoprotein lipase. A proportion of the infranatant from individual preparations described above was pooled and dialysed against Tris buffer for future use in this respect.

Infranatants from the above ρ<1.006 lipoprotein preparation were used as a source of lipoprotein deficient plasma which was required for the labelling procedure. The density of the infranatant was adjusted to 1.225g/ml by the addition of KBr (0.3517g/ml). 15.0ml of this solution was placed in Beckman tubes (25 x 89mm) and overlayered with 11.0ml of 1.225g/ml solution prior to 22 hours centrifugation at 140,000g, and 4°C in a Ti50.2 rotor. 11.0ml of the supernatant was carefully removed and discarded and the infranatants pooled and dialysed against an excess of Tris buffer.

2.5.3 Radiolabelling of ρ<1.006 lipoproteins

Triolein labelled with ^{3}H in the fatty acid moiety (Amersham) was incorporated into ρ<1.006 lipoproteins. Sufficient ^{3}H triolein for the entire experiment was washed to remove any contaminating free fatty acids. ^{3}H triolein, supplied in toluene, was placed in a glass tube and dried under nitrogen, then re-dissolved in 3ml of heptane. 7ml of 0.05M NaOH in ethanol: water (1:1, v:v) were added, the tube capped and shaken for 3 minutes, and the mixture allowed to separate. The heptane phase was removed and placed in aliquots of 135μCi in glass tubes and dried under nitrogen.

A 1.0ml portion of the isolated ρ<1.006 lipoprotein fractions (containing 30mmol/l triglyceride), and 0.5ml of the lipoprotein deficient plasma were added to these tubes, which were then incubated at room temperature for 4 hours, mixing occasionally. This mixture was then placed in ultracentrifuge tubes (Ultraclear 13x64mm) and overlayered with ρ<1.006g/ml solution, followed by ultracentrifugation at 110,000g, and 4°C overnight in a 40.3 rotor. Radiolabelled lipoproteins were collected from the surface of this preparation into a 1.0ml volumetric flask. Incorporation of label by this method is approximately 50%.[180]

2.5.4 Reconstitution of plasma with ρ<1.006 lipoproteins

In vitro lipolysis was conducted in an environment in which physiological cofactors and other materials normally present during lipolysis were provided in a controlled fashion. Radiolabelled lipoproteins were re-suspended in a pooled plasma fraction of ρ>1.006g/ml,

providing both lipoprotein and albumin to act as acceptor for liberated free fatty acid, and other lipoproteins to participate in the lipid and apoprotein exchanges which normally accompany lipolysis The pooled material had an LDL cholesterol of 3.4mmol/l, an HDL cholesterol of 1.15mmol/l, and a triglyceride concentration of 0.30mmol/l.

The triglyceride content and radioactivity of the labelled $\rho<1.006$ lipoproteins were measured and the specific activities calculated. A fixed activity of 500,000 disintegrations per minute (dpm) were added to a vial containing unlabelled $\rho<1.006$ lipoproteins derived from the same preparation as the labelled material, to give a final concentration of 1.0mmol/l $\rho<1.006$ triglyceride in 2.0ml of reconstituted plasma. Volumes were adjusted to 2.0ml with Tris buffer.

2.5.5 Preparation of lipoprotein lipase.

This enzyme was prepared by affinity chromatography from bovine milk. Enzyme from this source shows much similarity to that obtained from human post heparin plasma, and has been widely used as a model for the human enzyme. The procedure adopted was based on that of Iverius.[179] Purification of the enzyme was not the objective of this preparation however, and the procedure was only pursued as far as optimum recovery of lipase activity would allow.

Unpasteurised bovine milk was collected directly from the milking machine (Cockburn Farm Dairy) into prepared one litre bottles containing 300ml of glycerol in a phosphate buffer, to give a final concentration of 30% glycerol (v:v), 0.01M phosphate, pH7.4. The milk was kept cool and transported immediately to the laboratory where cream was separated by low speed centrifugation.

The infranatant was adjusted to 0.1M with solid trisodium citrate dihydrate and 500ml of this preparation stirred gently with 50ml of a heparin agarose gel (Sigma product H 5380) for 30 minutes at 4°C. The gel (to which LpL was adsorbed) is then separated on a sintered glass funnel and washed with 3x200ml volumes of ice cold 0.5M NaCl, 30% (v:v) glycerol, 0.01M phosphate(pH 7.4). It was then re-suspended in 100ml of 1.5M NaCl in 30% glycerol, phosphate buffer and stirred gently for 15 minutes, releasing the enzyme into solution.

This solution was collected after separation on a sintered glass funnel, and the gel washed with a further 100ml of 1.5M NaCl, 30%glycerol, phosphate buffer, and the wash added to the first collection. The pooled enzyme preparation was dialysed against 3.6M ammonium sulphate in 0.01M phosphate buffer and the enzyme precipitated by centrifugation at 1000g, 4°C, for 15 minutes. The supernatant was discarded and the precipitate dissolved in 1.0ml of 30% glycerol, 0.01M phosphate buffer, pH 7.4.

2.5.6 *In vitro* assay of lipolysis

Aliquots of radiolabelled substrate were incubated with purified lipase and enzyme activity assayed as ^3H free fatty acid liberated per minute per ml of enzyme.

Incubations: 1.0ml of the reconstituted plasmas containing $^3H\rho<1.006$ lipoproteins were placed in glass tubes and 0.15ml of purified milk LpL added. Blank tubes were set up containing 0.15ml of Tris buffer in place of enzyme. Total ^3H fatty acid activities were obtained by a direct count of the incubation mix prior to extraction, but in the presence of extraction solvents. Tubes were incubated in a shaking water bath at 37°C and 80 cycles per minute, and aliquots withdrawn at intervals of 5, 15, 30, 60, and 90 minutes for determination of ^3H free fatty acids.

Fatty acid extractions: [181] A 0.05ml aliquot was withdrawn from the incubation mixture and lipolysis terminated by addition to 3.25ml of methanol: chloroform: heptane (1.41: 1.25: 1, v:v:v). 1.05ml of 0.1M potassium carbonate-borate buffer, pH10.5, was added to these tubes and shaken vigorously for 5 minutes and then centrifuged (1000g) at room temperature for 15 minutes. A 1.0ml aliquot of the methanol: water upper phase was then added to 10ml of scintillation fluid (Cocktail T, BDH) and activity counted in an LKB Rackbeta.

2.5.7 Computations

After correction for blank values and dilution factors the activity of the enzyme was expressed as a percentage of total counts extracted with free fatty acids at each time point.

2.6 Data reduction and statistical analyses

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The full data set was mounted on a National Advanced Systems VL60, which forms part of Edinburgh University's Multi Access System (EMAS). Statistical analyses were performed by the Statistical Package for the Social Sciences (release 2.2 SPSS Inc.) except for multiple regression analyses for which the procedures of Minitab release 5 were used (Minitab Inc.).

Several of the parameters were positively skewed ($\rho<1.006$ components, HDL_2, retinyl esters, lipases) and so, unless otherwise indicated, non parametric procedures which make minimal assumptions about underlying distributions, were used for comparisons and correlations. Tabular data are presented as means ± standard deviations.

2.6.1 Statistical tests

For comparisons of paired data the Wilcoxon matched pairs signed rank test was employed. This test computes the differences between paired observations, ranks them, and sums the

positive and negative ranks, taking into account the magnitude of the differences. A test statistic Z is then assigned and a two-tailed P value calculated.

Unpaired data was compared by the Mann-Whitney U test, which tests whether two groups of unpaired data are drawn from the same population. It ranks all data in order of increasing size and computes a test statistic U, the number of times a score from group one precedes a score from group two. A two tailed P value is then calculated.

Spearman rank correlation (one tailed) was used to test for relationships between variables. This test ranks both sets of paired data and assigns a value to each rank. It then calculates the Spearman rank correlation co-efficient r, and calculates a one tailed P.

P values of more than 0.1 are not quoted in results, but are marked Not Significant (NS). The decision to adopt the 10% rather than the arbitrary, but more conventional 5% level for significance of P values was taken since several important aspects of the results would otherwise have been dismissed as insignificant.

Chapter 3

Alimentary lipæmia in normal subjects

3.1 Introduction and methods

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To assess quantitatively the effects of exogenous fat on lipoprotein metabolism *in vivo* the fat must be introduced into the bloodstream in some standardised fashion. Fat tolerance tests presently take several forms in terms of composition, quantity, and mode of application, and as yet their is no consensus regarding their design or implementation for purposes of quantitative comparison. For this reason investigators generally have to establish the limits of a particular fat tolerance test under their own standardised conditions, and within their chosen population.

The objectives of this part of the study were to characterise lipæmia in terms of alterations in the $\rho<1.006$ lipoprotein fraction and any co-ordinate changes in other lipoprotein classes. It was hoped to quantify these changes and to relate them to fasting lipoprotein profiles, thus providing a comprehensive description of lipæmia as it relates to lipoprotein metabolism in general.

Alimentary lipæmia was examined in a group of twenty-eight healthy volunteers (20 males and 8 females), recruited from the staff of Glasgow Royal Infirmary. None were prescribed any medication known to affect lipoprotein metabolism. None of the females used oral contraception. Two of the males and two of the females smoked approximately twenty cigarettes per day, and one of the males smoked a pipe (1/2 oz per week). The protocol described in Chapter 2 was followed throughout, with blood samples taken two hourly till eight hours, and again at twenty four hours. Samples for lipase estimations were obtained on a separate occasion. Lipoproteins were prepared by sequential flotation (section 2.4.1).

3.2 Results

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3.2.1 Fasting lipoproteins

Details of age, body mass index, and the fasting lipids & apoproteins in the serum of male and female groups are presented in Table 3.1. Males were on average 6 years older than females, and had lower levels of apoAI. Otherwise the differences were not significant.

Table 3.1 *Fasting serum lipids and apoproteins in normal subjects*

	Age	Quetelet Index	Cholesterol Free	Cholesterol Ester.	Trig.	Phos.	Apoproteins AI	AII	B	CIII	E
	(years)	(kg/m^2)	(mmol/l)				(mg/dl)				
Male (n=20)	31.9 ±6.9	24.3 ±2.8	1.76 ±0.56	3.01 ±0.54	1.28 ±0.60	2.68 ±0.43	144.1 ±29.2	36.7 ±10.4	94.6 ±24.9	5.0 ±2.1	7.8 ±4.1
Female (n=8)	26.0* ±5.7	24.2 ±2.1	1.63 ±0.37	2.95 ±0.57	0.91 ±0.21	2.81 ±0.30	168.5* ±15.2	35.6 ±9.7	85.0 ±24.0	4.9 ±1.4	7.3 ±4.2

Difference between males and females *P≤0.05

When fasting lipoprotein profiles were compared (Table 3.2) males were found to have more VLDL and less HDL_2 than females, but had similar amounts of IDL, LDL and HDL_3. The data in Table 3.2 regarding total lipoprotein mass was also representative of their individual lipid and protein constituents.

Lipoprotein and hepatic lipase activities in plasma were measured before and after the intra-venous administration of sodium heparin. This procedure is known to release these enzymes from their binding sites on the vascular wall into the bloodstream, thus increasing their activity and allowing direct assay in a blood sample. HL activities so obtained were greater in males, but LpL did not differ significantly (Table 3.3). *Pre*-heparin activities, although much lower than post-heparin, provided the same information in these comparisons. Pre and post heparin HL activities however, were not correlated, and the correlation between pre and post heparin LpL was only 0.54 (P≤0.01).

Table 3.2 *Fasting lipoproteins in normal subjects*

	VLDL	IDL	LDL	HDL_2	HDL_3
Males (n=20)	90.1 ±55.8	15.4 ±10.0	233.0 ±68.0	57.9 ±26.7	221.1 ±56.9
Females (n=8)	45.7* ±23.0	9.9 ±3.8	207.9 ±52.8	108.6** ±38.3	220.1 ±35.8

Total lipoprotein masses were calculated from the sum of their lipid and protein components expressed as mg/dl. HDL subfractions were derived from the analytical ultracentrifuge. Differences between males and females. *P≤0.05, **P≤0.01

Table 3.3 *Lipase activities in normal subjects*

	Lipoprotein lipase		Hepatic lipase	
	Pre-heparin	Post-heparin	Pre-heparin	Post-heparin
Males (n = 20)	0.52 ±0.24	196.7 ±55.7	0.20 ±0.15	171.6 ±61.6
Females (n = 8)	0.45 ±0.10	168.4 ±61.2	0.06* ±0.07	74.9** ±51.5

Units for lipase activity are mU/ml where 1mU = 1nmol fatty acid released per minute at 25°C.
Difference between males and females *P≤0.005, **P≤0.001

An examination of the inter-relationships between lipoprotein classes (Table 3.4), revealed a positive association between VLDL, IDL, and LDL levels, and a weak inverse relationship between VLDL and HDL_2. Quetelet index, a measure of obesity, was positively related to VLDL and negatively to HDL_2.

Total circulating VLDL mass (Table 3.2) was calculated by summation of the weights of its lipid and protein constituents as assayed in the ultracentrifugally isolated $\rho<1.006$ fraction. It should be remembered however that VLDL are heterogeneous in terms of size, and that differences in total mass may reflect variation in either the number of VLDL particles, the size of individual particles, or a combination of both. The more detailed specification of the VLDL fraction in Table 3.5 shows the similar (but not identical) particle compositions and average diameters in males and females, and indicates that the greater amount of VLDL in males was due primarily to a greater number of particles in the circulation. Pre heparin LpL activity was negatively related to fasting VLDL size ($r=-0.40$, $P\leq0.05$), but not to number.

3.2.2 Post prandial lipids and lipoproteins

Serum: Subsequent to consuming the fat load all subjects exhibited a rise in serum triglyceride of variable magnitude and duration. The other lipid constituents of the serum did not respond in such a predictable fashion, mean values tending to obscure an inter-individual variability which included cases of increase, decrease and stasis during lipæmia.

Table 3.4 *Correlations between fasting lipoproteins*

	Age	Quetelet	VLDL	IDL	LDL	HDL_2	HDL_3
Age	•						
Quetelet	0.30	•					
VLDL	0.08	0.52**	•				
IDL	-0.02	0.24	0.53**	•			
LDL	0.37*	0.45**	0.44**	0.50**	•		
HDL_2	-0.11	-0.45**	-0.35*	-0.05	-0.24	•	
HDL_3	-0.01	0.16	0.12	-0.14	-0.22	0.21	•

Correlations are between total lipoprotein masses in male subjects (n=20), *P ≤ 0.10, **P ≤ 0.05

Table 3.5 *Composition of fasting VLDL in normal subjects*

	Percentage composition				Particle	
	Chol.	Trig.	Phos.	Protein	Diameter Å	Number $x10^{13}$
Males (n=20)	13.4 ±2.8	58.7 ±7.5	20.5 ±4.2	7.4 ±3.1	39.9 ±8.2	2.83 ±1.29
Females (n=8)	14.3 ±2.9	54.1 ±9.0	21.0 ±2.2	10.6 ±6.4	35.4 ±7.7	2.02* ±0.70

Average particle diameters in individual preparations were estimated from a knowledge of their chemical composition as described in section 2.4.4. Figures quoted are the mean of these average diameters for male and female groups. Particle number was estimated as the total volume of lipoprotein present divided by the volume of a sphere of the calculated diameter. Differences between males and females, *P≤0.1.

Alterations in the lipids and apoproteins of un-fractionated serum are given in Table 3.6, and are further illustrated in Figures 3.1 & 3.2. The rise in serum triglyceride was less in females than in males, but showed the same single peak at 4 hours. The form of the phospholipid profile differed from that of triglyceride in that it persisted considerably longer. Phospholipid flux tended to be *greater* in females.

The overall trend was towards a rise in serum total cholesterol. In males this rise was positively correlated with the esterified cholesterol response at all stages of lipæmia (eg. areas r=0.74, P≤0.001). Free cholesterol response (Δ) was only correlated with total cholesterol flux at 6, 8, and 24 hours (r=0.51, 0.41, and 0.55 respectively, P≤0.05). Therefore total serum cholesterol in male subjects seemed to be a function of cholesteryl ester flux during the early stages of lipæmia, and a combination of free and esterified at later times.

Table 3.6 *Mean post prandial responses in serum*

	Hours post prandial	% change from fasting Males (n=20)	Females (n=8)
Total cholesterol	2-8	↑5*	-
Free cholesterol	2-8	-	↑8*
Esterified cholesterol	2-8	↑8*	-
Triglyceride	2-8	↑193**	↑140**
Phospholipid	2-8	↑11	↑12
Apo AI	2-8	-	-
Apo AII	2-8	↑8	-
Apo B	2-8	↑12*	↑6*
ApoCIII	2-4	↑16	-
	6	-	-
	8	↓16	↓21
Apo E	4-6	↑28	-
	8	-	↓16

The percentage figures given refer to the maximum mean deviation from fasting levels during the times indicated. ApoCIII and E showed a biphasic response to the fat load. Significance of difference from fasting levels, *P ≤ 0.05, **P ≤ 0.01.

In female subjects the rise in serum cholesterol was significantly less than in males, and could be attributed more to an increase in free rather than esterified cholesterol, the latter tending in fact to decline during lipæmia. Post prandial cholesterolæmia in these females differed therefore not only in magnitude from that of male subjects, but also in its molecular nature.

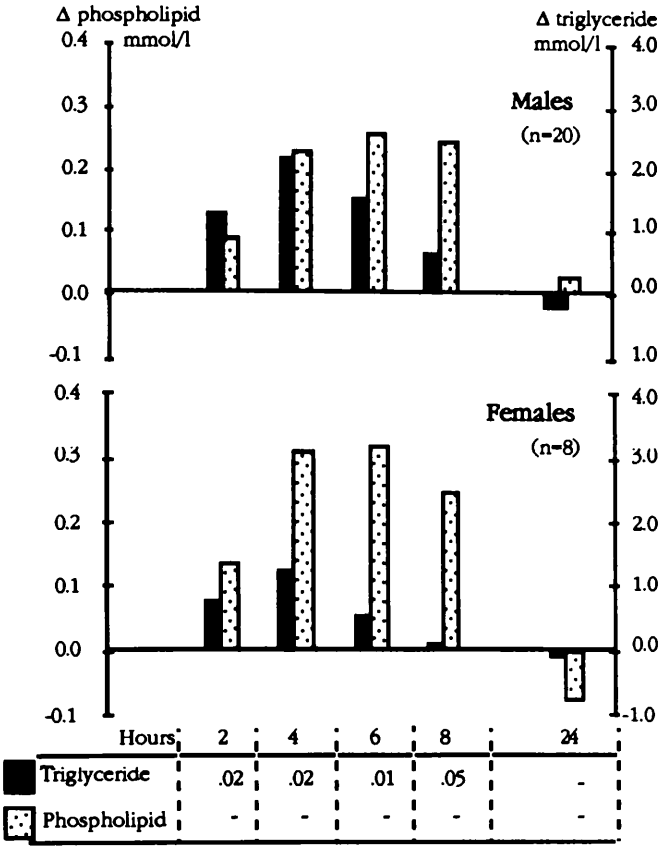


Figure 3.1 Serum tri-glyceride and phospholipid during lipæmia. P values refer to the significance of differences between male and female responses. See Table 3.6 for significance of differences from fasting levels.

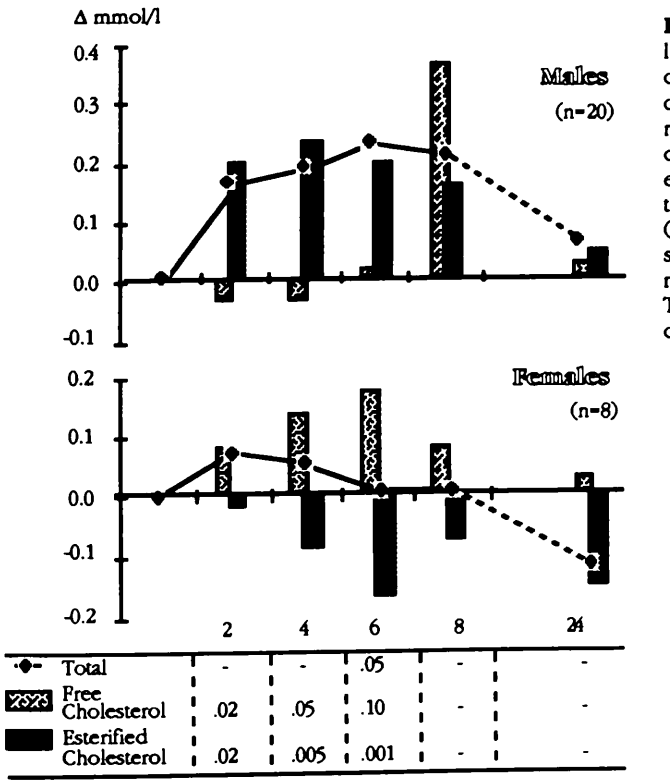


Figure 3.2 Serum cholesterol during lipæmia. Total cholesterol is depicted here to facilitate comparison with other studies, and represents the total amount of cholesterol present in both free and esterified form, not corrected for the fatty acid content of the ester (section 2.4.3).P values refer to the significance of differences between male and female responses. See Table 3.6 for significance of differences from fasting levels.

Lipoproteins of ρ<1.006g/ml: The influx of chylomicrons into this density range was the main feature of the changing lipoprotein profile during lipæmia, and is represented diagrammatically in Figure 3.3. All constituents of this lipoprotein fraction were significantly increased between 2 and 8 hours in both male and female subjects. The following description refers specifically to the males.

The increase in ρ<1.006 triglyceride was disproportionately greater (between 3 and 4 fold) than that of other components, and was reflected in an increased particle diameter. Thus in contrast to the variations found in fasting VLDL, post prandial triglyceridæmia was due mainly to fluctuation in particle size rather than in number.

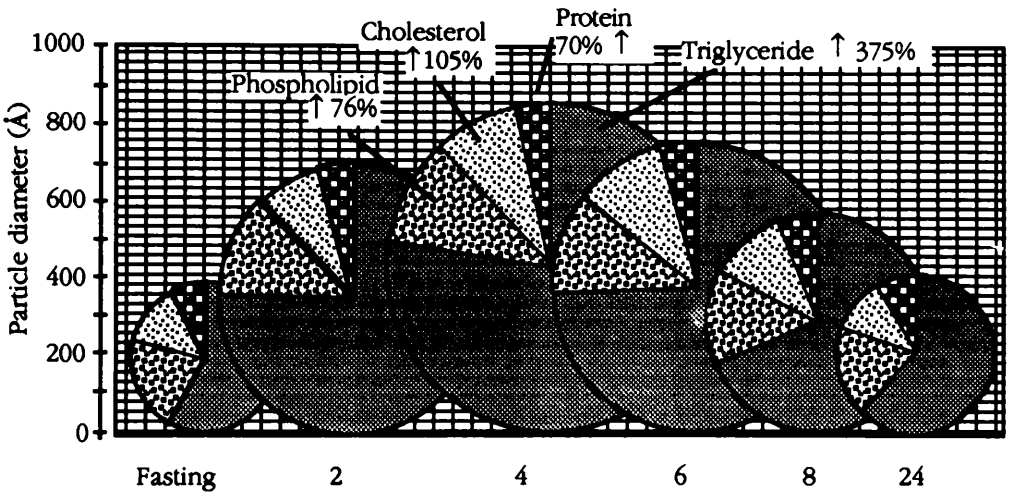


Figure 3.3 All components of the ρ<1.006 class of lipoproteins were raised during lipæmia. The percentage increases at the peak (4 hours), are shown above. The disproportionately greater increase in triglyceride led to an increase in particle diameter. Results are the average for twenty males.

Post prandial fluctuations were quantified as described earlier (Figure 2.1). Both types of measurement (area and Δ) are normalised to fasting levels, and are therefore designed to gauge *response* rather than to provide an absolute measure of lipæmia. The inter-relationships of these two measures (Table 3.7b), shows that ρ<1.006 triglyceride area was correlated with all Δ triglyceride between 2 and 8 hours. The decline in the strength of the association at 8 hours suggests that the major influence on the overall magnitude of triglyceridæmia was due to circumstances during early and peak periods. The Δ24 hour value appeared unrelated to the magnitude of lipæmia and will be discussed more fully later.

Individual responses to the fat load were highly variable, with ρ<1.006 triglyceride areas ranging from 1.50 to 38.4 mmol/l•8 hours in the male group. Reproducibility of response was examined in a group of 9 males who consumed the test meal twice (Figure 3.4). Although mean values for this group were remarkably similar on both occasions, there were three subjects (DB, DW and JMcK) in whom ρ<1.006 triglyceridæmic response was not so precisely reproduced. These deviations could not be ascribed to any specific factors although it was noted that they occurred among those with a greater response.

Table 3.7 *Correlation between measures of response in male subjects*

a). ρ<1.006	Δ 2	4	6	8	24	Areas
Retinyl Palmitate and Triglyceride	0.73***	0.77***	0.20	0.23	0.21	0.60**
b).	Triglyceride					
Triglyceride Area	0.79***	0.98***	0.78***	0.41*	-0.04	•
c).	Retinyl Palmitate					
Retinyl Palmitate Area	0.82***	0.88***	0.97***	0.73***	0.61**	•

Significance of correlations between the various measures of response, *P≤0.05, **P≤0.005, ***P≤0.001, n=20males.

Cholesterol and triglyceride responses in ρ<1.006 lipoproteins were highly correlated (eg. areas r=0.93, P≤0.001). The rise in ρ<1.006 cholesterol was also positively related to serum total and esterified cholesterol response Δ2-Δ8 (eg areas ρ<1.006 total cholesterol with serum total cholesterol r=0.52, P≤0.01, and with serum esterified cholesterol r=0.47, P≤0.02). Cholesterol flux in ρ<1.006 lipoproteins was unrelated to free cholesterol flux in whole serum. In terms of magnitude the rise in cholesterol in ρ<1.006 lipoproteins during the early stages of lipæmia could account for much of the rise in serum cholesteryl esters.

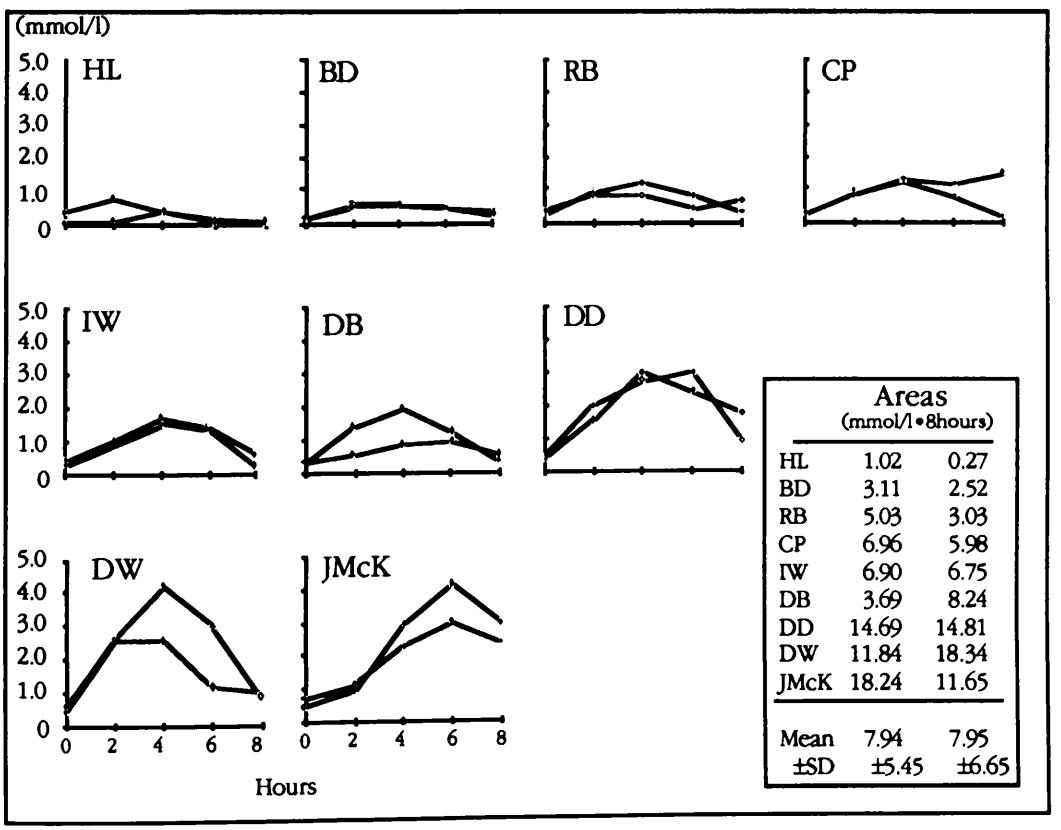
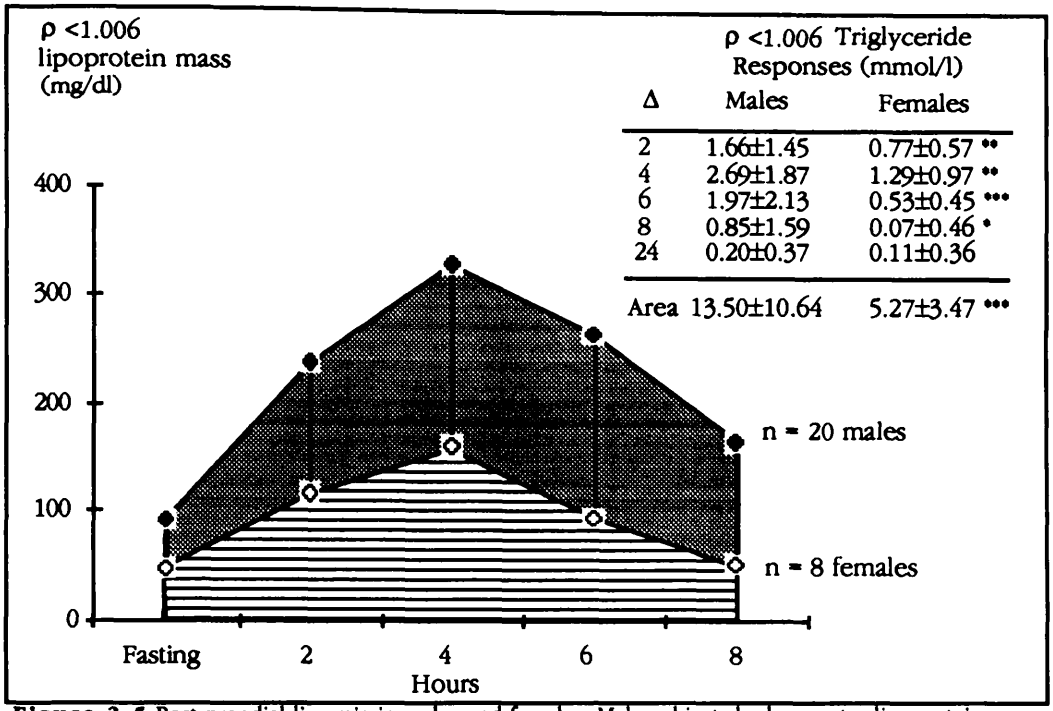


Figure 3.4 Reproducibility of the fat tolerance test. Results are ρ<1.006 triglyceride.



Δ	ρ <1.006 Triglyceride Responses (mmol/l) Males	Females
2	1.66±1.45	0.77±0.57 **
4	2.69±1.87	1.29±0.97 **
6	1.97±2.13	0.53±0.45 ***
8	0.85±1.59	0.07±0.46 *
24	0.20±0.37	0.11±0.36
Area	13.50±10.64	5.27±3.47 ***

Figure 3.5 Post prandial lipæmia in males and females. Male subjects had a greater lipoprotein mass in the ρ<1.006 fraction initially, and showed a greater increment (Δ) in response to the fat load. Significance of differences between male and female responses *P≤0.10, **P≤0.05, ***P≤0.01.

The differing profiles of lipæmia in male and female subjects are shown in Figure 3.5. Area and Δ values reveal that the greater lipæmia in males was due not only to higher initial VLDL mass, but also to an increased response to the fat load evident throughout all stages of lipæmia.

Retinyl esters in ρ<1.006 lipoproteins: Triglyceride and RP flux is illustrated in Figure 3.6, and responses in male subjects compared in Table 3.7a. RP rose more slowly than triglyceride and continued to rise after triglyceride had begun to decline. RP and triglyceride responses showed a degree of independence, particularly as lipæmia progressed. Although areas were correlated the relationship appeared to derive from Δ2-Δ4 rather than the entire range of Δ values.

Plasma RP concentrations were significantly less in females than in males at all time points, with the difference becoming more marked latterly (Table 3.8). The ratio of RP to

Table 3.8 *Retinyl palmitate flux in males and females*

	Fasting	2	4	6	8	24	Area
	(μmol/l)						(·8hour)
Males (n=20)	0.02 ±0.03	0.48 ±0.40	0.85 ±0.68	1.44 ±1.26	1.87 ±1.50	0.29 ±0.35	7.24 ±5.47
Females (n=8)	0.01 ±0.02	0.24* ±0.19	0.45* ±0.25	0.53** ±0.50	0.47** ±0.61	0.07** ±0.06	2.88*** ±2.08

Significance of difference between male and female groups, *P≤0.05, **P≤0.02, ***P≤0.01. See Figure 3.6 for profiles.

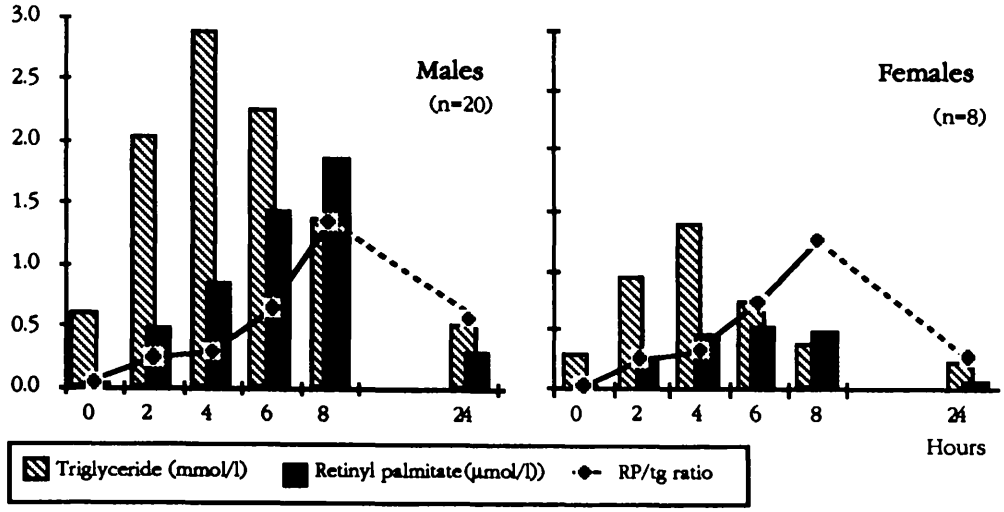


Figure 3.6 ρ<1.006 triglyceride and RP responses in males and females.

triglyceride, both residents of the lipoprotein core, provides an index of the relative enrichment of the ρ<1.006 fraction with retinyl ester as lipæmia progresses. This ratio was at its highest measured level in the last post prandial sample drawn (8 hours), and even at 24 hours was still as high as at the peak of lipæmia.

Despite significant gender differences in absolute levels of both RP and triglyceride, the RP/triglyceride ratios were quite similar (Figure 3.6). The implication is that the greater mass of RP in male subjects is distributed throughout a larger number of particles rather than present in greater concentration within the particle core.

Lipoproteins of ρ>1.006g/ml: Lipid flux during lipæmia was not limited to the triglyceride rich fraction, but was observed throughout the lipoprotein spectrum. Representation of the average changes is given in Figures 3.7-3.9 The following summary of these responses applies to the male group alone, although the pattern observed in females was qualitatively similar.

•*IDL* In the density fraction 1.006g/ml to 1.019g/ml phospholipid content rose (24-39%) and the cholesterol content fell (26-37%) during the first six hours of lipæmia. Triglycerides were not significantly altered.

•*LDL* The phospholipid content of this fraction rose continually throughout lipæmia and was at its highest level (↑12%) in the last post prandial sample drawn (8 hours). Triglyceride was also increased during lipæmia but peaked (↑9%) earlier, at 4 hours, and then declined. Mean levels of free and esterified cholesterol in LDL tended to fall initially and then rise later, although individual variations in the pattern of cholesterol flux meant that these values did not differ significantly from fasting.

•*HDL* The post prandial changes in HDL phospholipid, triglyceride, and free (but not esterified) cholesterol paralleled those of LDL. Phospholipid rose to its highest level (↑11%)

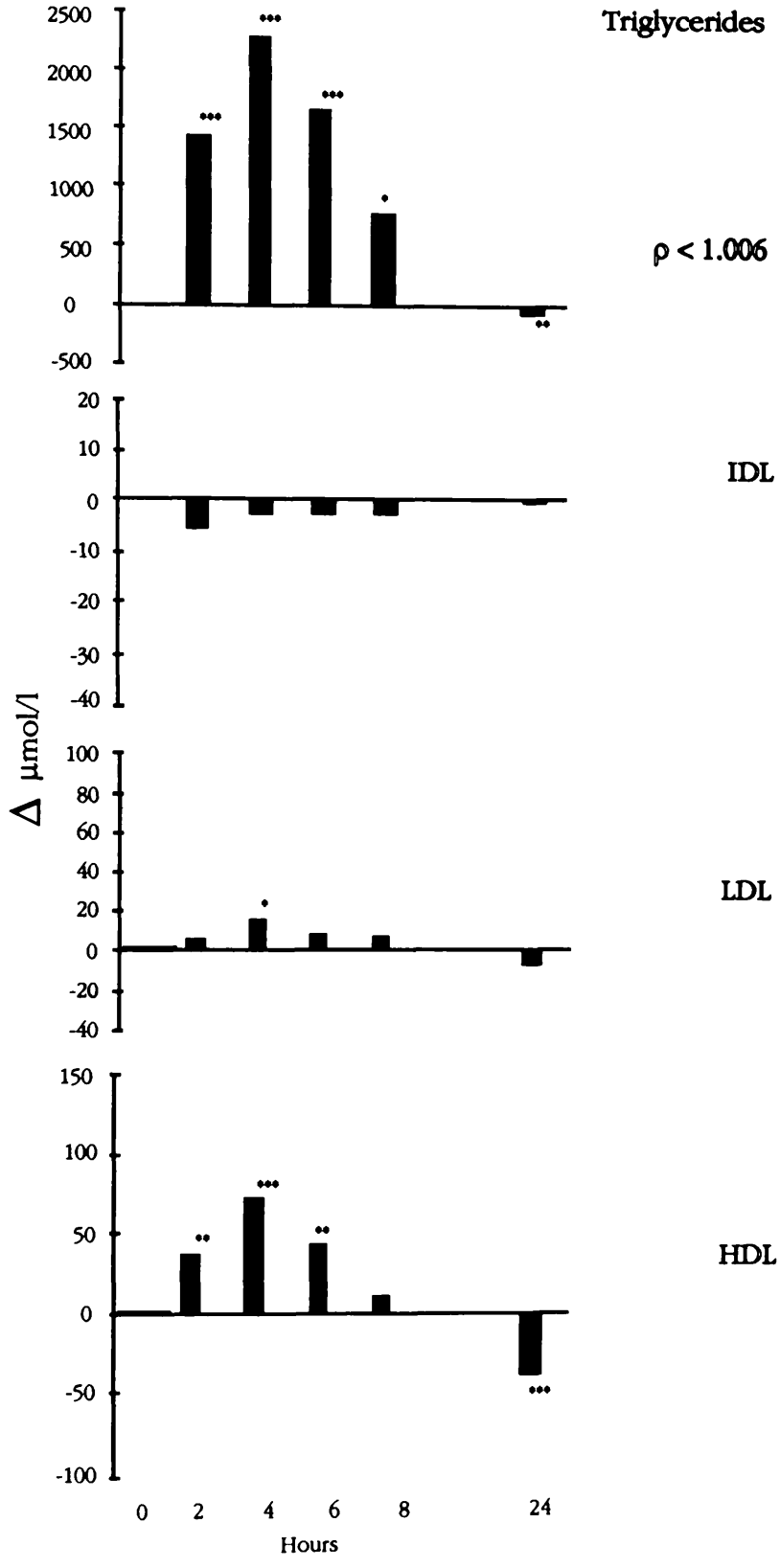


Figure 3.7 Changes in lipoprotein triglyceride during lipæmia. Zero marks fasting levels and vertical bars represent mean change from fasting. Significance of difference from fasting, *P≤0.05, **P≤0.01, ***P≤0.001. n=20 males.

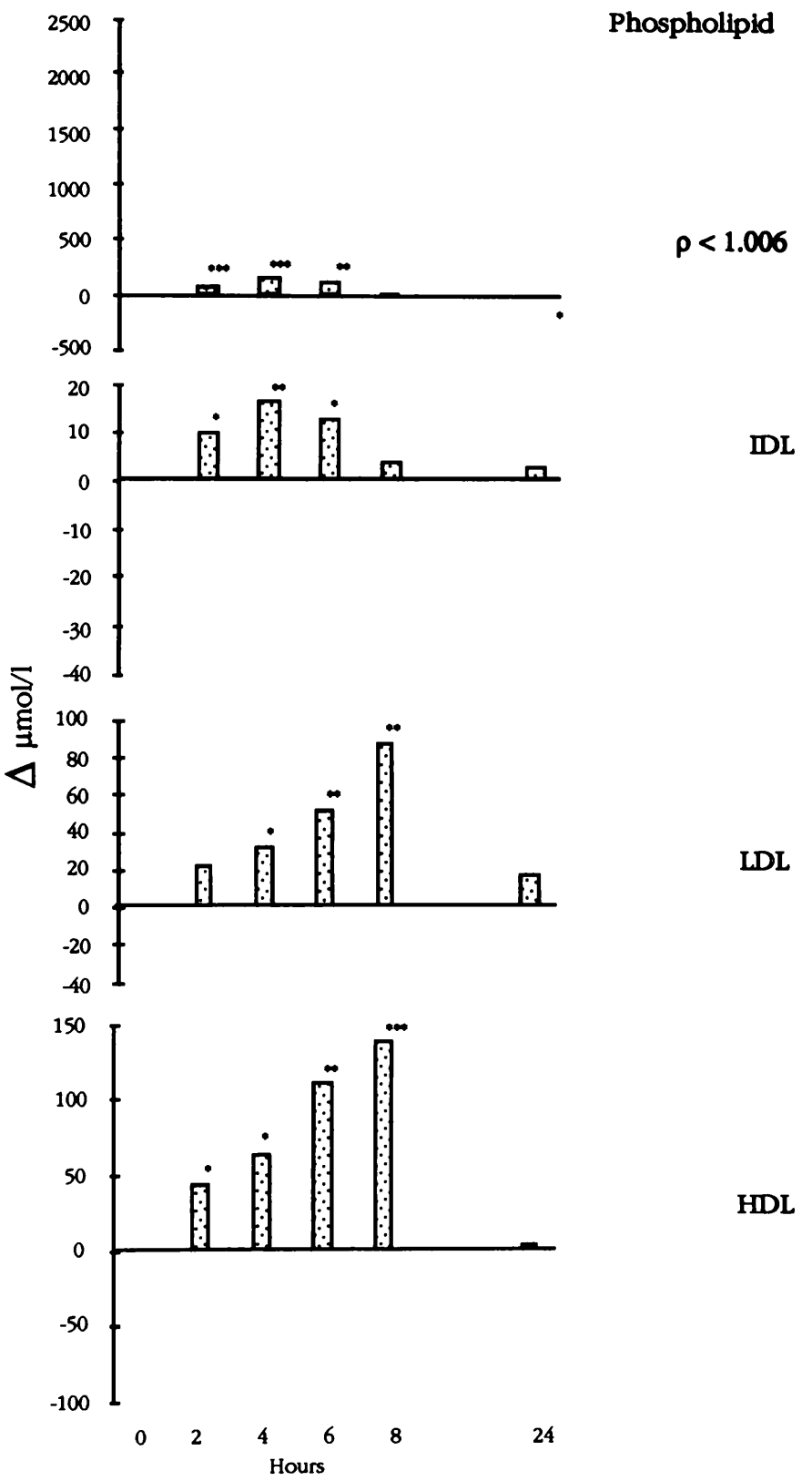


Figure 3.8 Changes in lipoprotein phospholipid during lipæmia. Zero marks fasting levels and vertical bars represent mean change from fasting. Significance of difference from fasting, *P≤0.05, **P≤0.01, ***P≤0.001. n=20 males.

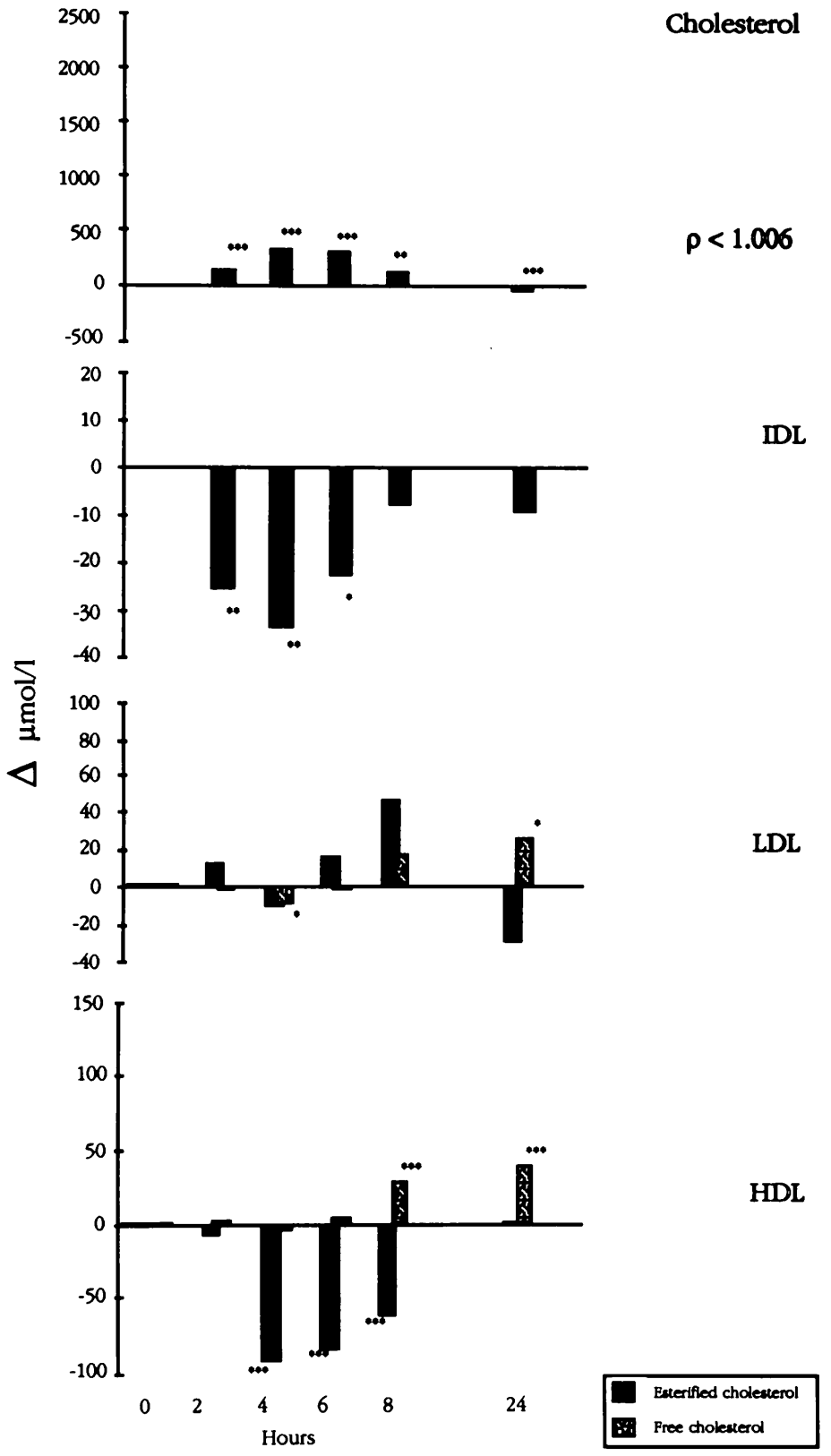


Figure 3.9 Changes in lipoprotein cholesterol during lipæmia. Zero marks fasting levels and vertical bars represent mean change from fasting. Cholesterol bars in ρ<1.006 fractions refer to total cholesterol. Significance of difference from fasting, *P≤0.10, **P≤0.05, ***P≤0.01. n=20 males.

by 8 hours, while HDL triglyceride peaked (↑63%) at 4 hours. Free cholesterol fell marginally at first but then rose gradually to be increased by 12% 8 hours after the fat load. HDL esterified cholesterol however, in contrast to the changes in LDL cholesteryl esters, fell consistently by between 5 and 8% throughout lipæmia.

The magnitude of post prandial triglyceridæmia was positively related to Δ LDL esterified cholesterol & triglyceride, and Δ HDL triglyceride, and negatively to Δ LDL free cholesterol and Δ HDL esterified & free cholesterol at various stages of lipæmia (correlations not quoted here). The pattern of changes in these lipids and post prandial lipæmia could be best understood by considering them as components of either core or surface domains.

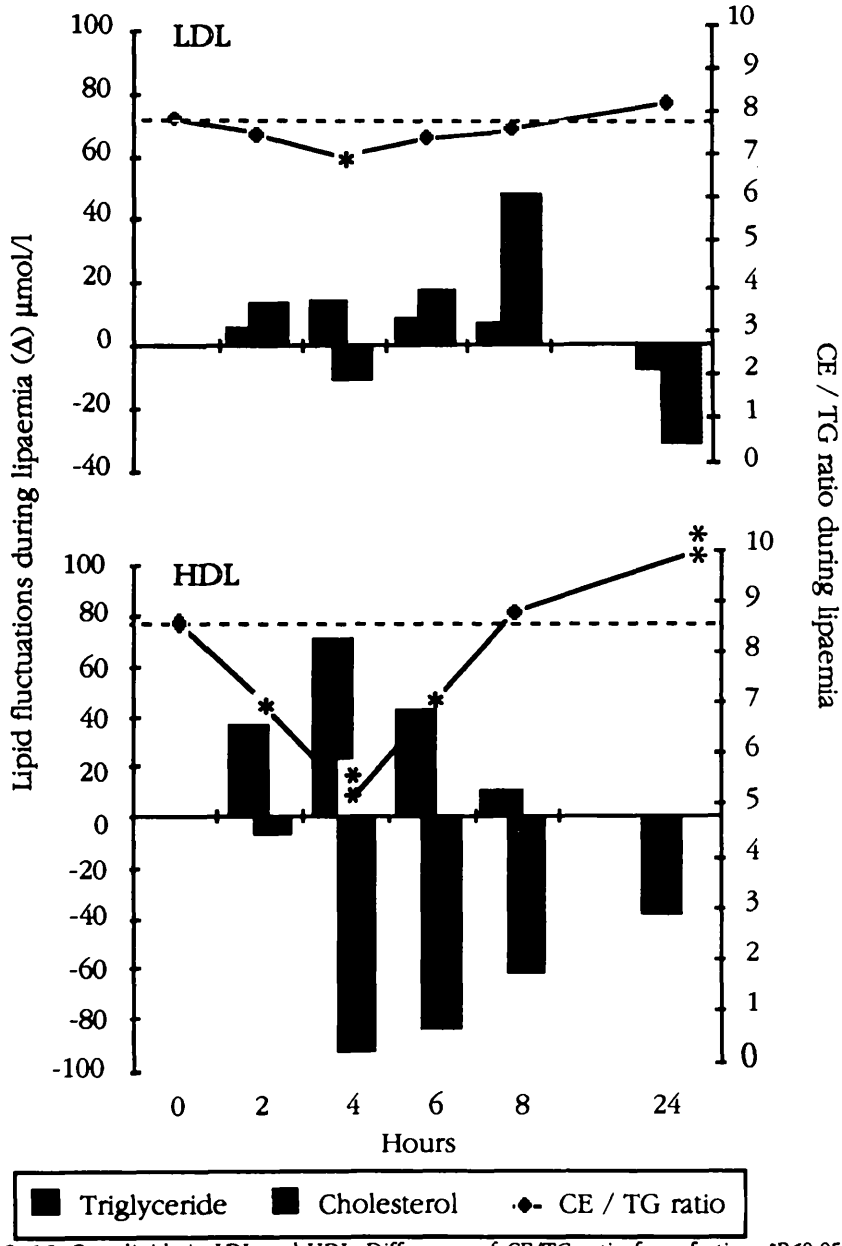


Figure 3.10 Core lipids in LDL and HDL. Difference of CE/TG ratio from fasting, *P≤0.05, **P≤0.01, n=20 males. See figures 3.7 & 3.9 for significance of Δ values.

Core: In both LDL and HDL the ratio of the core constituents cholesteryl ester and triglyceride (CE/TG) fell during lipæmia (Figure 3.10). In LDL, even when both lipids showed a net increase, the reduction in CE/TG ratio indicates that triglyceride influx exceeded that of cholesteryl ester. The same ratio in HDL fell by a much greater amount, from 8.8 to 5.5 (38%) at four hours, P≤0.01, and showed a strong negative correlation with ρ<1.006 triglyceridæmia (r=-0.71, P≤0.001). During lipæmia therefore the cores of both LDL and HDL became enriched in triglyceride, although only in HDL was this at the expense of cholesteryl ester.

By 8 hours the CE/TG ratio had risen again, and by 24 hours, despite little change in the absolute amount of cholesteryl ester, there was an increase in HDL of approximately 8%, P≤0.01.

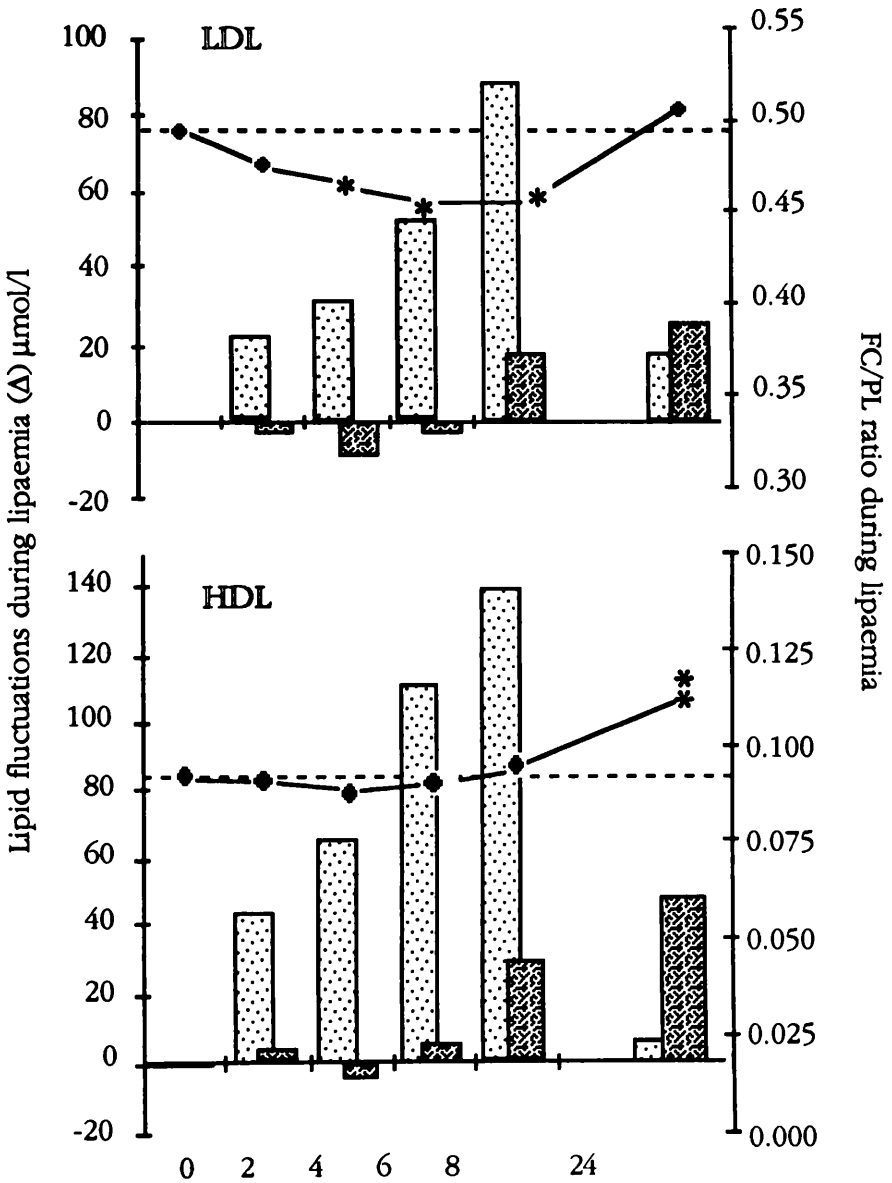


Figure 3.11 Surface lipids in LDL & HDL. Difference in FC/PL ratio, *P≤0.01, **P≤0.001, note the difference in scale for LDL and HDL , n=20males. See figures 3.8 & 3.9 for significance of Δ values.

Surface: The time scale of flux in free cholesterol and phospholipid, both associated with lipoprotein surfaces, differed from that of $\rho<1.006$ triglycerides, being at their highest levels in the latest samples drawn (8 hours).

The ratio of free cholesterol to phospholipid (FC/PL) was calculated as a measure of free cholesterol concentration at the lipoprotein surface (Figure 3.11). This ratio was approximately 5 times greater in LDL than in HDL. In HDL the ratio remained constant during lipæmia, but was increased by 20% at 24 hours (0.09 to 0.11, $P\leq0.001$). Figures 3.8 & 3.9 show that this late rise was due to the higher free cholesterol content of HDL since phospholipid levels were little changed at this time. Neither the rise in HDL free cholesterol, or FC/PL ratio at 24 hours were statistically related to the magnitude of the preceding post prandial triglyceridæmia.

The greater increase in phospholipid over free cholesterol in LDL led to a significant fall in the FC/PL ratio during lipæmia (8% at 6 hours, $P\leq0.005$). The free cholesterol content of LDL was apparently linked to the triglyceride content of $\rho<1.006$ lipoproteins. LDL free cholesterol levels were positively related to both fasting ($r=0.36$, $P\leq0.1$), and post prandial ($r=0.51$, $P\leq0.01$) $\rho<1.006$ triglycerides. However, post prandial ***changes*** in LDL free cholesterol, and in the FC/PL ratio were ***negatively*** related to both fasting and post prandial $\rho<1.006$ triglyceride (Table 3.9).

Table 3.9 *Correlations of post prandial triglyceride and free cholesterol flux*

$\rho<1.006$ Triglyceride	Δ LDL Free cholesterol 2	4	6	8	24
Fasting	-0.12	-0.31	-0.46*	-0.40*	-0.56**
Area	-0.10	-0.51*	-0.48*	-0.52**	-0.67**
			LDL FC/PL ratio		
Fasting	-0.64**	-0.42*	-0.58**	-0.58**	-0.63**
Area	-0.63**	-0.41*	-0.52**	-0.61**	-0.63**

Significance of the correlations between fasting and post prandial triglyceride response and the changes in LDL free cholesterol after the fat load, *$P\leq0.05$, **$P\leq0.01$.

The actual free cholesterol content of LDL and its flux during lipæmia were therefore both related to the magnitude of lipæmia, but with opposing sign. In fact there was a negative relationship between fasting levels and the response in LDL free cholesterol at 8 ($r=-0.49$, $P\leq0.02$), and 24 hours ($r=-0.55$, $P\leq0.01$), at which times LDL free cholesterol showed a mean rise (Figure 3.9). Those in whom lipæmia was greatest therefore tended to have higher fasting LDL free cholesterol, but were less likely to show a rise in free cholesterol during lipæmia.

3.2.3 Lipoproteins 24 hours after fat ingestion

Fasting blood samples obtained on the morning following the fat tolerance test differed in several respects from those obtained 24 hours earlier. Serum triglyceride in male subjects was lowered by an average 15%, P≤0.005, (Figure 3.1), mirroring a fall of similar magnitude in circulating VLDL mass (Table 3.10). The reduction in VLDL seemed primarily due to fewer particles, although composition was also altered in that relatively less cholesterol and more triglyceride was present compared with the previous days sample. It should also be recalled that a significant amount of RP continued to circulate in this density fraction at 24 hours (Figure 3.6).

The triglyceride content of HDL was 35% less (P≤0.001) than it was prior to the fat tolerance test, whereas HDL free cholesterol was increased by 17% (P≤0.02). Free cholesterol in the LDL fraction was also increased (4%, P≤0.1) (Figures 3.7-3.10). Significant and qualitatively similar changes were also observed in females.

Table 3.10 *The composition of fasting VLDL initially and at 24 hours*

	Total mass (mg/dl)	Percentage composition chol.	trig.	phos.	protein	Particle diameter Å	Nº x10^{13}
Initial	90.1 ±55.8	13.4 ±2.8	58.7 ±7.5	20.5 ±4.2	7.4 ±3.1	39.8 ±8.2	2.83 ±1.29
24 hours	73.1 ±50.0	11.4 ±2.8	61.8 ±5.4	18.7 ±2.4	8.1 ±4.2	42.3 ±6.8	1.97 ±1.11
	↓19%**	↓15%***	↑5%*	↓9%*	•	•	↓30%***

Significance of the changes at 24 hours, *P≤0.10, **P≤0.05, ***P≤0.01

3.2.4 Lipæmic response and fasting profile

To investigate the position of alimentary lipæmia as a component of an individuals lipoprotein constitution the principal markers of lipæmia (triglyceride and retinyl palmitate in ρ<1.006 lipoproteins) were correlated with fasting lipid profiles.

ρ<1.006 triglyceridæmic response: ρ<1.006 triglyceridæmic response correlated positively with all serum lipids, and with apoproteins B & CIII, and inversely with apo AI (Table 3.11). It also correlated positively with fasting VLDL, IDL, and LDL masses, the relationship to VLDL seemingly dependent on the size rather than the number of fasting particles. Fasting HDL_2 mass was inversely related to the magnitude of lipæmia, but HDL_3 mass was not. Two aspects of these correlations deserve further mention.

Firstly, regardless of sign, they were more pronounced during the first four hours, declined by six hours, and had disappeared by eight hours. Bearing in mind the interrelationships of Δ and area measurements in Table 3.7, suggesting a more significant influence of earlier Δ values on the overall magnitude of lipæmia, it would seem that the determinants

Table 3.11 *Correlations of fasting parameters with triglyceride response*

Fasting	Δ ρ<1.006 Triglyceride 2	4	6	8	24	Area
Free cholesterol	0.59***	0.50**	0.47**	0.12	0.16	0.53***
Ester. cholesterol	0.41**	0.35*	0.36**	0.03	-0.07	0.39**
Triglyceride	0.84***	0.88***	0.56***	0.15	-0.19	0.86***
Phospholipid	0.61***	0.59***	0.30*	0.04	0.16	0.58***
Apo AI	-0.31*	-0.39**	-0.35*	-0.25	0.00	-0.34*
Apo AII	0.11	0.25	0.25	0.34*	0.12	0.32*
Apo B	0.62***	0.51***	0.54***	0.18	0.08	0.53***
Apo CIII	0.41**	0.54***	0.34*	0.29	0.08	0.58***
Apo E	0.26	0.07	-0.23	-0.20	0.27	0.03
VLDL	0.83***	0.86***	0.58***	0.19	-0.32*	0.83***
..... Diameter	0.71***	0.77***	0.48**	0.11	-0.15	0.76***
..... Particle Nº	0.16	0.10	0.07	0.14	-0.16	0.09
IDL	0.49**	0.42**	0.17	-0.13	0.16	0.42**
LDL	0.54***	0.50***	0.49**	0.09	0.20	0.53***
HDL_2	-0.40**	-0.44**	-0.35*	0.22	0.45**	-0.39**
HDL_3	0.13	0.15	-0.06	0.02	0.10	0.10
LpL pre heparin	-0.33*	-0.31*	-0.09	-0.05	-0.13	-0.30*
LpL post heparin	0.17	-0.01	-0.08	-0.26	-0.07	-0.05
HL pre heparin	-0.38**	-0.57***	-0.58***	-0.33	0.08	-0.62***
HL post heparin	0.23	0.29	0.09	-0.26	-0.24	0.25

Significance of the correlation between various fasting parameters and triglyceridæmic response, *P≤0.1, **P≤0.05, ***P≤0.01·

of the earlier stages of triglyceridæmia in these subjects have more relevance to their fasting lipoprotein profile.

Secondly, in the case of fasting VLDL and HDL_2, the sign of the correlation with response is reversed at 24 hours. Therefore in those people initially possessed of high fasting VLDL and low HDL_2 there was a greater tendency for VLDL triglyceride to be reduced below original baseline levels following a fat load.

Post heparin lipase activities were unrelated to triglyceridæmic responses. There was however a weak negative correlation between pre heparin LpL activity and Δ2-Δ6 triglyceride, and a negative correlation between pre heparin HL and all phases of triglyceridæmia.

ρ<1.006 retinyl palmitate response: The relationship of RP flux to fasting profile resembled generally that of ρ<1.006 triglycerides, but differed from it in several important respects (Table 3.12). Whereas triglyceride responses beyond their peak declined in their associations with fasting profile, it was notable that even the later RP responses were related to fasting levels of serum triglyceride, phospholipid, VLDL and IDL.

Closer examination of the relationship of fasting VLDL to RP indicate that the initial rise in RP was related to fasting VLDL diameter, but not to particle number, whereas the opposite was true for later values of RP response. Therefore the factors governing fasting VLDL diameter appear more closely related to those involved in the initial rise in RP, and those involved in the determination of VLDL numbers more closely related to RP clearance.

Table 3.12 *Correlations of fasting parameters with retinyl palmitate*

Fasting	Δ ρ<1.006 Retinyl palmitate 2	4	6	8	24	Area
Free cholesterol	0.40**	0.37*	0.24	0.29	0.34*	0.33*
Ester. cholesterol	0.45**	0.44**	0.29	0.37*	0.09	0.39**
Triglyceride	0.62***	0.83***	0.68***	0.62***	0.72***	0.72***
Phospholipid	0.41**	0.44**	0.35*	0.57***	0.48**	0.49**
Apo AI	-0.31*	-0.54***	-0.38**	-0.16	-0.20	-0.37**
Apo AII	0.16	0.24	0.38**	0.08	0.40	0.33*
Apo B	0.31*	0.41**	0.21	0.27	0.26	0.28
Apo CIII	0.29	0.40**	0.38**	0.53***	0.60***	0.46**
Apo E	0.11	0.05	0.14	0.21	0.36*	0.19
VLDL	0.59***	0.79***	0.64***	0.71***	0.64***	0.72***
..... Diameter	0.74***	0.69***	0.48**	0.30	0.42**	0.61***
..... Particle Nº	-0.15	0.19	0.36*	0.59***	0.35*	0.28
IDL	0.31	0.27	0.39*	0.38*	0.49**	0.44**
LDL	0.44**	0.41**	0.23	0.12	0.26	0.32*
HDL_2	-0.40**	-0.38*	-0.28	0.07	-0.08	-0.29
HDL_3	-0.06	0.11	0.16	0.18	0.33*	0.14
LpL pre heparin	-0.01	-0.19	-0.04	0.13	-0.30*	-0.02
LpL post heparin	0.13	0.16	0.20	0.15	-0.01	0.20
HL pre heparin	-0.17	-0.25	-0.28	-0.45**	-0.37*	-0.34*
Hl post heparin	0.15	0.09	0.23	0.09	0.28	0.21

Significance of the correlation between various fasting parameters and response, *P≤0.1, **P≤0.05, ***P≤0.01

Δρ<1.006 RP at 24 hours was also related to initial fasting triglyceride, phospholipid, VLDL, and IDL concentrations. RP was more likely to be raised in those subjects with greater initial fasting levels of the VLDL and IDL classes.

Post heparin lipase activities were again unrelated to response, although a negative correlation did exist between pre heparin HL and Δ8 RP, and between both pre heparin enzyme activities and Δ24 hour RP.

3.3 Discussion

The nature of post prandial triglyceridæmia

The magnitude of post prandial triglyceridæmia has been described as the most variable of all parameters of lipid transport.[182] Within this normal population it was found to range in male subjects from 1.50 to 38.4 mmol/l•8hours, a 26 fold difference between individuals in ρ<1.006 triglyceride area. Although of lesser magnitude, fasting VLDL triglyceride also exhibits a high degree of variability, the corresponding figures to those above being 0.06 to 1.8 mmol/l; a 30 fold range. The results of this study illustrate however, a fundamental difference in the nature of fasting and post prandial triglyceridæmias.

The present findings confirm those of Redgrave and Carlson[175] which show that post prandial triglyceridæmia is accounted for primarily by an increase in the average size of ρ<1.006 lipoproteins, rather than an increase in their total number. The formation of larger particles by the intestine probably represents an adaptation for the conservation of surface materials (phospholipid and proteins) in the face of increased triglyceride flux after a fatty meal.[86]

Fasting triglyceridæmia on the other hand is a result of variability in the *number* of particles in the ρ<1.006 lipoprotein classes in both normo- and hyper-triglyceridæmic subjects,[175] manifest here in the difference in the number of VLDL particles in males and females. Size differences (as suggested by the slightly greater percentage of triglyceride in males) may make a minor contribution to fasting triglyceride variability. It has been calculated that 71% of the variation in fasting triglyceride is due to differences in particle number and 29% due to size.[183]

The present data suggest a degree of independence in the expression of size and number, affecting triglyceride rich lipoproteins of both hepatic and intestinal origin. Firstly, males and females differed not only in fasting triglyceride but also in their *response* to the fat load. Therefore in addition to a difference in the number of particles in the fasting condition, the magnitude of the post prandial increment (mainly due to size) also differentiated male and female groups.

Secondly, the increment in size was found to be a characteristic feature of an individual's metabolic profile, a finding supported by previous reports of the reproducibility of the fat tolerance test.[51,182,184] Lastly, post prandial triglyceridæmia was correlated with fasting VLDL

diameter, but not with particle number, suggesting a link in the physiological regulation of triglyceride rich lipoprotein size applying to hepatic as well as intestinal lipoproteins.

These findings agree with the concept of a competition between fasting and post prandial triglyceride rich lipoproteins for a common and saturable removal mechanism.[185,186] Since lipoprotein size in this density range is largely a function of triglyceride content, the importance of this variable suggests that it is the triglyceride burden of individual particles rather than simply total circulating triglyceride, which underlies the competition for triglyceride removal.

Grundy and Mok,[187] in discussing the discrepancy between chylomicron and VLDL catabolic rates, concluded that the type of particle in which fat was presented, rather than the total fat load was the important factor determining rates of hydrolysis. In addition Quarfordt and Goodman[188] have found that larger chylomicrons are catabolised faster than smaller ones. Although the determination of lipoprotein size will depend to some extent on the availability of apoprotein and other surface materials relative to the prevailing flux of triglyceride, it will also depend on individual capacity for triglyceride removal.

The enzyme responsible for triglyceride hydrolysis is LpL, and by virtue of a preference for larger and more triglyceride enriched particles its activity will tend to reduce average lipoprotein size. Individual variability in the capacity to hydrolyse triglyceride may therefore provide the link between fasting and post prandial lipoprotein size, since greater activity would alleviate competition for lipolytic sites, and thus maintain smaller sized VLDL and chylomicron populations. Such a relationship may be of more than academic interest in view of the divergent catabolism of large and small VLDL species.[189]

The clearing of lipæmia

The dual character of chylomicron catabolism was clearly illustrated by the differing profiles of retinyl palmitate and triglyceride. RP was added to the fat load as a tracer for chylomicron remnants. Its delayed rise, and its continued rise after the peak of triglyceridæmia, are evidence that its absorption is slower than that of triglyceride. The extended plasma lifetime of RP and the difference in RP & triglyceride clearance rates are in keeping with the concept of a biphasic catabolism of chylomicrons comprising an initial period of triglyceride lipolysis followed by uptake of the whole remnant. During the first catabolic phase triglyceride and RP responses were related, but during the second phase this relationship disappeared.

The first stage: The initial incorporation of RP into newly formed chylomicrons presumably contributes to the positive associations between RP and triglyceride during the early stages of lipæmia. In a study similar to the present one Weintraub *et al*[184] attempted to assay chylomicron and non-chylomicron (remnant) populations by measuring RP in post prandial lipoproteins isolated at $Sf>1000$ and $Sf<1000$ respectively. They found that RP and

triglyceride in chylomicrons were correlated but that there was no relationship in lipoproteins of S*f*<1000.

In the present study, in which RP was assayed in lipoproteins of S*f*>20 (ρ<1.006g/ml), the initial correlation between RP and triglyceride followed by a period in which they are not related may mark the passage from a stage of lipæmia in which chylomicrons predominate (ie. when absorption is still active), to a period when they are superseded in the circulation by remnant species.

Weintraub *et al*[184] found that RP in chylomicrons (S*f*>1000) was negatively related to LpL activity, the implication being that departure of RP into more dense fractions was contingent on rates of triglyceride hydrolysis. RP in the more heterogeneous mix of lipoproteins isolated in the present study did not show such a relation to LpL.

The second stage: The persistence of RP at densities of less than 1.006g/ml beyond triglyceridæmia, and until at least 24 hours after the original fat load, supports the concept of a second stage in chylomicron catabolism unrelated to triglyceride removal rates. Present evidence suggests that this second stage involves removal of the chylomicron remnant by a receptor mediated process taking place at the surface of hepatocytes.[139] The simplest explanation for the prolonged circulation of RP in the ρ<1.006 fraction would therefore be that chylomicron remnants can persist for long periods after a fatty meal.

Further illustration of the passage of RP from chylomicrons to remnants took the form of an early correlation of Δ RP with fasting VLDL size but not number, giving way to a later correlation with number but not size. As discussed above, variation in fasting VLDL pertains mostly to particle number, and so is presumably a function of both particle synthesis and removal rates. The correlation between RP and fasting VLDL number may reflect the similar mode of clearance of these particles, and suggests a possible competition between hepatic and intestinal remnants for the removal process.

The relationship to IDL which was confined to later Δ RP would follow from the above argument. The distinction between IDL and remnants on the basis of ultracentrifugation is an operational one, and since remnants will occupy a spectrum of flotation rates traversing the arbitrary boundary separating VLDL and IDL, higher levels of IDL may reflect to some extent the presence of remnant material. The correlation of RP with fasting triglyceride may be a further reflection of the importance of VLDL particle number (and remnant clearance) in the determination of triglyceride levels in the steady state.

It is implicit in the preceding argument that remnants are not the major source of triglyceridæmia in its later stages. Despite a positive Δ triglyceride, RP and triglyceride were not correlated latterly in either the mixture of lipoproteins of ρ<1.006g/ml in the present study, or in the non-chylomicron fraction of S*f*<1000.[184]

Continued, although lessened, triglyceride absorption and chylomicron formation at a time when the more slowly removed remnants are more plentiful, may lead to triglyceride

levels no longer directly related to amounts of intestinally derived particles, as described by RP levels. Alternatively triglyceridæmia may become contaminated with material of non-intestinal origins, perhaps through an augmented output of hepatic VLDL in response to the increased availability of fatty acid. Recycling of chylomicron triglyceride has been demonstrated in rats,[190] and hepatic VLDL secretion can be stimulated by both standard and triglyceride rich remnants.[191] A post prandial increase in apoB_{100} during alimentary lipæmia in humans has also been noted.[192,193] Recycling of exogenous material may underlie the biphasic triglyceride response reported by several groups.[193-195]

Effects at 24 hours

A lowering of plasma and VLDL triglyceride subsequent to a fat load has been noted frequently, but has remained an unexplained observation.[184,187,196] This effect may represent the lag phase of an adaptive response to the large influx of fat the day before, although whether this is due to stimulated lipolysis (LpL activity can be stimulated by an orally administered fat load[197]), or whether there is a temporary inhibition of endogenous triglyceride secretion is unclear.

In male subjects 24 hours after the fat load fewer particles circulated in the $\rho<1.006$g/ml density range, and in the average particle the amount of triglyceride was more, and the amount of cholesterol less than on the previous day. The evidence that a proportion of these particles were RP enriched remnants means that the 'average particle' represents a hybrid of hepatic VLDL and intestinally derived remnants, making compositional changes difficult to interpret. Nevertheless the restriction of the effect to particle number suggests a fall in VLDL production. The greater reduction in those with higher fasting VLDL and lower HDL_2 may perhaps reflect a more labile regulation of triglyceride metabolism in such subjects, of which a post prandial increase in hepatic VLDL production, as discussed above, may be another manifestation.

Lipases and lipolysis

Conventional methods for the assessment of LpL and HL activity involve the release of enzyme into the blood stream by the administration of heparin, permitting assay in plasma. Lipase activity in this study was also measured in fasting plasma obtained prior to the administration of heparin. Pre and post heparin lipase activities were much lower than, and were poorly correlated with post heparin measurements. Nevertheless, pre heparin activities showed similar gender differences and a greater relationship to other physiological variables than those obtained post heparin. It would seem therefore that free lipase, although not present in physiologically significant amounts, may still provide a marker of the potential pool of available lipase. [198,199]

A negative correlation between LpL activity and alimentary lipæmia has been reported,[184,200] although in the present normal subjects the relationship was of only marginal

significance. However, although it is now evident that LpL has a major role to play in the clearance of lipæmia, plasma lipase activity may offer only a crude guide to lipolytic capacity.

The measurement of post heparin lipase activity is known to be sensitive to the methodology used, and in particular to the amount of intravenous heparin administered.[201] Although correction was made here for body weight it is possible that the poor (or absent) correlation between pre and post heparin values is at least partly due to a semi- or non-quantitative release of enzyme from tissues. In addition LpL in plasma reflects enzyme activity released from several tissues, mainly adipose and skeletal muscle, but including heart and lung, each of which are independently regulated and may respond differently to such stimuli as fat feeding.[202]

Furthermore, as it progresses triglyceridæmia will become increasingly associated with remnants whose disposal from the circulation is seemingly less related to lipolysis. Redgrave & Carlson[175] concluded from their studies that a major proportion of post prandial triglyceridæmia was due to remnant particles rather than to chylomicrons.

The role of HL in lipoprotein metabolism is at present less well defined than that of LpL. A recent study has suggested that the triglyceride lipase activity of HL may be important when the capacity of LpL for triglyceride hydrolysis is exceeded, as for example occurs during fat absorption.[134] There is also evidence that HL has a role to play in the catabolism of smaller triglyceride rich lipoproteins. Conversion of small VLDL to IDL, and IDL to LDL is reduced in HL deficiency.[203]

The negative correlation between HL and lipæmia in the present study could be explained by either or both of these roles. Saturation of the lipolytic system is evident from the rise in triglyceride during lipæmia, and clearance of the excess may conceivably be related to HL activity. In addition, chylomicron catabolism is associated with the production of remnant particles whose smaller dimensions may place them in a population for which HL has greater affinity.

The negative relationship between HL activity and RP does not agree with the report of Berr[151] who stimulated lipolysis in humans by injecting heparin, but found no change in remnant clearance. Weintraub[184] however, did find a negative relationship between HL and non-chylomicron RP clearance. This negative correlation suggests a possible involvement of HL prior to, or coincident with, remnant uptake, and may hinge on the phospholipase activity of HL which could expose receptor re-cognition sites on apoE.

The concentration of apoCIII in fasting plasma showed an interesting relation to post prandial triglyceride and RP levels. This apoprotein has been suggested to modulate the activity of LpL,[135] and also to regulate the affinity of remnants for their hepatic receptor.[138] The association of apoCIII with Δ2-Δ6 triglyceride suggests that higher levels may indeed be associated with reduced lipolysis rates. The relationship to RP levels is confined to later responses, and is at its strongest with Δ24 RP. Again this is in agreement with the proposal that apoCIII is inhibitory in its effects on remnant uptake.

Lipæmia as a component of the lipoprotein profile

The apo B containing lipoproteins VLDL, IDL, and LDL form a metabolic cascade in which synthesis and catabolism of VLDL are important determinants of LDL levels. This relationship probably underlies the positive association of these lipoproteins and apoB in the fasting state. The positive association between post prandial triglyceridæmia and apo B containing lipoproteins may simply reflect the importance of lipolytic processes in determining the levels of both hepatic and intestinal lipoproteins, although the possibility that lipoprotein production in liver and intestine are under some form of co-regulation cannot be dismissed.

A reciprocal association between VLDL and HDL has been documented regularly in both population and clinical studies,[45] and has also been ascribed to differences in lipolytic activity. More efficient lipolysis, acting to maintain lower levels of VLDL will increase the transfer of surface materials (mainly phospholipid) into the HDL density range where they may either increase existing HDL mass, or act as a precursor in HDL formation. The negative relationship between post prandial triglycerides and HDL observed in this and in other studies[200] supports the operation of the above mechanism on chylomicrons as well as VLDL.

Lipid flux during lipæmia

A prolonged post prandial rise in plasma phospholipids, outlasting that of triglycerides, was noted initially by Havel[196] They concluded that there was a transfer of phospholipid into higher density lipoprotein classes which were removed from the circulation more slowly than the triglyceride rich fractions. The phenomenon of phospholipid transfer from triglyceride rich lipoproteins to HDL during lipolysis is thought to underlie the frequently observed reciprocal relationship between VLDL and HDL discussed above. The mechanism of transfer to higher densities involves either a specific phospholipid transfer protein, or a form of bulk transfer in which redundant surface materials forming subsequent to lipolytic reduction of the triglyceride core, fold outwards and eventually 'pinch' off, passing into the HDL density range.[133] A similar, but less widely documented transfer of phospholipid into LDL was observed in the present study.

In such a scenario more efficient lipolysis should therefore increase phospholipid flux through more rapid reduction of core volume, and indeed in females, in whom LpL activity was greater and triglyceride response less, the magnitude of phospholipid flux exceeded that of the male group.

It might be expected that free cholesterol, also a resident of surface regions, would accompany phospholipid into the higher density fractions during such bulk transfer, and in fact a rise in free cholesterol in LDL and HDL did occur. However this is not the only mechanism by which lipæmia might increase the free cholesterol content of the $\rho>1.006$ fractions.

The development of a reduced FC/PL ratio in LDL, presumably due to a greater influx of phospholipid compared with free cholesterol to the surface, may be instrumental in raising plasma levels of free cholesterol. Since free cholesterol can transfer spontaneously between cell membranes and lipoproteins, a decline in the FC/PL ratio would create a concentration gradient encouraging a net movement into plasma. Such a mechanism is thought to underlie the so-called 'reverse cholesterol transport' system by which cholesterol is returned from peripheral tissues to the liver. In the present study post prandial triglyceridæmia was negatively related to the magnitude of free cholesterol flux into LDL. An increase in triglyceridæmia, associated with reduced lipolytic rates and transfer of phospholipid into higher density lipoproteins, may therefore attenuate any decline in the FC/PL ratio, and so reduce the subsequent flux of free cholesterol into the LDL fraction.

This could explain why female subjects who demonstrated a lesser degree of triglyceridæmia also tended to have a greater amount of phospholipid entering plasma, accompanied by a greater increase in plasma free cholesterol during the later stages of lipæmia.

Interestingly, the concentration of free cholesterol in fasting LDL was *positively* related to lipæmia, and *negatively* to the flux of free cholesterol into LDL. However if it is indeed the ratio of free cholesterol to phospholipid which influences flux from cells, then an increased concentration of free cholesterol will tend to elevate that ratio in the face of similar phospholipid assimilation, and will therefore shorten the gradient driving cellular free cholesterol efflux.

Free cholesterol and phospholipid in HDL also increased, and in absolute terms the flux into HDL was greater than that into LDL. However the ratio of these lipids in HDL remained more or less constant. The stability of the FC/PL ratio may be a result of LCAT activity which, in plasma, is mostly associated with the HDL fractions. This enzyme, using both free cholesterol and phospholipid as substrate, and activated by apoAI, catalyses the formation of cholesteryl esters. Due to their hydrophobic nature these esters then move inwards to the core of the HDL particle. The combination of a decline in FC/PL ratio and LCAT activity would thus propagate a continuous movement of free cholesterol from cells into cholesteryl esters in HDL.

Despite this activity cholesteryl ester does not accumulate in HDL. In fact there is a highly significant fall leading, in combination with a reciprocal rise in triglyceride, to a marked change in the composition of post prandial HDL core lipids. This change is presumably a result of lipid transfer protein mediated hetero exchange of these lipids between lipoproteins,[204] in which cholesteryl esters transfer into particles with the lowest CE/TG ratio, and triglyceride moves in the reverse direction. The large influx of dietary triglyceride into the $\rho<1.006$ fraction will considerably steepen this gradient and encourage a fall in HDL cholesteryl ester.

The acquisition of triglyceride at the expense of cholesteryl ester is thought to render HDL susceptible to HL action, leading to the formation of a small HDL species, depleted of both cholesteryl ester and triglyceride. By this means post prandial lipæmia may cause a redistribution of HDL towards the smaller HDL_3 subfraction, and thereby contribute to the low levels of HDL_2 associated with exaggerated alimentary lipæmia.[200]

The position of LDL in core lipid transfer is less well defined. The positive association between Δ cholesteryl ester and triglyceride cannot be explained by simple, gradient driven hetero exchange of these lipids, particularly since the CE/TG ratio in HDL fell well below that of LDL.

It has been suggested[205] that LDL are in fact the major acceptors of LCAT derived cholesteryl esters, at least during fasting, and argues that the FC/PL ratio may make an important contribution to enhancing transfer rates. These authors suggest further that by providing an acceptor for transferred cholesteryl ester the post prandial decline in LDL FC/PL ratio will release inhibition on LCAT activity giving rise to the previously documented stimulation of this enzyme during lipæmia[206]

The free cholesterol content of lipoproteins can influence protein binding,[21] and since transfer activity seems to depend on the binding of transfer protein to lipoprotein substrates,[207] it would seem feasible that this mechanism would enhance transfer activity. The influx of phospholipid to lipoproteins has also been reported to enhance lipid transfer.[208] These findings are perhaps two aspects of the same phenomenon, the ratio of the lipids being the important factor.

Enhanced transfer activity however, need not necessarily affect the *direction* of lipid flux. The ultimate changes in LDL core lipids may be a composite effect of several transfer processes. Triglyceride from the $\rho<1.006$ fraction and LCAT derived cholesteryl esters from HDL may both enter LDL. LDL however may also lose cholesteryl ester by transfer to the $\rho<1.006$ lipoproteins, and also to IDL, with a proportion of this lipid subsequently returning to the LDL density range as a result of VLDL and IDL catabolism.

Although LDL may provide an acceptor for transferred cholesteryl ester in the post absorptive state, it would be reasonable to expect that the large influx of cholesteryl ester-poor chylomicrons during lipæmia would provide an additional route for transfer. Furthermore the efficient removal of remnants by the liver subsequent to accepting transferred cholesteryl ester might provide an explanation for the lowering of plasma cholesterol observed in some subjects (females) during lipæmia.

Lipæmia in males and females

Several features of lipoprotein metabolism distinguished male and female groups in this study. The lower fasting VLDL and greater HDL further polarised the reciprocal relationship governing the concentrations of these lipoprotein classes the in plasma. The magnitude of triglyceridæmia was less in females than in males, and the activity of LpL was greater. There

was an increased flux of free cholesterol into plasma, and a post prandial reduction in plasma esterified cholesterol. In addition retinyl palmitate levels were less in females at all time points, but particularly at 24 hours. All of these features are compatible with an enhanced rate of catabolism of triglyceride rich lipoproteins in females.

Chapter 4

Alimentary lipæmia in coronary artery disease

4.1 Introduction and Methods

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Plasma lipid abnormalities derive much of their interest from their association with coronary and peripheral atherosclerosis, although many of those developing CHD do not have clinical hyperlipidæmia. To explain the deposition of lipoprotein cholesterol in the arteries of normolipidæmic individuals alimentary lipæmia was proposed by earlier workers as a kind of 'latent' form of hyperlipidæmia, not apparent from normal fasting assessments. Based on then available techniques, these studies[14] concentrated on the particulate nature of chylomicrons in the aetiology of the disease. In this part of the study the relationship of alimentary lipæmia to atherosclerotic disease was re-examined using the retinyl palmitate fat tolerance test and modern analytical techniques.

The severity of atherosclerosis in a group of middle aged men was determined by coronary angiography, a common technique in the assessment of coronary artery stenosis. These arteries are particularly susceptible to atherosclerotic occlusion leading to the condition known as coronary artery disease (CAD), in which lipolysis of vital areas of the myocardium induces tissue necrosis (infarction) and cardiac dysfunction.

The extent of arterial stenosis is quantified by passing a radio-opaque material through the coronary arteries and taking an X-ray photograph. The lumen of the artery and the presence of any narrowing are thus revealed. The X-ray is then assessed and scored in standardised fashion by trained personnel. Angiogram scores thus obtained were compared with the incidence of established lipid and non-lipid risk factors in these men, and also with the lipid profiles of alimentary lipæmia.

4.1.1 Subjects

Males aged between 40 and 60, referred for assessment of CAD and admitted to the Department of Medical Cardiology at Glasgow Royal Infirmary for routine coronary

angiography, were asked to participate in the study. Upon consenting to undertake a fat tolerance test details of age, height, weight, resting blood pressure, smoking habits, and any family history of heart disease were obtained, in addition to a routine biochemical and hæmatological screen.

The use of drugs which block ß adrenoceptors has been found to interfere with lipid and lipoprotein metabolism.[209] Since these drugs are commonly prescribed to hypertensives and angina sufferers a large proportion of referrals for angiography are already on long term ß blocking therapy. In view of this and the dangers involved in stopping such treatment at a time of stress for patients, particularly when participating voluntarily in a research project, it was decided that use of these drugs should not exclude subjects from taking part.

4.1.2 Protocol

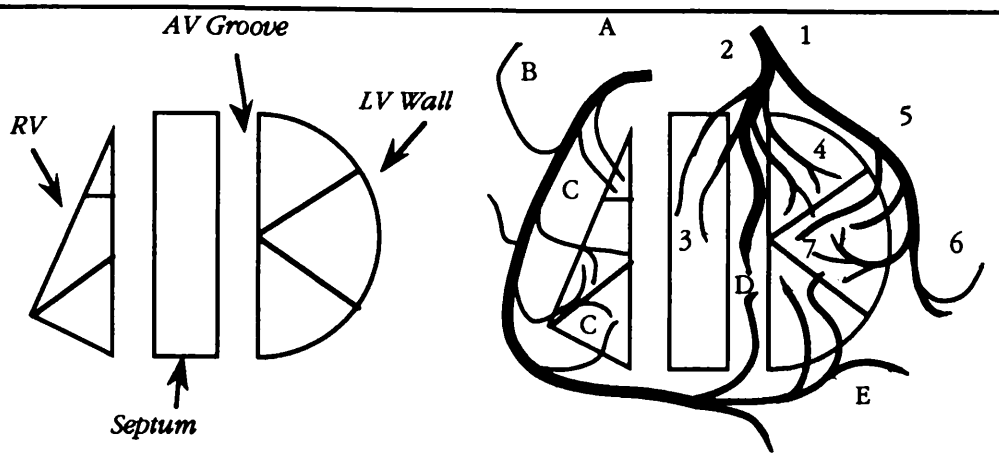
CAD subjects were admitted to the ward in the evening two days before, and administered the fat tolerance test one day before angiography. The test was conducted as described in section 2.3 and lipoproteins prepared by ß quantification. In a subgroup of 8 men lipoproteins were additionally prepared by sequential flotation ultracentrifugation. Subjects re-attended on a separate occasion for the estimation of lipase activities.

Smoking habits and any family history of heart disease were scored by a procedure adopted from Angaard *et al*[210] (Table 4.1). Angiography was performed by staff of the Department of Medical Cardiology and scoring of angiograms carried out by Dr JH Maclean of that Department using the method of Brandt *et al*.[211] (Figure 4.1).

Table 4.1 *Risk scoring* for smoking habits and family history of heart disease*

Smoking habits	**Score**
Non smoker > 1 year	0
0-10 cigarettes/day, pipe, cigar, non smoker>6 months	1
Heavy smoker stopped <6 months	2
11-20 cigarettes/day	3
21-40 cigarettes/day	5
40+ cigarettes/day	7
Family history	
No IHD# in relatives	0
For each family member with diagnosed IHD	1
For each family member with MI¶ or dying from IHD	2

*Adapted from reference 210, #Ischæmic heart disease, ¶Myocardial Infarction



Myocardial Score

Percentage Obstruction	*Myocardial value* 1	2	3	4	5	6	7	8	9	10
100	1	2	3	4	5	6	7	8	9	10
90-99	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0
75-89	0.6	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6.0
50-74	0.4	0.8	1.2	1.6	2.0	2.4	2.8	3.2	3.6	4.0
<50	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0

1. Left main coronary
2. Anterior descending
3. Septal branches
4. Diagonal branches
5. Left circumflex
6. Left atrial
7. Marginal branches

A. Right coronary
B. Right atrial
C. Right ventricular
D. Posterior descending
E. Left ventricular inferior branches

Figure 4.1 Angiogram scoring. Stenoses are assigned a *myocardial value* on the basis of their position, the importance of the area of the myocardium served, and the presence of any collateral pathways. The degree of stenosis is then graded according to cross sectional area loss and a *myocardial score* derived from the table.

4.2 Results

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4.2.1 Angiogram score and conventional risk markers

Angiogram scores ranged from 0 to 17 from a possible total of 36. The relationship of this score to some common risk factors is given in Tables 4.2 & 4.3. Of the non lipid factors only body mass index approached significance. Angiogram score was positively correlated with fasting serum and LDL cholesterol, and negatively with HDL cholesterol (Table 4.3). HDL_3, as measured in the analytical ultracentrifuge also showed a negative relationship. Fasting levels of triglyceride, whether in serum or in VLDL, were not significantly related to angiogram score.

Table 4.2 *Non lipid risk factors in CAD subjects*

	Mean ±SD	Correlation with angiogram score
Age (years)	50.1 ±7.3	0.06
Quetelet index (kg/m^2)	2.63 ±0.30	0.35*
Blood pressure (mmHg)		
Systolic	126.9 ±13.7	0.23
Diastolic	80.2 ±12.7	0.23
Smoking score	•	0.08
Family history score	•	-0.06

n=27 , *Mean scores not quoted, *P≤0.10

Table 4.3 *Fasting lipids in CAD subjects#*

mmol/l	Mean ±SD	Correlation with angiogram score
Serum cholesterol	6.73 ±1.35	0.45**
Serum triglyceride	2.29 ±0.87	0.18
VLDL triglyceride	1.27 ±0.57	0.17
LDL cholesterol	4.65 ±1.30	0.58**
HDL cholesterol	1.05 ±0.25	-0.37*
HDL_2 (mg/dl)	32.3 ±15.2	-0.22
HDL_3 "	236.0 ±46.9	-0.39*
Lipoprotein lipase (mU/ml)	154.2 ±57.2	0.20
Hepatic lipase (mU/ml)	¶122.5 ±49.4	0.30

n=27, ¶One mU = one nmol fatty acid released per minute at 25°C, *P≤0.05, **P≤0.005

Table 4.4 *The relationship of post prandial ρ<1.006 triglyceride and retinyl palmitate response to angiogram score*

		Δ						
ρ<1.006	Area	2	4	6	8	10	12	24 hours
Triglyceride (mmol/l)	18.3 ±13.4	0.80 ±0.67	1.92 ±1.10	2.46 ±1.81	1.89 ±1.93	1.45 ±1.44	1.22 ±1.18	-0.18 ±0.33
	0.32*	-0.12	0.17	0.42**	0.51***	0.25	0.16	-0.35**
Retinyl palmitate (μmol/l)	44.9 ±29.1	0.39 ±0.47	1.78 ±1.68	3.88 ±3.89	6.05 ±4.39	7.47 ±5.07	6.32 ±4.54	1.03 ±1.37
	0.05	-0.04	-0.08	-0.14	0.22	-0.03	0.11	0.56***

Correlation with angiogram score *P≤0.1, **P≤0.05, ***P≤0.01, n=27

4.2.2 Angiogram score and post prandial lipæmia

The profiles of triglyceride and RP in the CAD group are depicted in Figure 4.2. Although the correlation of ρ<1.006 triglyceride area with angiogram score was of only marginal significance (Table 4.4), Δ values revealed a greater correlation beyond the peak of triglyceridæmia, on the steepest part of the descending limb of the chylomicrograph (Δ6-Δ8). Table 4.5 shows that the area under the triglyceride curve was also more strongly related to peak and later Δ values than to those during the rise in triglyceridæmia.

RP rose and peaked later than triglyceride, and levels during lipæmia were unrelated to angiogram score (Table 4.4). RP was also unrelated to Δ triglyceride during the first 6 hours of lipæmia, but became increasingly related at 8, 10, 12, and 24 hours (Table 4.5).

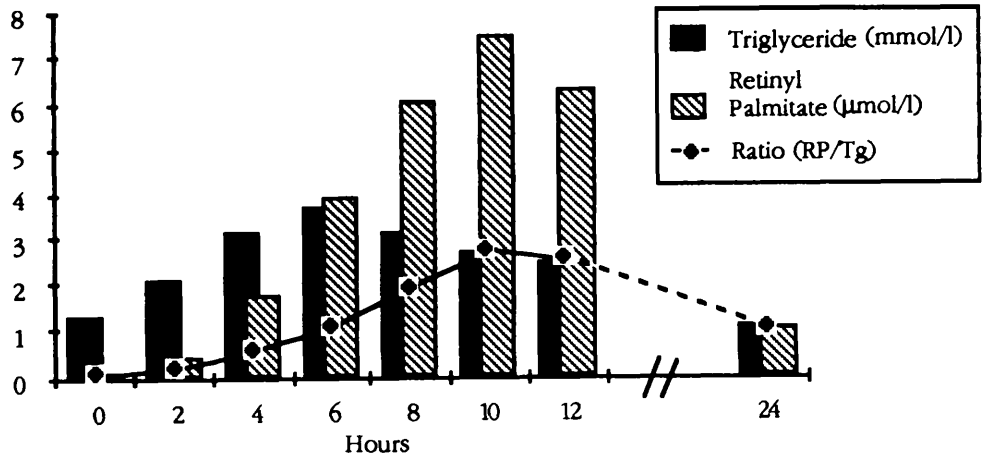

Figure 4.2 ρ<1.006 triglyceride and RP profiles in CAD subjects

Table 4.5 *Correlation between measures of post prandial response in CAD subjects*

a). ρ<1.006	Δ 2	4	6	8	10	12	24	Areas
RP & Triglyceride	0.14	0.16	0.03	0.35*	0.44**	0.48**	0.34	0.45**
b).	Triglyceride Δ							
Triglyceride Area	0.68***	0.76***	0.93***	0.83***	0.89***	0.84***	-0.10	
c).	Retinyl Palmitate Δ							
RP Area	0.29	0.59***	0.54**	0.67***	0.49**	0.46**	-0.10	

*P≤0.05, **P≤0.01, *** P≤0.001, n = 27

4.2.3 24 hours after fat ingestion

VLDL triglyceride fell by 14%, P≤0.02 at 24 hours. Serum triglyceride was also lowered (19%, P≤0.002), as were serum cholesterol (5%, P≤0.01), and phospholipid (8%, P≤0.025). The magnitude of Δ24 VLDL triglyceride was negatively related to angiogram score, and so a fall in VLDL triglyceride was more likely in those with a higher angiogram score.

RP remaining in the ρ<1.006 fraction 24 hours after the fat load was positively related to angiogram score (Table 4.4). Although RP was also detected in the ρ>1.006g/ml fraction at this time (0.47 ± 0.30μmol/l, mean ± SD), no relationship was observed between this amount and angiogram score. 24 hours after the fat load therefore, the VLDL fraction was relatively depleted in triglyceride, but enriched in RP to a degree that was related to angiogram score.

4.2.4 Predictors of angiogram score

Linear regression analysis was performed to determine the best predictors of angiogram score among the various parameters measured. Before this procedure was undertaken skewed data (fasting & post prandial triglycerides, RP, and HDL_2) were transformed to their natural logarithms. Assayed individually the best predictors of score were RP at 24 hours and LDL cholesterol (Table 4.6), although serum cholesterol and Δ8 hour triglyceride were also positive, and HDL_3 a negative predictor of angiogram score.

None of the non-lipid risk markers demonstrated significant ability to predict score, nor did HDL_2, HDL cholesterol, fasting triglyceride or any other of the postprandial responses.

Risk factors with significant R^2 values were then assayed in combination by multiple linear regression. In multiple regression models only LDL and RP at 24 hours retained significance, although when these two variables were included in the same model the significance of RP 24 hours was lost. In these equations the maximum R^2 obtained was 31.6% for the model containing Δ8 hour triglyceride plus LDL cholesterol. In models containing more than 2 parameters none retained significance.

Several of the parameters showing a relationship to angiogram score were themselves inter-related. LDL cholesterol was positively correlated with RP at 24 hours (r=0.59, P≤0.001),

Table 4.6 *Predictors of angiogram score*

Predictor	R2 (%)#
Univariate	
RP 24 hours¶***	19.8
LDL cholesterol***	19.3
Serum cholesterol**	15.7
HDL_3 (-ve)**	13.8
Δ8 hour ρ<1.006 triglyceride¶**	13.1
Multivariate	
LDL cholesterol** + Δ8 hour ρ<1.006 triglyceride	31.6
LDL cholesterol* + RP 24 hours	26.9
RP 24 hours** + Δ8 hour ρ<1.006 triglyceride	26.7
LDL cholesterol** + HDL_3	25.3
RP 24 hours** + HDL_3	21.0

Coefficient of determination corrected for degrees of freedom, ¶ After conversion to natural logarithms, *P≤0.05, **P≤0.025, ***P≤0.010

and HDL_3 was negatively related to Δ8 hour triglyceride (r=-0.49, P≤0.01). The correlations of post prandial triglycerides with non-lipid parameters and with fasting lipids are given in Tables 4.7 & 4.8. Lipæmia was negatively related to age and positively to systolic blood pressure, fasting VLDL triglyceride and LDL cholesterol. HDL cholesterol and HDL_2 were negatively related to lipæmia. The activity of LpL was not related to fasting lipoprotein levels, but HL activity was inversely related to HDL (HDL_2 r=-0.70, P≤0.01, & HDL_3 r=-0.44, P≤0.05). Neither LpL or HL showed significant relation to lipæmia.

Table 4.7 *Correlation of lipaemic response with non-lipid parameters in CAD subjects#*

ρ<1.006 triglyceride	Area	Δ 2	Δ 4	Δ 6	Δ 8	Δ 10	Δ 12	Δ 24	24 hour RP
Age	0.34**	-0.35**	-0.40***	-0.38**	-0.09	-0.22	-0.20	0.15	0.22
Quetelet index	0.02	-0.29	0.01	-0.04	0.13	0.18	-0.05	0.07	0.14
B.P.Systolic	0.41***	0.27*	0.37**	0.44***	0.22	0.32*	0.30*	-0.14	0.06
B.P. Diastolic	0.20	0.06	0.27	0.25	0.16	0.09	-0.04	-0.06	0.14
Smoking score	0.27	0.31*	0.15	0.34**	0.21	0.09	0.22	0.16	0.10
Family history	0.08	-0.15	0.15	0.06	0.17	0.08	0.06	0.19	0.39**

#n = 27, *P≤0.1, **P≤0.05, ***P≤0.025

Table 4.8 *Correlation of lipæmic response with fasting lipids in CAD subjects*[#]

	ρ<1.006 triglyceride	Δ							24 hour
Fasting	Area	2	4	6	8	10	12	24	RP
VLDL triglyceride	0.54**	0.36*	0.51**	0.67***	0.39*	0.40*	0.42**	-0.14	0.34
LDL cholesterol	0.36*	-0.14	0.39*	0.49**	0.46**	0.28	0.26	0.07	0.59***
HDL cholesterol	-0.48**	-0.13	-0.20	-0.45**	-0.62***	-0.47**	-0.41**	0.00	-0.43**
HDL_2	-0.60***	-0.27	-0.49**	-0.62***	-0.45**	-0.45**	-0.46**	0.15	-0.35*
HDL_3	-0.30	0.10	0.14	-0.22	-0.49**	-0.43**	-0.28	0.14	-0.32

[#]n=27, *P≤0.05, **P≤0.01, ***P≤0.001

4.2.5 The effects of ß blockade

Patients on ß blocking drug therapy were not excluded from the study. One volunteer was prescribed propranolol, three atenolol, five metoprolol and one was prescribed Trasidrex, a combination of ß blocker and thiazide diuretic. These drugs have been found to interfere with lipid metabolism and in particular they seem able to raise triglyceride and lower HDL cholesterol levels. Kruskall Wallis one way analysis of variance failed to support any significant influence of these drugs on angiogram score, total & LDL cholesterol, or fasting & post prandial triglycerides (Table 4.9). There was an indication that HDL levels were lower in those on ß blockade. In addition, although there was no statistically significant difference in RP levels at 24 hours between those taking ß blockers and those on no therapy, subjects taking metoprolol appeared to have greater amounts of RP at 24 hours. Elimination of the 6 subjects taking metoprolol from the statistical analyses however, did not reduce the correlation of 24 hour RP with angiogram score.

4.2.6 Sequential flotation analyses

In a subset of 8 patients lipoproteins were prepared by the sequential flotation technique as well as by ß quantification, providing a more detailed analysis of lipid fluctuations. These are summarized in Figures 4.3 to 4.6.

In these men the mean response was a rise in triglyceride and phospholipid in all lipoprotein classes. Cholesterol in the ρ<1.006 fraction was also raised. Cholesterol levels in the ρ>1.006 fraction (IDL, LDL, HDL) fell between 2 and 8 hours. (Free and esterified cholesterol were not separately measured in this part of the study).

Closer examination of Figure 4.6 reveals that Δ cholesterol in LDL and HDL were reciprocal rather than parallel during lipæmia, and in fact LDL and HDL cholesterol areas were correlated negatively ($r = -0.74$, $P < 0.05$), ie a greater fall in HDL cholesterol was associated with a lesser fall (or a rise), in LDL cholesterol.

The post prandial rise in HDL triglyceride tended to parallel that in the ρ<1.006 fraction. Δ LDL triglyceride however, was not related to other triglyceride fluctuations, but was related

Table 4.9 *The effects of ß blockers on angiogram score and risk factors*

Drug	n	Angiogram Score	Blood pressure Sys (mmHg)	Blood pressure Dias (mmHg)	Serum Cholesterol (mmol/l)	Serum Triglyceride (mmol/l)	VLDL Triglyceride (mmol/l)	LDL Cholesterol (mmol/l)	HDL Cholesterol (mmol/l)	HDL_2 (mg/dl)	HDL_3 (mg/dl)	ρ<1006 Triglyceride areas	Retinyl Palmitate
None	16	4.94 ±5.84	125.0 ±12.7	78.0 ±13.3	6.71 ±1.52	2.26 ±0.96	1.31 ±0.65	4.60 ±1.52	1.13 ±0.21	36.1 ±17.1	251.4 ±40.8	18.7 ±15.5	0.76 ±0.76
Propranolol #	1	3.00	120.0	80.0	6.05	2.62	1.36	4.39	0.62	22.0	180.0	17.0	0.50
Atenolol #	3	8.33 ±7.23	125.0 ±17.3	82.5 ±9.6	6.34 ±1.30	2.29 ±0.92	1.28 ±0.53	4.48 ±1.30	0.91 ±0.21	27.8 ±8.5	253.8 ±38.5	19.8 ±12.4	0.60 ±0.32
Metoprolol #	6	9.50 ±5.43	133.3 ±16.3	85.0 ±15.2	7.12 ±1.21	2.45 ±0.79	1.19 ±0.50	5.01 ±0.98	0.93 ±0.25	26.7 ±12.1	192.5 ±40.7	16.4 ±13.1	2.41 ±2.49
Trasidrex #	1	0.00	130.0	70.0	6.87	1.64	0.90	4.21	1.40**	32.3	236.1**	16.0	0.19
Total on drug treatment ¶	11	7.73 ±5.96	127.1 ±16.8	799.4 ±12.4	6.75 ±1.15	2.34 ±0.76	1.21 ±0.45	4.71 ±1.00	0.94* ±0.27	26.6 ±9.9	213.6** ±47.9	17.6 ±10.8	1.38 ±1.87

Kruskall Wallis one way analysis of variance, ¶ Unpaired t-test aginst no drug, *P≤0.1, **P≤0.05

positively to Δ LDL cholesterol in the later stages of lipæmia (Δ6 hours r = 0.86, P ≤ 0.01, and Δ8 hours r = 0.75, P ≤ 0.05), and at 24 hours (Δ24 hours r = 0.75, P ≤ 0.05).

24 hours after the fat load all components of VLDL, and the triglyceride content of HDL were lower than on the previous day (Figures 4.3 to 4.6). The fall in HDL triglyceride at 24 hours was negatively related to the magnitude of the previous day's lipæmia, (ΔHDL triglyceride and ρ<1.006 triglyceride area r=-0.77, P≤0.05) and also to the post prandial decline in HDL cholesterol (ΔHDL triglyceride and HDL cholesterol area r=0.72, P≤0.05). Increased post prandial triglyceridæmia is therefore associated with a greater decline in HDL cholesterol during lipæmia, and a greater decline in HDL triglyceride at 24 hours.

4.3 Discussion

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Fasting lipids and lipoproteins in this group illustrate the well characterised profile of the 'at risk' individual. Greatest risk was associated with serum and LDL cholesterol, with average levels in a range at which risk increases steeply.[32] In addition the rather low average levels of HDL cholesterol[212] were a negative risk marker, by now a well established finding.[213]

Notably however, although mean fasting triglyceride also fell into a range meriting dietary advice,[38] individual levels did not show any direct relationship to CAD severity. The controversy surrounding the role of triglyceride in the pathogenesis of atherosclerosis was discussed in section 1.3.3. When presented with the challenge of an oral fat load the resultant triglyceridæmia in these subjects was a positive indicator of angiogram score.

In agreement with earlier studies[50,51] the greatest predictive power of the chylomicrograph, and the majority of the influence on overall post prandial triglyceridæmia, coincides with a time when *"the elimination of fat is greater than the addition, and the steepness of the curve indicates the relative excess of the reduction."* In other words risk is associated with a slower clearance of triglyceridæmia.

An obvious explanation for this would be reduced activity of LpL as a feature of CAD. Although not confirmed by this study, LpL has been reported by others to have an inverse relationship to alimentary lipæmia.[200] The intravenous fat tolerance test, using radio labelled synthetic emulsions to measure lipolysis rates *in vivo*, can discriminate between CAD positive and negative populations.[214]

The data do not exclude the possibility that re-cycling of dietary triglyceride into hepatic VLDL occurs to a greater extent in CAD. The possibility of this pathway was mentioned in the previous chapter. Earlier studies[51,184] concluded that exaggerated lipæmia in CAD was the result of a high initial fasting VLDL, leading to competition for triglyceride removal. Such a mechanism was postulated to explain the positive relationship commonly observed between fasting VLDL and post prandial lipæmia. Fasting VLDL triglyceride was not itself related to angiogram score. Nevertheless an increase in VLDL production during lipæmia would place

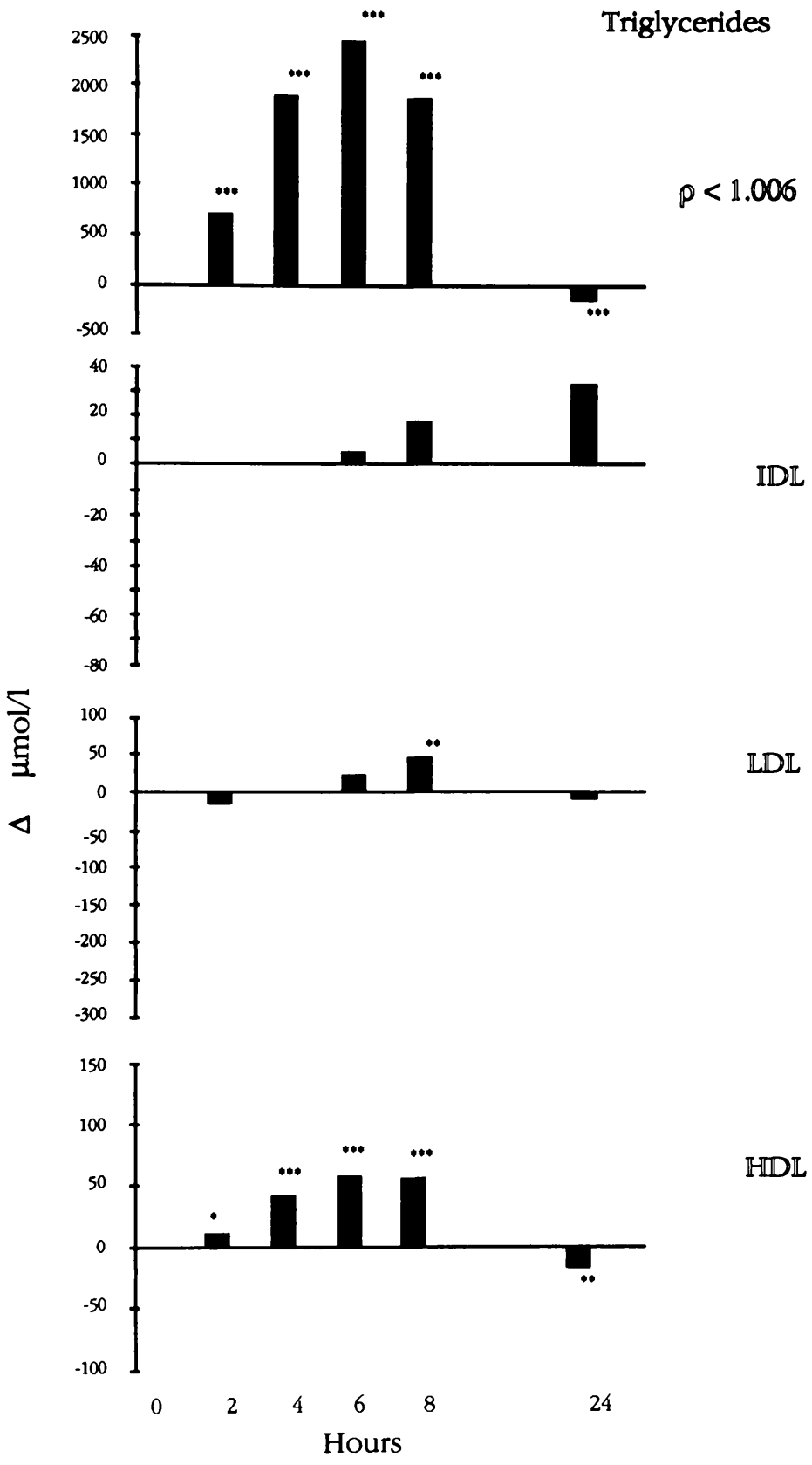

Figure 4.3 Changes in lipoprotein triglycerides in CAD subjects. Zero marks fasting levels and bars represent mean change from fasting, *P≤0.1, **P≤0.05, ***P≤0.01, n=8.

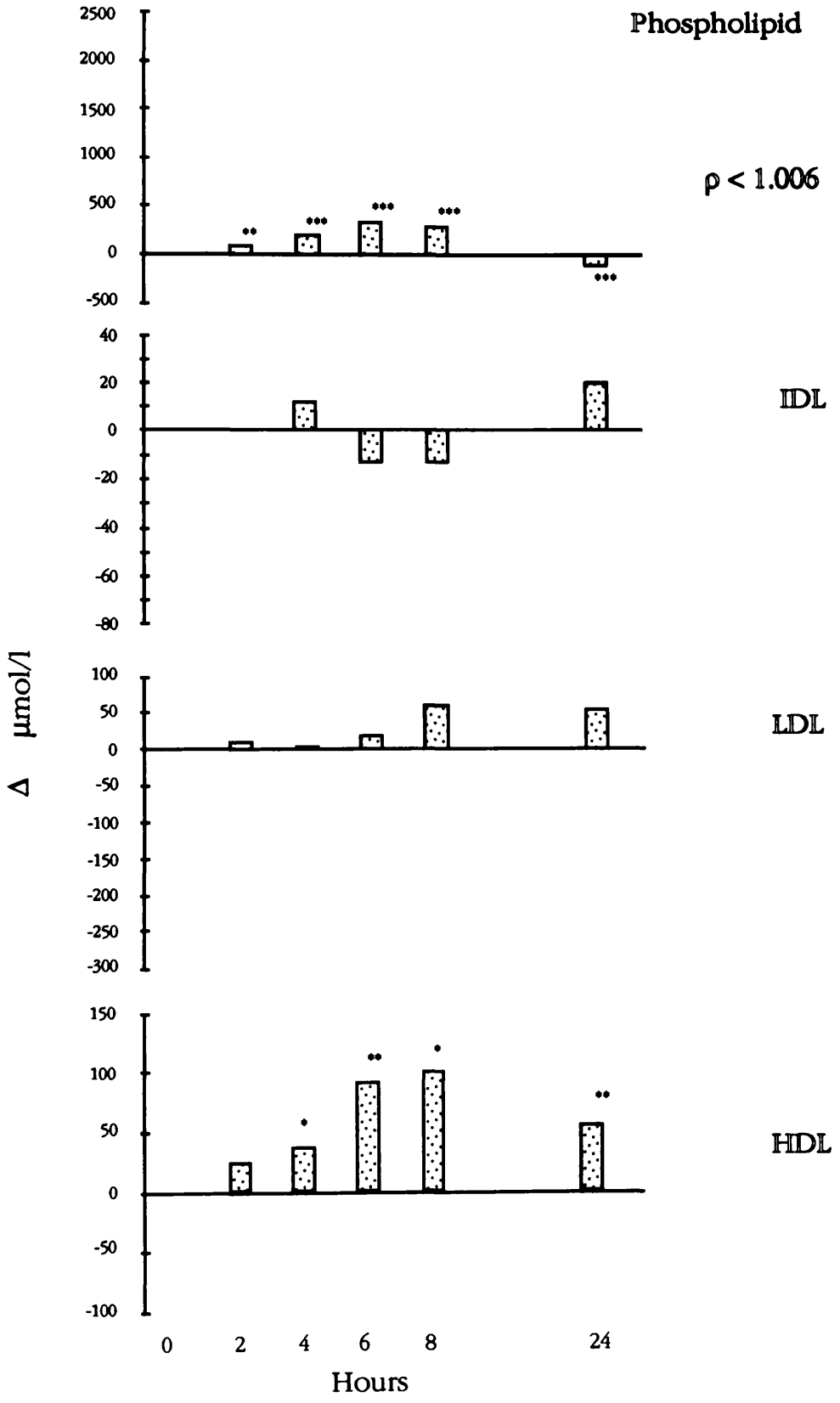

Figure 4.4 Changes in lipoprotein phospholipid in CAD subjects. Zero marks fasting levels and bars represent mean change from fasting, *P≤0.1, **P≤0.05, ***P≤0.01, n=8.

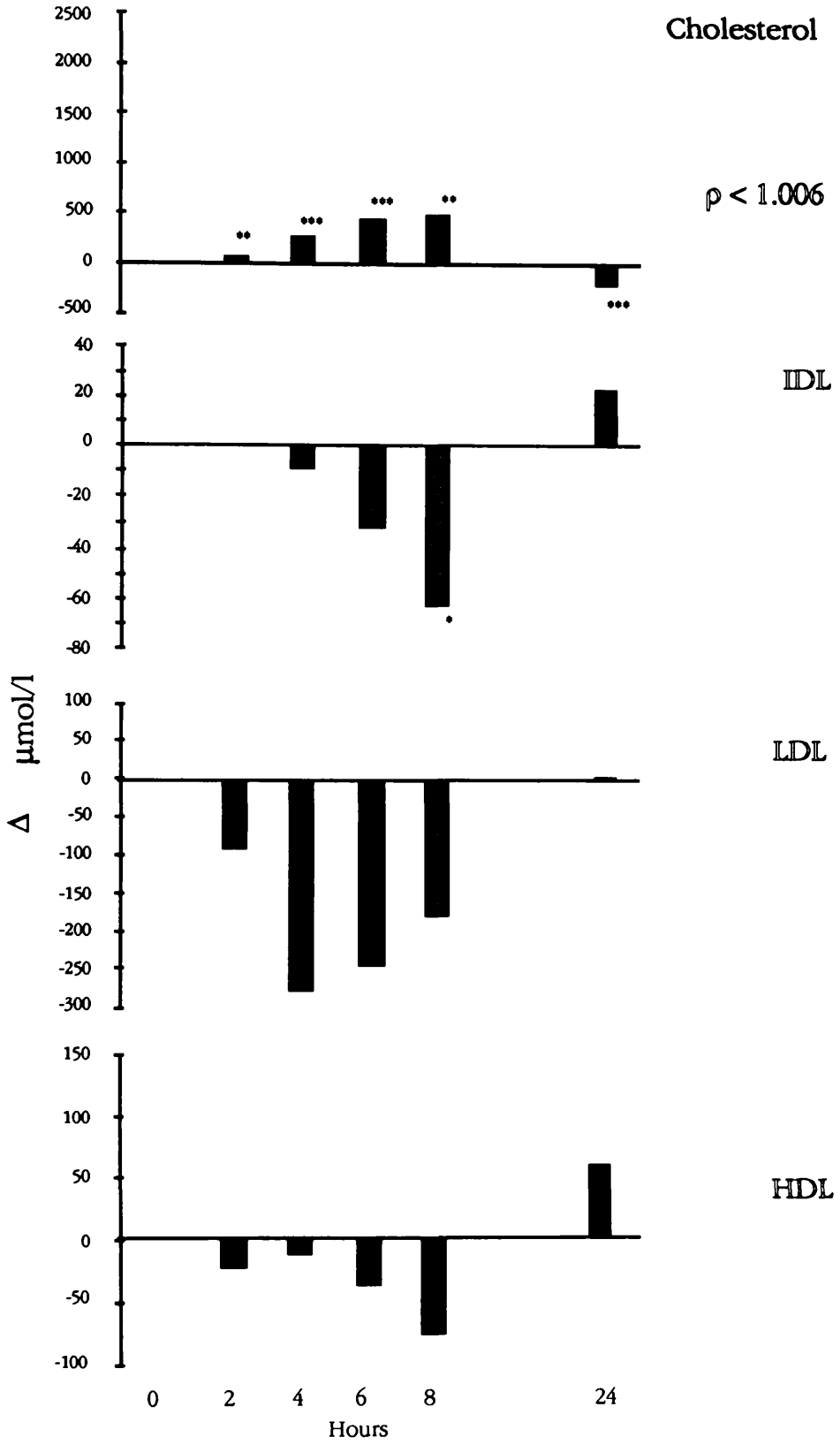

Figure 4.5 Changes in lipoprotein cholesterol (total) in CAD subjects. Zero marks fasting levels and bars represent mean change from fasting, *P≤0.1, **P≤0.05, ***P≤0.01, n=8.

increased pressure on the lipolytic system,invoking greater competition for lipolytic sites. Whether or not this became excessive would depend on the stringency with which VLDL production rates are controlled during periods of high substrate availability. A more labile responsiveness to fatty acid influx may occasion a greater output of recyled triglyceride in VLDL.

A poorly regulated responsiveness in VLDL production could also explain the phenomenon of a fat load induced over compensation in fasting levels of VLDL at 24 hours. This was also found to be related to the severity of CAD in these subjects. The reduction in serum cholesterol and triglyceride observed 24 hours after the fat load may also be reconciled with this model if a temporary lowering of VLDL synthesis reduces the entry of hepatic lipid to the circulation.

An alternative possibility is that triglycerides remaining at this time are associated with an increased number of chylomicron remnants which are relatively resistant to further lipolysis. Evidence for this comes from the increasing correlation between post prandial triglycerides and RP in the later stages of lipæmia.

RP was added to the fat load to trace remnant clearance. During lipæmia individual levels of RP were not significantly correlated with angiogram scores. In a study based on paired comparisons however, Krauss *et al*[215] found an increasing divergence between CAD positive and negative populations at 10, 12, and 14 hours after a fat load. In the present study the amount of RP remaining in the $\rho<1.006$ fraction at 24 hours was the single most powerful predictor of angiogram score. The simplest explanation for this is that chylomicron remnants are cleared more slowly in CAD.

As a risk marker the ability of RP 24 hours to predict score was greater than that of LDL cholesterol, although when combined in a multiple regression model only LDL cholesterol remained significant.

The clearance of both LDL and chylomicron remnants from the circulation are both accomplished by a receptor mediated process, although different receptors are believed to be involved in each case. It now seems however that the scope of the apo B/E receptor extends beyond strict affinity for LDL alone,[64,216] and has been implicated in the clearance of VLDL remnants from the *Sf*20-60 density range.[216] In view of the present uncertainties concerning the identity of the chylomicron remnant receptor, the positive relationship between LDL and remnants may feasibly have its basis in either an element of co-regulation of LDL and remnant receptors, or a degree of competition for uptake by the same receptor.

The inverse relationship between HDL and Δ triglyceride is not surprising in view of the dynamic processes linking VLDL lipolysis and HDL production.[45] The influx of post prandial triglyceride penetrated all lipoprotein classes during lipæmia, and was accompanied by a shift of cholesterol from lipoproteins of density less than 1.006g/ml to those of density greater than 1.006g/ml This pattern of events has been well described for HDL,[217] and under the

direction of the LTP is thought to lead to a redistribution of LCAT derived cholesteryl esters from HDL to $\rho<1.006$ lipoproteins, a process considered to have atherogenic potential. A concomitant enrichment of HDL with triglyceride and renders it susceptible to hydrolysis by HL.[200] A negative correlation between HDL and HL activity has been reported previously.[218] By virtue of both triglyceride and phospholipase activity HL may play a major part in the conversion of HDL_2 to HDL_3 and in the regulation of HDL levels.

The position of LDL and IDL in this scheme is less clear. The flux of phospholipid into LDL was not significant, and the changes in LDL were not so obviously related to lipæmia as were those in HDL.

It was of interest to note the reciprocal relationship between the flux of cholesterol in LDL and HDL, and also the positive association between Δ triglyceride and cholesterol in LDL. Clearly a simple exchange of LDL core lipids does not occur. Musliner *et al* have reported the formation of VLDL/LDL complexes during lipolysis,[219] and that complex formation can be inhibited by HDL.[220] Since it seems that binding of lipoproteins in a complex with LTP is an integral part of the transfer process it is likely that the relative amounts of these lipoproteins will affect its direction and efficiency.

In addition, if exaggerated lipæmia were to lead to a greater loss of cholesteryl ester from HDL, and if an increased amount were transferred to VLDL as well as to chylomicron remnants, then, since VLDL are catabolised to LDL, more cholesteryl ester would find its way into LDL. Such a process could conceivably lead to a reciprocal relation between the change in HDL and LDL cholesteryl esters. The suggestion, discussed above, that VLDL production may be affected by lipæmia complicates the picture even further, and may amplify the reciprocal effect.

In summary, the features of alimentary lipæmia which marked CAD were an exaggerated triglyceridæmia, particularly in the later stages, an indication that VLDL production was more easily affected by acute dietary intake of fat, and evidence for a persistence of chylomicron remnants. In the small number of subjects in whom more detailed information was available, no relationship of angiogram score to lipid flux in lipoproteins of density greater than 1.006g/ml was found.

Chapter 5

Acute dietary effects in alimentary lipæmia

5.1 Introduction and Methods

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A dietary component attached to both the susceptibility to atherosclerosis, and to the regulation of lipoprotein metabolism is well recognised. In individuals identified as 'at risk' by virtue of elevated plasma lipids the first line of therapy is always dietary advice, even if drugs are eventually also required.

Dietary recommendations concentrate initially on a reduction of total caloric intake and the correction of obesity, which may in itself help to alleviate any hyperlipidæmia. These recommendations however also pertain to specific dietary constituents, namely the amount of cholesterol and the amounts and types of fat consumed.[38]

Although the role of dietary cholesterol and fat has been a subject of great interest for many years, most studies have concentrated on longer term effects on fasting lipoproteins and cholesterol homeostasis. Relatively little information is available on the acute effects on post prandial lipoproteins of altering the composition of the diet. In this section the effects of adjusting the cholesterol content and the fatty acid composition of the fat tolerance test were studied.

5.1.1 The effects of increasing the cholesterol content of the fat load

It has been proposed that the inclusion of cholesterol in a fatty meal may be essential to the formation of cholesterol enriched remnants during the post prandial phase.[61] In rabbits and several other animal species fat feeding alone produces only chylomicronæmia, whereas the inclusion of cholesterol leads to high levels of cholesterol rich remnants. On this evidence Zilversmit suggested a fat-plus-cholesterol tolerance test for studies of remnant metabolism. To pursue this suggestion further the cholesterol content of the standard fat load was raised and post prandial lipoprotein responses assessed as before.

500mg of cholesterol were added to the standard fat load bringing the total cholesterol content to 900mg. This was administered to a group of 8 male volunteers, and post prandial responses compared to those obtained when the original standard fat load was administered to the same subjects. Standard and cholesterol enriched meals were administered in random order, with an interval of 2 weeks between.

5.1.2 The effects of fatty acid saturation on alimentary lipæmia

The physical and chemical characteristics of dietary triglycerides are highly dependent on the nature of their component fatty acids. These characteristics are in turn dependent on the number of carbon atoms in the fatty acid, and on the presence and positioning of double bonds along the carbon chain.

Fatty acids with no double bonds are referred to as *saturated*. These molecules are readily synthesized endogenously from acetate by animals, and dietary saturated fats are mostly derived from animal tissue. Increasing amounts of saturated fats in the diet raises plasma cholesterol, and is positively associated with CHD.[31]

The inclusion of double bonds between the carbon atoms of fatty acids eliminates their hyper-cholesterolæmic effect and the positive association with heart disease is no longer seen. Fatty acids containing double bonds are termed *unsaturated*. Mono-unsaturates have only one double bond. The most common example of these is oleic acid which has 18 carbon atoms and one double bond (18:1), positioned at the 9th carbon from the methyl terminal (ω9).

Fatty acids with two or more double bonds are poly-unsaturated (PUS). PUS fatty acids with a double bond at the ω6 position cannot be synthesized by animals, and since they are biologically essential they must be obtained from plant materials in the diet. Linoleic acid (18:2 ω6) is the most common representative of this type of fat.

Replacement of dietary saturated fats with ω6 PUS has been found to *lower* plasma cholesterol,[221] and low tissue levels of linoleic acid seem to be associated with an increased risk for CHD.[222]

A further series of PUS fatty acids have the first double bond at the ω3 position. These are also essential in the diet since, although they are required for several important biological functions, they cannot be synthesized by terrestrial animals and are derived ultimately from marine plankton. They enter the human diet through the consumption of fish. ω3 fatty acids have also been found to influence lipoprotein metabolism, particularly by reducing the amounts of triglyceride rich lipoproteins,[223] and have also been associated with a reduction in cardiovascular risk.[224]

As with manipulations of dietary cholesterol, studies on the influence of fatty acid saturation have tended to concentrate on fasting lipoproteins, and few have examined their effects on post prandial lipid flux. This part of the study was undertaken to examine the

effects of replacing a proportion of the saturated fats in the fat tolerance test with unsaturated fat.

7 males consumed two fat loads of differing fatty acid composition. The standard fat load, described in section 2.3.1 derived its fat from dairy cream, and was designated the 'saturated' fat load. An 'unsaturated' meal took the form of a delicious mayonnaise in which the major source of fat was safflowerseed oil. This meal was made according to the following recipe.

30g of egg yolk, 10g of caloreen, 20ml of vinegar and a quarter teaspoon of suet were placed in a blender. With the blender at full speed 125ml of safflowerseed oil was poured in slowly. This mixture was consumed with 200g of potatoes and one small white bread roll. 80ml of natural yoghurt was eaten separately. A comparison of the composition of the two meals is given in Table 5.1. The combined carotenoid and vitamin A contents of the meals was 2mg for the saturated and 0.2mg for the unsaturated load,[225] plus 165mg added as described earlier. It was noted that most subjects had more difficulty in consuming the unsaturated meal, although all managed to complete both and none suffered any serious discomfort as a result.

Table 5.1 *The composition of oral fat loads*

	Saturated		Unsaturated
Protein	8.8g		8.8g
Carbohydrate	31.0g		30.0g
Fat	135.0g		135.0g
		%	
	58	saturated	12
	38	mono-	20
	4	poly-	68
		unsaturated	
Cholesterol	394.0mg		384.0mg
Retinyl esters	167.0mg		165.2mg
Calories	1371		1368

5.1.3 *In vitro* lipolysis of lipoproteins of differing fatty acid composition

In an extension to the above experiment the susceptibility to lipolysis of $\rho<1.006$ lipoproteins generated after consumption of the different fat loads was examined. This section of the study was done in collaboration with Mr Keith Dyson, a medical student conducting a project for honours biochemistry

The enzyme lipoprotein lipase is responsible for the hydrolysis of triglycerides in triglyceride rich lipoproteins, and its activity is one of the major determinants of lipæmia. This enzyme has not generally been found to have a preference for any particular class of fatty acids, although the experiments performed to examine specificity have mostly used synthetic substrates, either *in vitro*, or given as a bolus injection intravenously.

The true biological substrates of this enzyme however are endogenously synthesized particles whose fine structure is determined by physiological as well as physical factors, not easily reproduced *in vitro*. To further investigate the influence of dietary fatty acid saturation

on the process of lipolysis, the activity of a partially purified bovine milk LpL was assayed *in vitro* with naturally synthesized post prandial triglyceride rich lipoprotein substrates, prepared *in vivo*.

In outline the protocol for this experiment was as follows. Fat loads of differing fatty acid compositions as described above, were administered to a group of 9 healthy male volunteers. 5 hours after consumption of this meal a blood sample was withdrawn and $\rho<1.006$ lipoproteins prepared by ultracentrifugation. These lipoproteins were then labelled with radioactive triolein and incubated with a bovine milk LpL preparation and the extent of hydrolysis compared with the fatty acid composition of the substrate as determined by gas chromatography (section 2.4.7).

5.2 Results

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5.2.1 The effects of increasing the cholesterol content of the fat load

Figure 5.1 illustrates $\rho<1.006$ cholesterol fluctuations in this group. The inclusion of an additional 500mg of cholesterol in the fat load produced no systematic or statistically significant changes in the post prandial responses of the 8 male subjects studied.

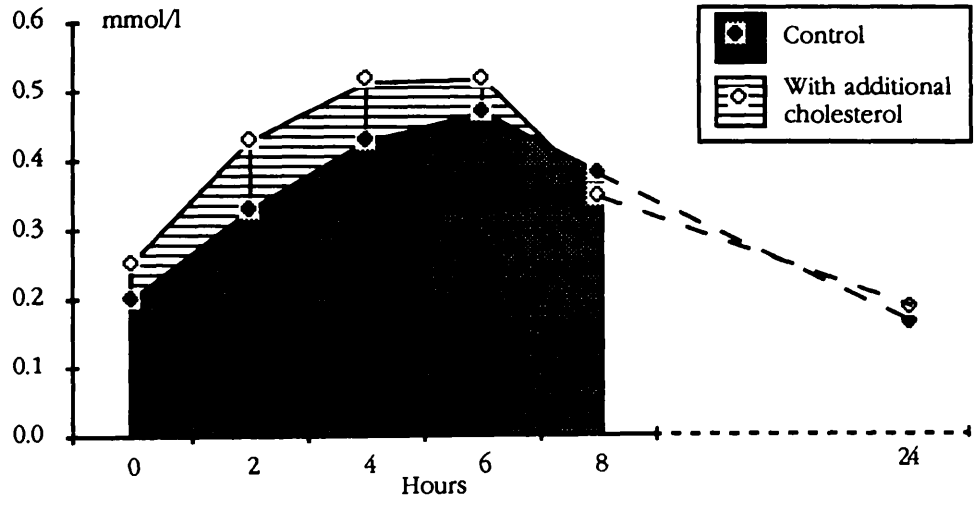

Figure 5.1 The effects of additional dietary cholesterol on $\rho<1.006$ lipoprotein cholesterol content. The control load contained 500mg of cholesterol and the test load contained 900mg. The amount of cholesterol passing through the triglyceride rich fraction was not significantly different in either case.

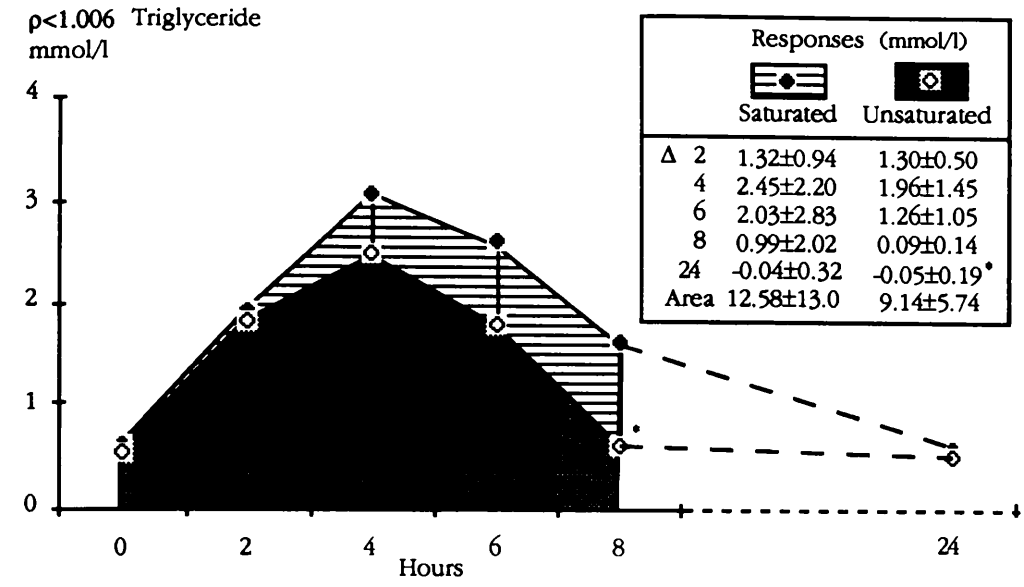

Figure 5.2 ρ<1.006 triglyceride after fat loads of differing fatty acid composition. *P≤0.05, n=7males.

5.2.2 The effects of altering the fatty acid saturation of the fat load

Post prandial responses: Mean ρ<1.006 triglyceride profiles for the saturated and unsaturated fat loads are given in Figure 5.2. Responses to the two types of fat diverged during the later stages of lipæmia, although only achieving statistical significance at 8 hours. ρ<1.006 triglyceride areas were not significantly different. Retinyl palmitate responses however differed markedly in the two fat load tests (Figure 5.3). Replacement of saturated fat by unsaturated resulted in an RP profile which rose earlier, to greater peak levels, and started to decline sooner. There were no observable differences in the lipæmic responses in the other lipoprotein classes.

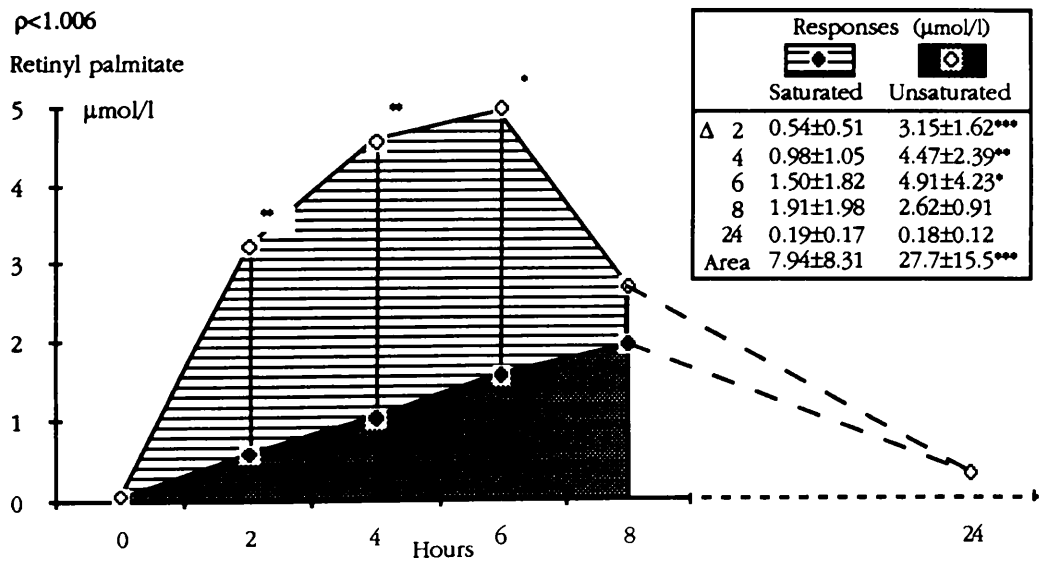

Figure 5.3 The flux of retinyl palmitate was greater when consumed with the unsaturated compared with the saturated fat load, *P≤0.02, **P≤0.002, ***P≤0.001, n=7males.

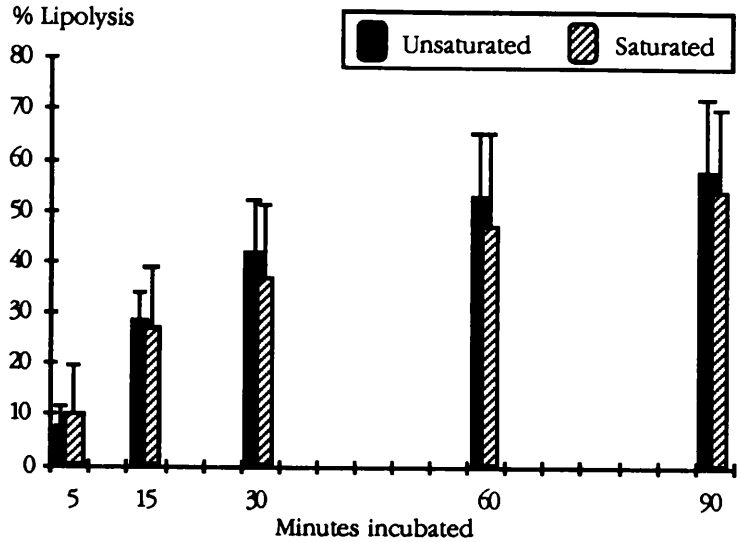

Figure 5.4 Chylomicrons formed after saturated and unsaturated fat loads were isolated at 5 hours post prandially, labelled with tritiated triolein and incubated with partially purified bovine milk LpL. The extent of lipolysis was estimated as liberated free fatty acid bound tritium. 9 subjects consumed both fat loads. Error bars are standard deviations. No significant differences were found.

In vitro lipolysis: Radioactive free fatty acids liberated during lipolysis were extracted, and lipolytic activity expressed as a percentage of total added radioactivity. The mean percent hydrolysis from 9 subjects are plotted in Figure 5.4. Paired comparisons of these values failed to reveal any differences in the extent of hydrolysis of either substrate.The fatty acid compositions of fat loads and $\rho<1.006$ lipoprotein substrates are summarised in Figure 5.5. Some general points can be made from this data.

Firstly, within a specified fat load the fatty acid compositions of triglycerides and phospholipids were broadly similar. It can be seen however that consumption of a fatty meal does not give rise to lipoproteins of identical fatty acid composition to the meal fed. Analysis of lipid classes reveals that triglyceride fatty acids *do* resemble those of the fat load, therefore triglyceride fatty acids are noticeably different in each of the two groups of substrate.

Phospholipid compositions however did not change with the fat load to the same extent. They differed considerably from the composition of the fatty meal and were similar in the two substrates. Fat loads therefore acutely affected the fatty acid composition of triglycerides in $\rho<1.006$ lipoproteins, but did not influence phospholipid fatty acids so markedly. These alterations did not significantly alter their susceptibility to lipolysis *in vitro* by a purified bovine milk LpL. There was a negative correlation between the percentage of 16:0 in substrate phospholipid & % lipolysis (-0.83, $P\leq0.005$), and a positive correlation between the percentage of 18:2 & %lipolysis (0.76, $P\leq0.02$).#

Results quoted for 5 minute samples also held at other time points.

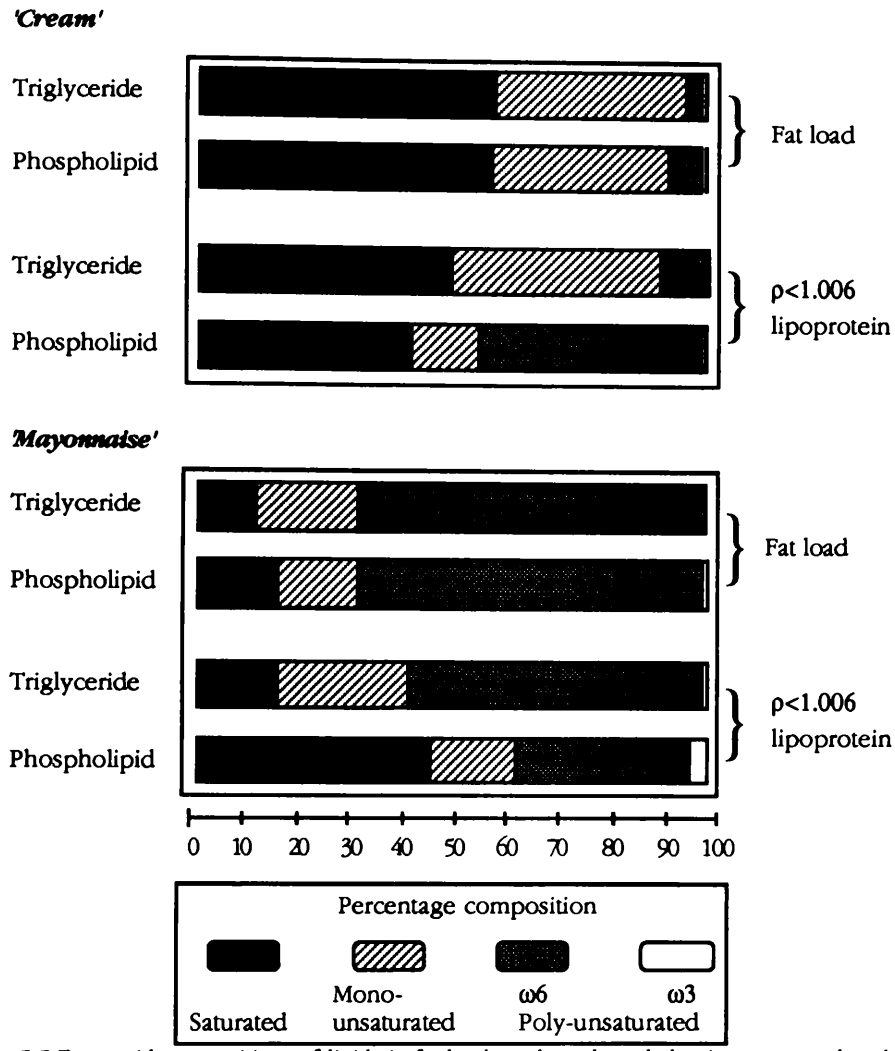

Figure 5.5 Fatty acid compositions of lipids in fat loads and product chylomicrons as analysed by TLC. Lipoprotein compositions are the mean of 9 preparations.

Discussion

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5.3 The cholesterol content of a fat load

Supplementation of the diets of certain animals with cholesterol has been found to lead to marked hypercholesterolæmia as well as to the formation of abnormal lipoprotein species.[54] Although controlled metabolic ward studies indicate that increased dietary cholesterol can raise plasma cholesterol in man, the effect is not as great as that found in experimental animals.[226] An increase of 500mg of cholesterol in the fat load did not materially alter the magnitude or direction of post prandial cholesterol flux in the present experiment.

It was hypothesized that additional dietary cholesterol, by expanding the cholesterol content of newly secreted chylomicrons, might enhance the formation of cholesteryl ester enriched remnants in plasma. Several factors probably contributed to an attenuation of the impact of additional cholesterol on post prandial lipoproteins.

Firstly, much of the cholesterol absorbed from the gut is derived from endogenous sources (bile and gut endothelial cell debris). Thus while 400-500mg is the average daily intake of dietary cholesterol, a further 800-1200mg per day enters the intestine in bile.[227] Therefore increasing the cholesterol load by 125% (from 400mg to 900mg), may effectively increase the amount of cholesterol traversing the gut by only 35%.

Secondly, cholesterol absorption from the gut is not quantitative. Estimates vary widely between 25 - 75%.[226,227] Therefore of the 35% extra passing through the gut only a rather poorly defined proportion will be absorbed and enter the bloodstream.

Thirdly, nascent chylomicrons seem to contain considerably less cholesterol than their plasma counterparts, most being acquired by transfer from cell membranes and other lipoproteins upon entering the plasma compartment.[21] The proportion of chylomicron cholesterol derived from newly absorbed material may therefore be relatively small, and increasing this amount by a small percentage will make minimal impact on the total cholesterol content of this plasma fraction.

A clearer determination of the significance of the cholesterol content of a fat load in post prandial lipoprotein metabolism will require more sensitive techniques than those applied here.

5.3.2 The fatty acid saturation of the fat load

A reduction in the degree of saturation of the fat load reduced the triglyceridæmic response only marginally. Two similar studies,[228,229] published recently, also found that alteration of the fatty acid composition of a fat load has, by itself, little effect on lipæmia *when administered against a background diet which is relatively rich in saturated fats.* The importance of the chronically fed fat was emphasized by both of these studies.

The free living Scottish diet, against which the the present study was conducted, has a P/S ratio in the region of 0.3,[230] which lies between the experimental 'saturated' diets instituted by Harris *et al* (0.5), and Weintraub *et al* (0.07)*. Therefore the habitual diets of these subjects may have minimised any differences in lipæmia induced by changing acutely the composition of the fat load.

Nevertheless there was a reduction in triglyceridæmia biased towards later Δ values. This suggests a more efficient clearance of un-saturated fat loads, and led to the hypothesis that chylomicrons formed after a saturated fat load may be more susceptible to lipolysis by LpL than those formed after an unsaturated load. Studies on the fatty acid specificity of LpL, have however yielded contradictory results. It seems in general that when synthetic substrates are examined LpL exhibits a preference for short chain, unsaturated fatty acids at the 1 or 3

* The potential role of mono-unsaturated fats should be borne in mind when comparing the results of these studies. Whereas the 'saturated' loads had reasonably similar P/S ratios in both studies (0.07), Weintraub *et al* specifically examined the effects of replacing saturated with poly-unsaturated fat, whereas the present study explored the effects of reducing the saturated fat content. Therefore the relative contributions of mono and poly unsaturated fats were not considered.

position of triglycerides,[231,232] but when lipoprotein substrates are assayed preferences are not so evident.[233-235]

Although synthetic emulsions can be useful for assaying lipolytic activity (eg in the intravenous fat tolerance test), they cannot be expected to reproduce the subtle interaction of components which guide the metabolism of lipoproteins *in vivo*. For this reason we chose to manufacture chylomicrons *in vivo* by administration of different fat loads. By this means they were endowed with the full complement of apoproteins and co-factors, in physiological combination with naturally derived lipid components endogenous to the individuals studied, and so reflecting the sum of the processes of chylomicron formation and transport in their response to differing fat loads. These were then labelled with ^{14}C trioleate and incubated with LpL.

The purpose of the incubation therefore was not to test the specificity of LpL for particular fatty acids, but to assay the susceptibility to lipolytic attack of chylomicrons whose bulk composition was determined by oral intake of different fats. No major differences were found.

This finding might appear to contradict the *in vitro* lipolysis data of Weintraub *et al*[28] who performed a similar experiment and found greater overall fatty acid release when fat loads were poly-unsaturated. They found this to be an acute effect, not dependent on the nature of the long term diet, and suggest that it may be due to a greater susceptibility of poly-unsaturated triglyceride to LpL activity. The conflicting evidence regarding this possibility has been mentioned above, and these authors themselves concede that it is contrary to their finding of no difference in the clearance of saturated and poly-unsaturated fat loads while on a saturated fat diet.

Differences in the methodologies employed by these studies may provide a clue to these apparently contradictory results, and may also help to explain the differing chronic and acute dietary effects on alimentary lipæmia. The rate of release of fatty acid during lipolysis, as assayed by Weintraub, depends not only on the reaction between LpL and triglyceride, but also on the rate of the preceding interaction between the enzyme and the chylomicron surface. It is the combined influence of the multiple interactions at this earlier stage, and the effects of dietary variations on them, which were assayed in the present study.

Therefore while poly-unsaturated triglycerides may be preferred by LpL, if chylomicrons differ *only* in their triglyceride composition then the initial interactions between enzyme and substrate, which are dependent on surface considerations, may not differ, and the overall impact on lipæmia may be reduced. The importance of the fatty acid composition of phospholipid was underlined by the correlations observed between %18:2, %16:0, and %lipolysis. Their influence on lipolytic rates has been demonstrated previously.[236-238] Emulsions carrying saturated phosphatidyl cholines seem to be poor substrates for LpL compared with those containing mixed chains (egg yolk)[238]

Although phospholipid fatty acid composition is readily altered by dietary glycerides, yet metabolic controls on phospholipid composition seem to be more stringent than those on triglyceride,[239] and they are therefore more resistant to acute dietary changes.[84] This may be due to a quantitatively greater endogenous contribution to chylomicron phospholipids.[69]

When acute effects alone are investigated *in vitro,* then any differences will be due mostly to triglyceride preferences. If the end point of the assay is the release of the same fatty acid from both species of chylomicron (ie radio-oleic acid in the present study) then this difference will be obscured, whereas a measure of the total release of fatty acid (as in reference 228) would be expected to allow differences to be more easily detected.

When the effects of chronic feeding are investigated *in vitro* the influence of surface composition will be more evident. Such changes may be more apparent when assaying with a single labelled triglyceride, but not so when triglyceride release is the end point.

An alternative explanation for improved clearance after a poly-unsaturated meal would be the swifter removal of remnants. Evidence for this should have come from the RP data. However the increased response of RP after an unsaturated load is difficult to explain, and contrasts with other reports.[228] Dietary fatty acid composition has not been found to affect RP absorption.[228,240] It is possible that the different format of the mayonnaise (semi-solid as opposed to liquid) affected digestion and absorption of RP.

Chapter 6

Lipid lowering drugs in alimentary lipæmia

6.1 Introduction and Methods

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Pharmacologic intervention is used as a second line of treatment when dietary measures fail to correct plasma lipid abnormalities. At least two large studies have shown that such intervention not only improves the lipid profile, but can lower the risk of suffering from CHD.[33,35] Although the main use of such drugs is obviously in the correction of lipid abnormalities, their biochemical effects are now sufficiently well described to allow their use as probes of lipoprotein metabolism. Thus in this part of the study two classes of lipid lowering drugs with known hypo-triglyceridæmic potential were administered to normal volunteers in order to further elucidate the nature of post prandial lipæmia.

Eight males, mean age 29.4 ± 3.7 years, took 300mg of fenofibrate (isopropyl 2-[p-(p-chlorobenzoyl) phenoxyl-2-methyl propionate) (Laboratoire Fournier) per day as three 100mg tablets. A separate group of eight males, mean age 34.3 ± 9.5 years were given 3g of nicotinic acid (Napp Laboratories) in the form of two 500mg tablets 3 times per day. In each of these studies fasting & post prandial lipoproteins and lipase activities were assayed before, and again six weeks after embarking on the course of drug treatment. Subjects were asked not to alter their dietary or exercising habits for those six weeks.

6.2 Results

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6.2.1 Fenofibrate

Six weeks of treatment with fenofibrate reduced serum cholesterol from 5.00±0.63 to 4.29±0.59 mmol/l (↓14%, P≤0.005), and fasting triglyceride from 1.29±0.68 to 0.88±0.31 mmol/l (↓32%, P≤0.05). The fasting lipoprotein profile in Table 6.1 shows a reduction in the mass of apo B containing lipoproteins, and an increase in HDL_3. Post heparin LpL activity was increased from 196.4±67.0 to 268.1±77.0 mU (↑36%, P≤0.05), but HL activity was not changed.

Table 6.1 *Fasting lipoproteins after fenofibrate treatment*

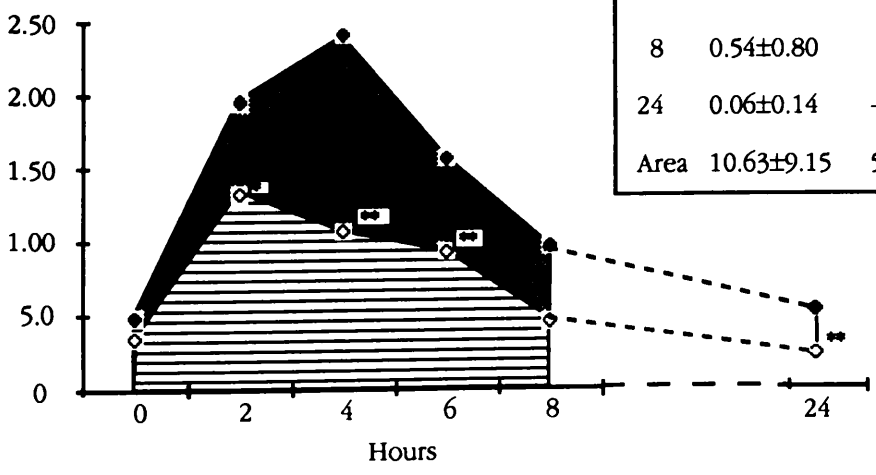
	VLDL	IDL	LDL	HDL_2	HDL_3
			(mg/dl)		
Control	80.7 ±54.6	18.7 ±14.4	252.5 ±55.3	68.8 ±31.3	228.1 ±30.0
Fenofibrate	59.0 ±29.7	11.9 ±5.7	195.9 ±40.9	56.6 ±35.6	271.4 ±33.0
	↓27%*	NS	↓22.4%**	NS	↑19%**

Significance of changes in total lipoprotein mass following 6 weeks treatment with fenofibrate. * P≤0.1, **P≤0.01, NS = not significant. n=8 males.

The fall in fasting VLDL mass was of only marginal significance, and in VLDL triglyceride (0.55±0.42 to 0.39±0.21mmol/l), it was not statistically significant. The effects on post prandial p<1.006 triglyceride were more pronounced. The overall response to the fat load was reduced by 50% (Figure 6.1), affecting particularly Δ4 to Δ6. RP levels on the other hand were if anything increased by fenofibrate treatment (Figure 6.2).

Among the associated post prandial changes in lipoprotein lipids the magnitude of the decline in HDL cholesteryl ester during lipæmia was virtually eliminated for the group as a whole (HDL cholesteryl ester areas -33.1±34.0 to 0.4 ±23.1, P≤0.05).

Figure 6.1 Triglycerides in the ρ≤1.006 fraction after 6 weeks on fenofibrate. Fasting levels were not significantly less but the post prandial response was reduced. *P≤0.1, **P≤0.05, n=8 males.



	Post prandial response	
	Control	Fenofibrate
Δ		
2	1.66±1.58	1.11±1.09**
4	2.18±1.62	0.81±0.31*
6	1.21±1.16	0.66±0.67**
8	0.54±0.80	0.11±0.29*
24	0.06±0.14	-0.13±0.14
Area	10.63±9.15	5.26±3.64**

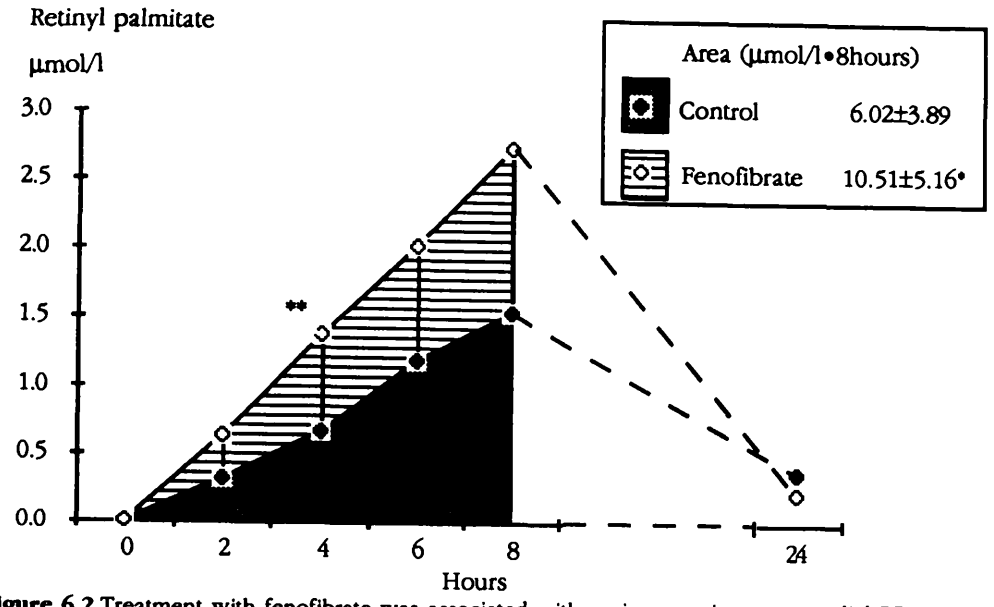


Figure 6.2 Treatment with fenofibrate was associated with an increase in post prandial RP response. *P≤0.1, **P≤0.05, n=8 males

6.2.2 Nicotinic acid

Nicotinic acid treatment led to a reduction in fasting serum cholesteryl ester, triglyceride, and phospholipid (Table 6.2). Alterations in the distribution of fasting lipoprotein mass in Table 6.3 shows a reduction in VLDL and LDL, and an increase in HDL_2. LpL activity fell from 210.1±54.5 to 178.9±70.6mU (↓9.5%, P≤0.02), but HL was not significantly altered. Nicotinic acid also lessened the post prandial triglyceride response to the fat load (Figure 6.3), although RP flux was not significantly affected by drug treatment (Figure 6.4).

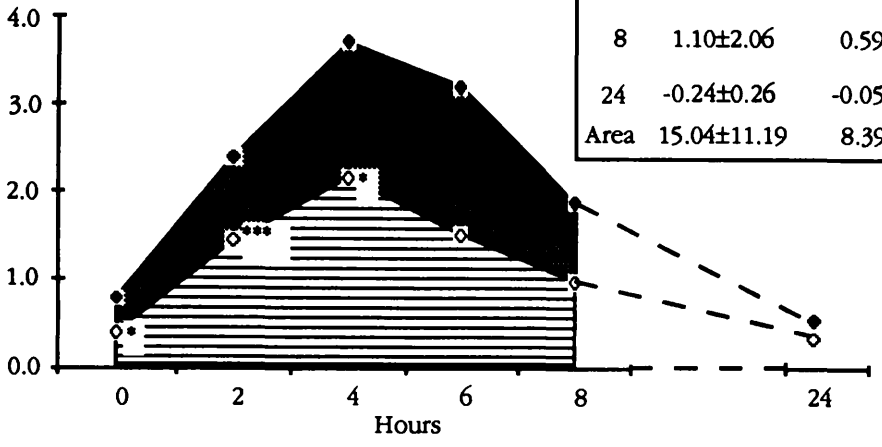
Despite the absolute changes in LDL and HDL, the response to the fat load, ie the rise in CE/TG and the fall in FC/PL ratios during lipæmia, were not affected by nicotinic acid.

Table 6.2 *The effects of nicotinic acid on fasting serum lipids*

	Cholesterol		Triglyceride	Phospholipid
	Free	Esterified		
			mmol/l	
Control	1.55 ±0.70	3.14 ±0.68	1.47 ±0.55	2.68 ±0.50
Nicotinic acid	1.44 ±0.50	2.28 ±0.48	0.88 ±0.42	2.22 ±0.41
	NS	↓27%***	↓40%**	↓17%***

Significance of changes in serum lipids following 6 weeks treatment with nicotinic acid. *P≤0.05, **P≤0.001, NS = not significant, n=8males.

Figure 6.3 The effects of nicotinic acid on ρ<1.006 triglycerides. Both fasting levels and the post prandial response were reduced, *P≤0.1, **P≤0.05, ***P≤0.02, n=8 males.



	Post prandial response	
	Control	Nicotinic acid
Δ		
2	1.60±1.08	1.03±0.84**
4	2.94±1.82	1.75±1.69*
6	2.43±2.65	1.12±1.07*
8	1.10±2.06	0.59±0.54
24	-0.24±0.26	-0.05±0.18***
Area	15.04±11.19	8.39±6.50**

6.3 Discussion

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The two classes of drug used in this part of the study were selected for their known ability to lower triglyceride, and because both have seen many years of clinical use, during which time much has been learned regarding their pharmacology and their hypotriglyceridæmic mechanisms.

Nicotinic acid is thought to act by reducing VLDL synthesis.[241] It does this by lowering the activity of hormone sensitive lipase in adipose tissue, inhibiting triglyceride mobilisation and so circulating free fatty acid falls sharply. This in turn reduces triglyceride formation and incorporation into VLDL in the liver. Fenofibrate on the other hand is considered to act by promoting triglyceride clearance at the periphery through a stimulation of LpL. [242] Both classes of drug effectively lowered post prandial triglyceridæmia in line with these proposed mechanisms of action.

Fenofibrate raised post heparin LpL. At the same time there was a reduction in fasting VLDL and a rise in HDL_3. This is in line with an increased flux of ρ<1.006 lipoprotein surface materials into HDL subject to an increase in lipolytic activity. The reduction in VLDL and in post prandial triglycerides also means that there is less opportunity for transfer of cholesteryl esters from HDL into ρ<1.006 lipoproteins, and so the post prandial decline in HDL cholesterol was eliminated.

Nicotinic acid produced a similar reduction in post prandial triglycerides without a rise in LpL activity. This effect may be secondary to the reduction in hepatic VLDL which will ease competition for the lipolytic pathway, and so enhance the throughput of post prandial tri-

Table 6.3 *Fasting lipoproteins after nicotinic acid*

	VLDL	IDL	LDL	HDL_2	HDL_3
			(mg/dl)		
Control	108.3 ±58.2	11.5 ±4.6	218.9 ±81.2	44.3 ±18.1	224.1 ±82.9
Nicotinic acid	59.1 ±48.5	11.4 ±7.1	175.0 ±60.7	82.3 ±26.3	196.4 ±46.3
	↓45%*	NS	↓20%**	↑86%*	NS

Significance of changes in total lipoprotein mass following 6 weeks treatment with nicotinic acid.
* P≤0.02, ** P≤0.005, NS = not significant. n=8 males.

glycerides. Without an increase in lipolysis however one would not expect an increase in HDL. On the contrary the smaller amount of VLDL substrate should provide less material for transfer to HDL. Although there was a fall in HDL_3 it was not statistically significant. HDL_2 however was virtually doubled.

These changes in HDL _ an increased HDL_2/HDL_3 ratio due to a fall in HDL_2 and a rise in HDL_3 _ are a frequent consequence of nicotinic acid treatment,[243,244] and serve to illustrate the point that these drugs may have effects other than on triglyceride turnover.

Both fenofibrate and nicotinic acid lowered LDL. In the case of nicotinic acid this may stem from the fall in production of VLDL precursor. Fibrates on the other hand may promote LDL clearance.[242] Despite the fall in levels of LDL neither drug altered the post prandial compositional changes

Gemfibrozil, a fibrate related to fenofibrate, was found to reduce RP in Type IV and Type IIa hyperlipidæmia,[184] although the effect was more striking in the chylomicron (Sf>1000) than in the non-chylomicron (Sf<1000) fraction. There is also evidence that gemfibrozil may be able to promote uptake of VLDL remnants by apoE receptor mediated pathways.[245] There was no evidence from this study that either fenofibrate or nicotinic acid could reduce the amounts of circulating chylomicron remnants. The cardioprotective influence of these drugs with regard to chylomicron metabolism remain to be established.

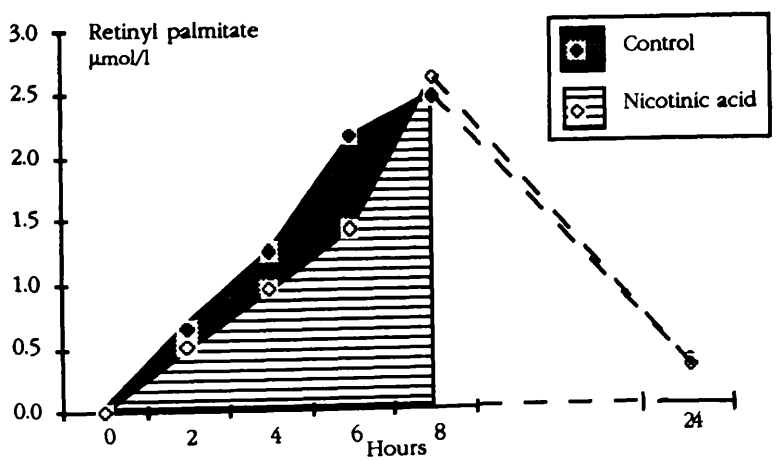

Figure 6.4 Retinyl palmitate response after treatment with nicotinic acid.

Chapter 7

General discussion

7.1 On the fat tolerance test

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It is a principle function of the plasma lipoproteins that they distribute newly ingested dietary fats to the tissues; a function in which they are engaged for a major part of daily life. Since dietary fat intake therefore represents a significant component of the lipoprotein system, and since both dietary fats and lipoprotein metabolism are strongly linked to the development of coronary heart disease, it is perhaps surprising that research has focussed largely on lipoprotein metabolism in the fasting state. This is particularly so since early studies clearly identified alimentary lipæmia as a feature of CHD.[13,14,48-51]

The reasons are probably mostly operational, deriving from the difficulties in designing and applying a uniform test in a reproducible way, and then in the interpretation of the widely varying results. The advent of the ultracentrifuge, the discovery of an endogenous lipoprotein system, and the subsequent identification of distinct disorders, easily recognised in fasting sera, may have distracted attention from the less well defined 'alimentary lipæmia'. More recently however, the increasingly detailed knowledge of the morphology of the lipoprotein system, of the enzymes involved in its regulation, and the growing appreciation of its essentially dynamic nature, have led to a resurgence of interest in post prandial metabolism, and have highlighted the necessity for some sort of function test in addition to existing static assessments of fasting, 'steady state' levels.

Although the problems of standardisation attending the implementation of fat tolerance tests remain, it is hoped that this study has shown how post prandial metabolism may be related to the fasting lipoprotein profile of an individual, and has indicated some features to which attention might be directed in the future.

It is worth considering first some general features of the test itself, and how some of its problems may be reduced or accounted for. To some extent these will depend on the resources available, and the detail with which the subject is to be studied.

The background to the test: Obviously, as with any physiological system, individual genotype in combination with environmental & behavioural circumstances combine in determining lipoprotein response to a fat load. In addition to any explicit dys-lipoproteinæmias or other metabolic conditions, it may be informative to include measurement of apoE isoforms when conducting fat load tests. Although not addressed specifically in this report it has been shown to affect the pattern of post prandial response.[150]

It is clear that the habitual diet of individuals, in particular the fatty acid composition, is equally, if not more important than the composition of the fat load in determining response. Therefore careful dietary assessment, or perhaps the implementation of standard diets prior to testing, combined with fatty acid analysis of lipoprotein lipids, may eventually simplify interpretation.

It may also be helpful to assess levels of fitness in addition to age, body mass etc, since exercise can influence lipæmia,[246] lipase activities, and lipoprotein levels.[247]

The form of the test: Tolerance to fat depends on many processes, from digestion to remnant clearance, and it is possible to modify the test to make it more specific to particular aspects. Thus rates of absorption can be tested more directly by intra-duodenal infusion, and rates of clearance can be studied by intra-venous administration of emulsions. The oral fat tolerance test is the least specific, but the most physiologic of these methods since it tests the combined efficiency of all of these processes.

The objective of the present study was to assess lipoprotein changes during lipæmia, without particular regard to the factors regulating its magnitude. In this context the oral route was the most suitable since by this means the endogenous lipoprotein system was exposed to exogenously derived material in the form of chylomicrons manufactured according to the natural physiology of the individual.

The composition of the fat load: This can be tailored to the investigations being undertaken, with due consideration to the background long term diet. It may be representative of the normal diet, or it may be experimental in design. It should however contain carbohydrate and protein in amounts sufficient to allow physiological digestion to take place. Future work may perhaps involve the use of a 'mixed meal' containing carbohydrate, fat and protein in proportions representative of realistic diets. Such meals will call into play all of the control mechanisms of intermediary metabolism, and may require assessments of 'non lipoprotein' parameters such as glucose or insulin responses. The results may be more difficult to interpret but would however, have the advantage of being more relevant to real life.

Regarding the amount of fat given, it is necessary to provoke a measurable response in an individual, and to allow discrimination of those with impaired or poor ability to cope with exogenous fat. Even with the comparatively large loads given in this study (of the order of a days supply of fat calories) some of the subjects demonstrated triglyceridæmia barely exceeding fasting levels. Such is the variation even in a normal population however, that others experienced massive and prolonged lipæmia after the same fat load. It is important to remember nevertheless, that the total quantity of fat passing through the lipoprotein system was the same in each case, and that it is the relative rates of appearance and disappearance that account for the different levels of triglyceridæmia.

It has been common practice to administer fat according to body mass or surface area, so that larger individuals receive more.[162,184] There is little evidence however that larger people can accommodate greater amounts of fat. On the contrary there seems to be a negative correlation between body mass and the magnitude of lipæmia, so that giving less fat to smaller individuals may only bias results.

The conduct of the test: Standardisation of the fat tolerance test is facilitated by starting at a set time of day, probably in the morning after an overnight fast. This fits with a normal physiological situation, and also circumvents any problems arising from the diurnal variations known to affect triglyceride levels.[248] To continue the fast after administration of the fat load introduces a further problem however, since such a situation is neither physiological nor practical. A compromise may be to allow standard low fat meals at appropriate times.

Samples should be taken more frequently and for longer than they were in this study. It seems that triglyceridæmia in the more extreme cases may still be significant up to 12 hours after consuming the fat, and that remnant particles can persist in significant amounts at least as late as 24 hours. More frequent (hourly) sampling would reduce the danger of missing the narrow or biphasic peaks described by some studies.[192,195] This may be even more important when considering the rapidly changing responses affecting flux of lipid between lipoprotein species.

Measurements: Although triglyceridæmia is the most obvious and well recognised change in post prandial plasma, it is not the only one. Free cholesterol changes for example, were inversely related to triglyceridæmia. Therefore in those who suffered the least lipæmia a post prandial rise in free cholesterol was more easily detected. It remains to be established whether it is simplistic only to measure triglycerides, or whether some other estimates may say more about the efficiency of post prandial lipid transport. The next section, in describing some of the features of post prandial lipoprotein metabolism, may suggest some areas to which attention could be directed.

7.2 Post prandial lipoprotein metabolism

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7.2.1 Post prandial lipoprotein metabolism

Oil and water, according to the commonly held maxim, don't mix. Since 'oils' must travel through an aqueous environment to satisfy the bodies requirements for lipid material, physiological systems have evolved which permit not only the transport of lipid, but the fine regulation of its distribution according to the requirements of the tissues. This system integrates the movements of polar and non-polar lipids, in conjunction with specific specialized proteins, by exploiting the amphiphilic character of the polar lipids in the dissolution of the non-polar triglycerides and cholesteryl esters. The polar lipids enclose the non-polar in a spherical particle, and the proteins direct their movements.

It is an inevitable consequence of this arrangement however, that the metabolism of these lipid groups have become interdependent to a degree, so that flux in one will influence that of others. One should remember nevertheless, that the common transport system does not necessarily signify a common origin, destination, nor function for these hydrophilic molecules. Thus the operation of the triglyceride transport system, designed primarily for the distribution of fuel materials, can affect the movements of cholesterol and phospholipid whose turnover in the tissues are not strictly related to energy requirements. This effect is nowhere better observed than during post prandial lipæmia. The following scheme may account for many of the observations made in this and in other studies.

The first and most obvious feature characterising lipæmia is an increase in the average size of the triglyceride rich lipoproteins, producing the characteristic turbidity of the serum. This increase in size distinguishes alimentary from endogenous triglyceridæmia, which is associated with an increased *number* of circulating hepatic VLDL.[175] The periodic influx to the bloodstream of these large triglyceride enriched particles perturbs transiently the 'equilibrated' state of fasting lipoprotein transport by altering the composition of both the polar and non-polar phases of circulating lipid.

Firstly the non-polar core of the chylomicron is initially relatively poor in cholesteryl ester, and has a lower ratio of cholesteryl ester to triglyceride compared with surrounding membranes and other lipoprotein species. Secondly, the surface phospholipid film is relatively deficient in free cholesterol, and has a lower ratio of free cholesterol to phospholipid. The concentration gradients thus developed induce several processes which run in parallel during the lifetime of the chylomicron and its remnant. These are illustrated in Figure 7.1.

Lipolysis: Upon entering the circulation chylomicron triglycerides are hydrolysed by LpL, free fatty acids are released, become bound to albumin, and are removed from the lipo-

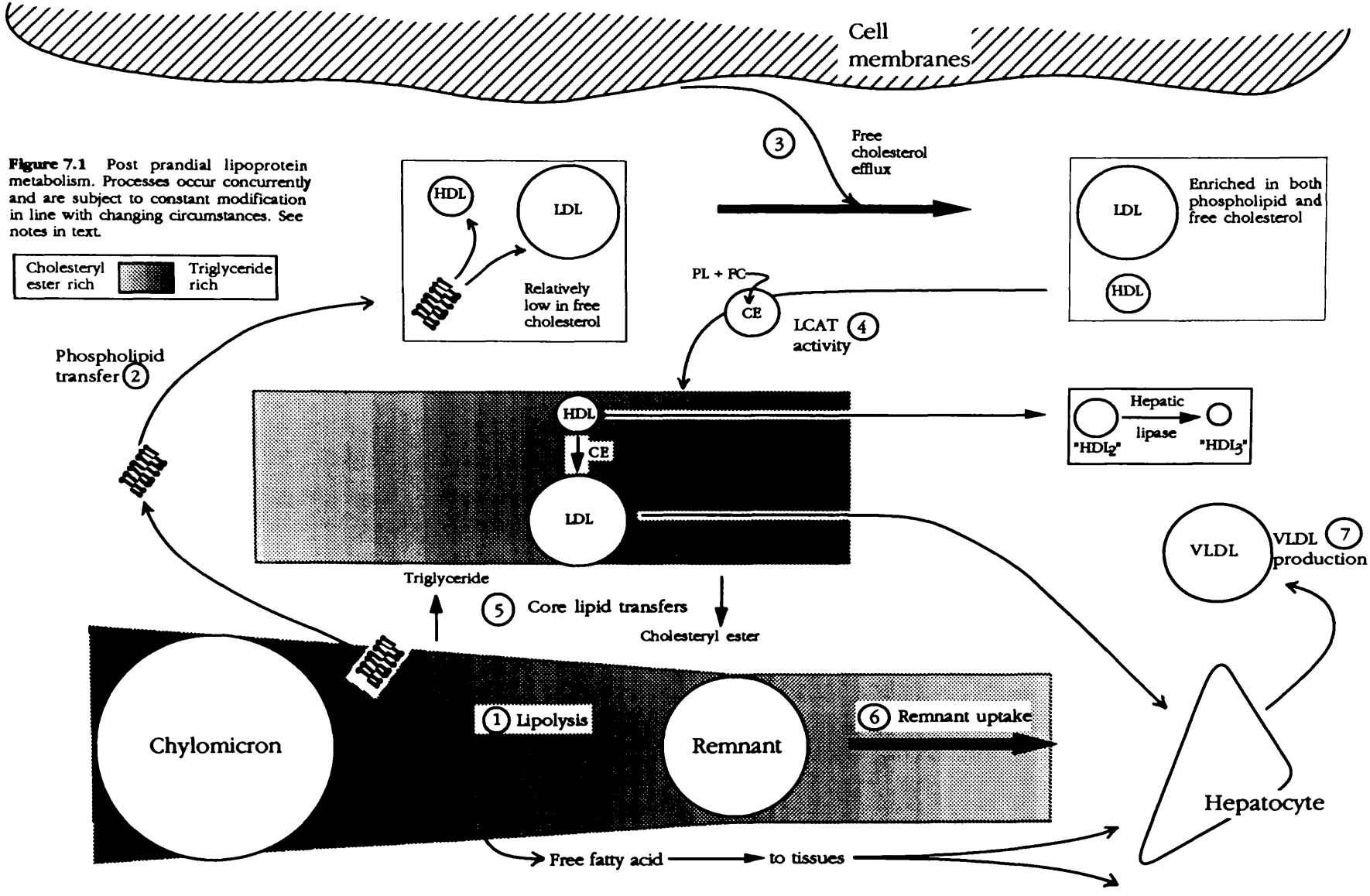

Figure 7.1 Post prandial lipoprotein metabolism. Processes occur concurrently and are subject to constant modification in line with changing circumstances. See notes in text.

lytic site. As the triglyceride core diminishes surface phospholipids become superfluous and are also released into the circulation. The pattern of lipid flux observed as this proceeds is a rise followed by a decline in the triglyceride and phospholipid content of the $\rho<1.006$ fraction, with a peak at around 4 hours. In whole plasma however levels of phospholipid do not fall away after this peak, but continue to rise, in many individuals till at least as late as 8 hours after consuming the fat load. The greater the rate of lipolysis the more phospholipid will enter the $\rho>1.006$ fraction, and since LDL and HDL are turned over more slowly than $\rho<1.006$ lipoproteins the more prolonged the phospholipid peak will be.

Free cholesterol flux: The result of phospholipid uptake by these lipoproteins is a reduction in the ratio of free cholesterol to phospholipid in the surface regions, leading to the formation of a free cholesterol gradient between cell membranes (mainly red blood cells) and circulating LDL and HDL. Since free cholesterol can diffuse between lipoprotein and tissues there is a subsequent movement into the circulation where it becomes associated with both LDL and HDL. Since this flux depends on the uptake of phospholipid by these lipoproteins the rise in free cholesterol comes even later than the rise in plasma phospholipid. The greater the transfer of phospholipid the steeper the free cholesterol gradient will be. A greater flux of free cholesterol would therefore be expected to accompany more efficient lipolysis.

LCAT activity: Although their is evidence that LCAT may act on LDL,[156] quantitatively the majority of plasma activity is associated with HDL. The transfer of both phospholipid and cholesterol to these lipoproteins will therefore provide substrate for the known increase in post prandial LCAT activity,[206] the cholesteryl esters so formed enriching the core of HDL. The extent to which this occurs in LDL is unknown. Despite the assimilation of free cholesterol & phospholipid and the activity of LCAT, there was no absolute rise in cholesteryl esters in LDL or HDL. On the contrary their was a highly significant *fall* in HDL cholesteryl ester during lipæmia.

Core lipid transfer: Lipolysis takes place against a background of LTP activity which strives to bring about equilibrium in the distribution of core lipids between the lipoprotein fractions. The introduction of a large triglyceride pool initiates a period of core lipid flux in which triglyceride was seen to penetrate all of the lipoprotein classes in parallel with the rise and fall in the $\rho<1.006$ fraction.

Cholesteryl esters on the other hand should move in the opposite direction, leaving HDL (and LDL) and entering the triglyceride rich fraction. This phenomenon has been described before and is most evident in the HDL.[152,153,217] Having become enriched in triglyceride at the expense of cholesteryl ester the HDL apparently acquire affinity for hepatic lipase which

hydrolyses triglyceride (and phospholipid) leaving a small sized HDL_3 like particle. This scenario is thought to underlie the reciprocal link between lipæmia and HDL levels. The position of LDL in the scheme is less clear.

Remnant formation and clearance: Hydrolysis of chylomicron triglyceride is extensive but not complete. At an indeterminate stage in its catabolism the chylomicron loses affinity for lipase and is broken down no further. At this stage the whole remnant is removed from the circulation by a hepatic receptor. The present study suggests that a significant proportion of these particles remain in the circulation for up to 24 hours after ingestion of fat.

VLDL production: Evidence that levels of hepatic VLDL rise during lipæmia has accumulated steadily.[190-195,249,250-251] Material for the production of VLDL would presumably be derived from FFA which are not effectively cleared at the periphery,[252] and which have been found to rise during lipæmia, and from breakdown products delivered to hepatocytes in the form of chylomicron remnants.[191]

The preceding diagram and notes represent a simplified and idealised description of the processes operating during post prandial lipæmia. The situation *in vivo* however, is clearly one in which they work in an integrated and interdependent fashion, and the observed pattern is therefore a compound one.

7.2.2 Factors influencing the pattern of post prandial lipæmia

The regulation of post prandial lipoprotein metabolism may be viewed on several different levels. The environmental/behavioural effects of diet, alcohol consumption, exercise habits, etc, are superimposed on genetic influences acting through the medium of apoprotein polymorphisms, receptor activities and enzyme production rates (Table 1.1). These are the factors most commonly identified by clinical or epidemiological study. It is important to remember that lipoprotein metabolism takes place within the context of whole body metabolism, and that nervous, hormonal, or metabolic conditions perhaps only indirectly related to fat metabolism may also have repercussions during the post prandial phase.

The above factors impinge on one or more of the metabolic processes discussed throughout this thesis. At this 'systems' level the relative rates of lipolysis (LpL & HL), lipid transfers, and LCAT activity, in conjunction with the pool sizes and lipid carrying capacities of all the lipoprotein classes combine in determining the pattern of events.

The central process here would seem to be the rate at which chylomicrons traverse the lipolytic cascade since this determines not only the size of the circulating triglyceride pool, but also the production rate of remnants and the rate of phospholipid flux into $\rho<1.006$

fractions. These in turn influence free cholesterol efflux, compositional changes, and LCAT activity. Since chylomicrons are turned over much faster than other lipoprotein classes the rate of their removal will largely determine the period available for transfers to take place.

A second key event is the conversion of chylomicron to remnant. This transition represents the end of lipolysis and a change from a fast to a slower phase of catabolism. The suitability of the remnant as a ligand for its hepatic receptor at this stage will be crucial in determining the plasma residence of the remnant and its subsequent fate. If, at the cessation of lipolysis, the remnant is only poorly identified by its receptor, then further remodelling in the plasma may eventually preclude hepatic uptake leaving clearance to take place by other routes.

Physical methods are unable to isolate such remnants, and metabolic methods are only approximate. Notwithstanding these difficulties the question of what regulates the transition to remnant and permits its uptake by receptors is important and should certainly provide a focus for future investigation.

In approaching these problems we may benefit from an examination of the many fine variations in affinity between lipids, apoproteins, enzymes, and cofactors etc. In a dynamic process involving constant flux and interchange of components even small fluctuations in affinity may be fundamental in directing the course of events.

On this more subtle level there are several examples of such regulations, and room for speculation on others. The cholesteryl ester content of the chylomicron,[236] the nature of the fatty acid components of phospholipid at the chylomicron surface,[237-238] or the build up of lipolytic products (FFA and lyso phospholipid) in the micro environment around the lipolytic site may affect the rate of lipolysis.[137] In this circumstance the ability of plasma factors to clear these materials, or the capacity of the tissues to assimilate them may be important.[253]

Other possibilities may include the change in curvature at the surface, or a change in the composition of the core as triglyceride substrate is replaced by cholesteryl ester. At the critical stage of catabolism which depends on the ordered departure of apoCII and CIII, and the activation of apoE even small changes in affinity between chylomicron, apoproteins and enzyme may be important.

Similar factors may apply to core lipid transfer rates where the binding of lipoprotein and transfer protein appears to be an essential step in the process.[207] The raised phospholipid and FFA content of HDL,[208] and the increase in LDL free cholesterol[205] have both been proposed as enhancing transfer activity, and of having as a consequence a stimulating effect on LCAT activity.

7.2.3 Alimentary lipæmia and atherosclerosis

There seems little doubt that alimentary lipæmia, certainly as defined by post prandial triglyceridæmia, is positively associated with coronary heart disease. From this and other studies several aspects emerge whereby a more massive and prolonged lipæmia may be causally related to the development of the disease. Although these mechanisms are not necessarily directly related to the triglyceridæmia itself, they stem from the influence that a delayed clearance of triglyceride rich particles has on the distribution of cholesterol among the tissues and other lipoprotein fractions.

1. A prolonged lipæmia will be marked by a reduced flux of phospholipid into $\rho>1.006$ fractions, and hence a reduced flux of free cholesterol from tissues into the lipoprotein system. Such a decrease in the efficiency of free cholesterol clearance from tissues may have the further repercussion of inhibiting receptor mediated uptake there and thus raising plasma levels of LDL cholesterol.

2. Cholesterol which does enter the bloodstream may be directed into metabolic pathways with atherogenic potential.
a). Cholesteryl esters may increasingly accumulate in relatively long lived remnant particles whose ultimate disposal is not physiologically controlled by receptor systems.
b). HDL levels may be lowered and subfraction distribution shifted towards the smaller HDL_3 by virtue of triglyceride accumulation and cholesteryl ester depletion followed by hepatic lipase activity. [200]

3. Chylomicron remnants seem to persist longer in plasma in those with CHD. The risk that cholesteryl ester enriched remnants represent has been discussed previously. The factors underlying the prolonged lifetime of these remnants are not known. Apo E isoforms may contribute a genetic component.[150] Cholesteryl ester enrichment or related compositional changes may themselves lead to a lengthening of their circulation.

4. It may be that metabolic control over VLDL production is less stringent in those at risk for CHD, so allowing a rise in production when supplied with an abundance of substrate. Inefficient clearance or utilisation of lipolytic products in peripheral tissue may be an integral feature of this phenomenon.

5 Although not addressed in the present study post prandial triglycerides may be related to a hyper coagulable condition. Thus as well as contributing to the long term development of the disease, a rich fatty meal may provide a focus for the acute event.

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