SHORT TITLE:

The Catabolism of Very Low Density Lipoproteins

THE CATABOLISM OF HUMAN AND RAT VERY LOW DENSITY LIPOPROTEINS BY PERFUSED RAT HEARTS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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TO MY PARENTS

AND JANICE

for their encouragement, support and patience.

ABSTRACT

The production of remnants from rat and human TG-rich lipoproteins by the perfused rat heart was investigated. The rat heart is consistently able to remove more TG from rat and human chylomicrons than serum VLDL of either species. When the catabolism of rat and human ^{125}I-VLDL were compared, it was found that TG was removed to a greater extent than protein, leaving remnants, smaller in size, containing relatively more apoB and less apoC. The extent of TG removal from the rat and human VLDL was similar and appeared to saturate the lipoprotein lipase. The loss of TG correlated with the loss of apoC, which was recovered in the d = 1.063 - 1.21 g/ml range of the perfusate, suggesting the formation of LpC. Phospholipid was the dominant lipid of this fraction. Iodinated lipoproteins of d = 1.019 - 1.063 g/ml were also produced in the course of VLDL degradation, containing largely cholesterol and cholesteryl ester. Very little of this material however was produced from low (physiological) concentrations of rat VLDL, most of the lipoprotein being removed by the heart by an apparently specific, saturable process. Lipoproteins of this density were formed from human VLDL at all concentrations. Human lipoproteins were also taken up by the heart by an apparently non-specific process. Agarose gel filtration of the human VLDL heart perfusate lipoproteins revealed large aggregates containing LDL-like particles in size and apoprotein composition, as well as a fraction containing predominantly apoC.

The subsequent uptake of the rat lipoprotein remnants by the perfused liver was significantly greater than the uptake of those of the human, suggesting some specificity in the hepatic uptake. These data suggest that at normal concentrations, rat VLDL are completely catabolized by the heart and the liver without significant formation of LDL. Low density lipoprotein is produced from human VLDL at all concentrations.

RESUME

Par suite de la perfusion d'un coeur de rat avec des lipoprotéines riches en triglycérides provenant soit de l'homme, soit de rat, la formation par le coeur de restes lipoproteiques a été étudiée. Le coeur du rat peut enlever davantage de triglycérides des chylomicrons de l'homme ou du rat que des VLDL de l'une ou l'autre espéce et ce, de façon consistante. L'étude du catabolisme des ^{125}I-VLDL de l'homme ou du rat a montré qu'une plus grande quantité de triglycérides que de protéines était enlevée résultant dans la formation de restes lipoproteiques plus petits et contenant relativement plus d'apoB et moins d'apoC. La même quantité de triglycérides est enlevée des VLDL du rat que de celles de l'homme et semble saturer la lipoprotéine lipase. La perte en triglycérides correspond à la perte en apoC. ApoC se retrouve dans la fraction du perfusat ayant une d=1.063-1.21 g/ml ce qui tend à suggérer la formation de LpC. Les phospholipides sont les principaux lipides de cette fraction. Pendant la dégradation des VLDL, des lipoprotéines iodinées (d=1.019-1.063 g/ml) contenant principalement du cholestérol et des esters de cholestérol, sont aussi produites. Cependant à de faibles (physiologiques) concentrations de VLDL de rat il se forme très peu de ce genre de lipoprotéines, le coeur enlevant la majorité des lipoprotéines par un processus apparemment spécifique et saturable. Des lipoprotéines de cette densité sont cependant formées à toutes concentrations de VLDL humaines. Les lipoprotéines humaines sont aussi assimilées par le coeur mais par un processus apparemment non-spécifique. La filtration sur gel d'agarose d'un perfusat de lipoprotéines obtenu après perfusion du coeur avec des VLDL humaines révèle la présence d'aggregats contenant des particules qui, par leur dimension et par leur composition en apoprotéines, s'apparentent aux LDL ainsi que la présence d'une fraction contenant prin-

cipalement apoC.

L'assimilation subséquente des résidus lipoprotéiques par le foie perfusé est significativement différente selon que ces résidus proviennent de lipoprotéines d'homme ou de rat se qui tend à suggérer une spécificité d'espèce pour l'assimilation hépatique. Ces données suggérent qu'à des concentrations normales, les VLDL du rat sont catabolisées complètement par le coeur et le foie sans que soient formées des quantités significatives de LDL, alors qu'à toutes concentrations de VLDL humaines, des LDL sont produites.

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LIST OF ABBREVIATIONS

d :	density
S_f :	flotation rates in a medium of d = 1.063 g/ml
TG :	triglyceride (triacylglycerol)
MG :	monoglyceride (monoacylglycerol)
DG :	diglyceride (diacylglycerol)
LPL :	lipoprotein lipase
VLDL :	very low density lipoproteins (d < 1.006 g/ml)
IDL :	intermediate density lipoproteins (d = 1.006 - 1.019 g/ml)
LDL :	low density lipoproteins (d = 1.019 - 1.063 g/ml)
HDL :	high density lipoproteins (d = 1.063 - 1.21 g/ml)
SDS :	sodium dodecyl sulfate
PAGE :	polyacrylamide gel electrophoresis
LCAT :	lecithin-cholesterol acyltransferase
NMR :	nuclear magnetic resonance
CHD :	coronary heart disease
Lp :	lipoprotein
EDTA :	ethylenediamine tetraacetic acid (disodium salt)
DTNB :	5,5'-dithionitrobenzoic acid
HMG-CoA :	3-hydroxy-3-methylglutaryl Coenzyme A

CHAPTER I

INTRODUCTION

A. GENERAL LIPID METABOLISM

Lipids are not only the largest and most efficient (1) source of metabolic energy available to the body, but also serve as the precursors of many important structural and regulatory molecules. It is essential then that dietary lipids be transported from their sites of absorption and synthesis to sites of utilization and storage. It is also important that during periods of fasting, stored lipids be readily mobilized and delivered to tissues in need. And finally, an organism should possess a mechanism enabling it to remove certain lipids, such as cholesterol, from tissues unable to catabolize them, to alternate sites for use or disposal.

In the fasting state, the stored TG in the adipose tissue is broken down by a cAMP-activated, hormone sensitive lipase and the fatty acids are released directly into the circulation, where bound to albumin, they can supply 50 - 90% of the body's total energy needs (2). As the turnover rate of the free fatty acids in the serum is very high, the bulk of the lipid in the plasma at any one time is present as neutral lipid: TG, cholesterol and cholesteryl esters. To overcome the extreme insolubility of neutral lipids in aqueous media such as serum, they are transported in association with specific proteins and polar lipids, forming rather large lipoprotein complexes. A significant amount of carbohydrate is also present in lipoproteins as part of the protein

moiety. There are several classes of lipoproteins present in the plasma, reflecting the variety of the lipids to be transported as well as the function of the transport mechanism itself.

During the fed state much of the lipid in the intestinal lumen is present as insoluble oily globules or emulsified particles. In addition to TG; cholesterol, derived chiefly from the diet as well as several endogenous sources (bile, saliva, gastric secretions and degraded mucosal cells) is present, mostly in the unesterified form. The action of pancreatic lipase on the surface of the TG emulsions releases fatty acids and MG. The small amount of cholesterol ester present is also hydrolyzed into cholesterol and fatty acids. When bile salts, secreted by the liver, are present in sufficient quantities, the fatty acids, MG, and small amounts of DG are solubilized in mixed bile salt - lipid micelles. The presence of fatty acids and MG in turn enhances the solubilization of cholesterol and fat-soluble vitamins in the micelle (3). The diameter of the micelles is about 40 - 50 Å (4), and thus the conversion of fats from an emulsion phase to a micellar one provides an approximately 100-fold reduction in particle diameter accompanied with an over 10,000-fold increase in surface area (5).

The actual mechanism of the penetration of the micellar lipids into the mucosal cells is not firmly established. Their size enables them easy penetration of the intermicrovillous spaces. It seems unlikely that the intact micelles penetrate the cell membrane, since the uptake of lipids and bile salts occur in different regions of the small intestine (6). The close proximity of the micelle to the membrane however, provides a high concentration of lipids necessary for the maintenance of a concentration gradient, replacing lipid molecules

diffusing from the intervening aqueous film into the cell membrane (6). In the absence of micelle formation, as occurs during bile diversion or obstruction, absorption can proceed only through diffusion from the oily phase across the aqueous medium. The importance of the micellar phase for lipid absorption varies with the type and solubility of the lipid molecule. Thus cholesterol, which is insoluble in aqueous media, is totally dependent on micellar solubilization for absorption, while the medium and short chain fatty acids are virtually independent of it.

Following absorption, fatty acids and MG are reesterified by the mucosal cells to TG. The reesterified TG is packaged together with cholesterol, cholesteryl ester, phospholipid, and some specific proteins into chylomicrons, which are secreted into the circulation via the lymphatic system. Medium and short chain fatty acids and monosaccharides enter the circulation via the portal system. Chylomicrons can deliver their TG load to any tissue possessing LPL. These include the muscle and heart tissue which use liberated fatty acids for their energy needs; the mammary glands, utilizing fatty acids in milk production during lactation; and adipose tissue, which stores the fatty acids as TG. The physiological state of the organism determines the amount utilized by each tissue. Significantly depleted of their lipids, the chylomicrons, at this stage usually referred to as remnants, deliver the remainder of their lipid load (TG, cholesteryl ester and phospholipids) to the liver (7-9).

In addition to the residual lipids in chylomicron remnants, the liver also receives a load of carbohydrate and uses both to satisfy its own energy demands and synthetic needs, storing the excess as glycogen. Since the storage capacity of the liver is limited, glucose can

be converted into TG. This endogenous TG is packaged with cholesterol, cholesteryl ester, phospholipid and specific proteins into VLDL. When in circulation, VLDL TG is also available to tissues containing LPL. Most of it is , however, stored in the adipose tissue.

It is evident then that both chylomicrons and VLDL function to redistribute lipids from the intestine and liver to adipose tissue for storage or to other tissues for energy utilization or membrane maintenance. Like chylomicrons, VLDL are good substrates for the LPL; but while chylomicron remnants are removed by the liver, the nature and fate of the VLDL catabolites are more complex. Tracer experiments, using ^{125}I-labelled VLDL suggest that human VLDL are converted to LDL and HDL in the circulation (10-13), possibly after passing through an IDL stage (11). Rat VLDL on the other hand, may be rapidly cleared by the liver after the initial depletion of its TG content without significant formation of LDL (14-17). Indeed, normally LDL is not a significant constituent of rat lipoproteins.

Both LDL and HDL, the latter being also synthesized de novo by the intestine and liver, have cholesteryl esters and phospholipids as their major lipid components. The fate of these components is not clear, but it has been suggested that LDL and HDL may be the main suppliers of cholesterol to the extrahepatic tissues as well as shuttles for removing the excess cholesterol from these tissues. The role of these lipoproteins may be different in the human and rat, since unlike the human, in the rat HDL is the major cholesterol-carrying lipoprotein.

While LDL appears to be associated with atherogenesis, HDL seems to have a protective effect (18). Since the catabolism of VLDL, at least in the human, results in the production of LDL and HDL, the underlying

mechanism of this process is of considerable interest. Unfortunately, the individual steps of VLDL catabolism and the immediate products formed are difficult to isolate in in vivo experiments using ^{125}I-labelled VLDL. The data obtained from these studies provide little information about the importance of the various organs, such as the liver, and the enzymes and various substances present in the plasma that may act as acceptors of the protein and lipid moieties of VLDL in the process of its degradation. The interpretation is further complicated by the exchange of some of the apoproteins and lipids between the lipoproteins present in the serum (19). The use of purified LPL may circumvent some of these problems, but the system is not physiological and the enzyme is very unstable (20,21).

To overcome these difficulties, our laboratory developed a two-stage heart and liver perfusion system for the study of rat thoracic duct chylomicron catabolism (9). This system was used in the present study to clarify some of the aspects of VLDL catabolism, after establishing its suitability for the study of the catabolism of human and rat serum chylomicrons. While the kinetics of VLDL TG hydrolysis by the LPL have been studied extensively (22-24), no attention has been given to the various products formed in this process. In the present study, attention is focused primarily on the products formed during rat and human VLDL degradation by the action of LPL in a physiological, membrane-bound state. The uptake of these catabolic products by the perfused rat liver was also investigated and some preliminary results are presented.

B. NATURE OF LIPOPROTEINS.

1. Analytical and Preparative Methods in Lipoprotein Isolation

Lipoproteins can be isolated from the serum and separated from each other for analytical and preparative purposes by several techniques that utilize their physico-chemical properties. The nomenclature of lipoproteins evolved with the techniques used to isolate or detect them and is descriptive of their properties. One of the quickest methods to assess the lipoprotein composition of the serum is electrophoresis. This method is particularly useful for clinical applications.

a) Electrophoresis

The presence of many polar components in the lipoproteins predispose them to movement in an electric field. Their relative mobilities depend on the medium used. In paper electrophoresis of serum (25), two protein fractions, the α- and β-globulins were greatly enriched in cholesterol and phospholipid in comparison to the other protein fractions and were thus named α- and β-lipoproteins (26). Subsequently, two other TG-rich fractions were found, one at the origin and one at the pre-β position, corresponding to chylomicrons and pre-β lipoproteins,(VLDL), respectively. Electrophoretic separation of lipoproteins has since been expanded to many different media. These include starch (27), cellulose acetate (28,29), agarose (30,31) and Geon-Pevicon (32). While electrophoresis is a useful clinical tool, it is not suitable for preparative purposes. The method of choice for large-scale preparations of lipoproteins is ultracentrifugation.

b) Ultracentrifugation

Analytical ultracentrifugation was first applied to serum lipoproteins

by Gofman et al. (33). This method takes advantage of the flotation of lipoproteins in a medium adjusted by the addition of salt (NaCl), to a density higher than that of the lipoproteins. To quantitate the flotation characteristics of lipoproteins, the S_f value was introduced (34), and defined as the rate of flotation of a lipoprotein under unit centrifugal field in a solution of d = 1.063 g/ml at 26°C. The method was further refined when the Schlieren patterns, obtained by analytical ultracentrifugation were correlated with the densities of each lipoprotein species (35,36). It was assumed that the lipoprotein species resolved by the analytical ultracentrifuge represented metabolically and structurally distinct species. Thus, four main classes of lipoproteins were distinguished, in order of increasing densities: chylomicrons, VLDL, LDL and HDL. The correspondence of these density classes of lipoproteins with those separated by agarose or paper electrophoresis is well established (see Figure 1).

A variation of this technique, where the different lipoprotein classes of increasing densities are isolated in a single run, is density gradient ultracentrifugation (37-39), or rate zonal ultracentrifugation (40).

c) Other Techniques

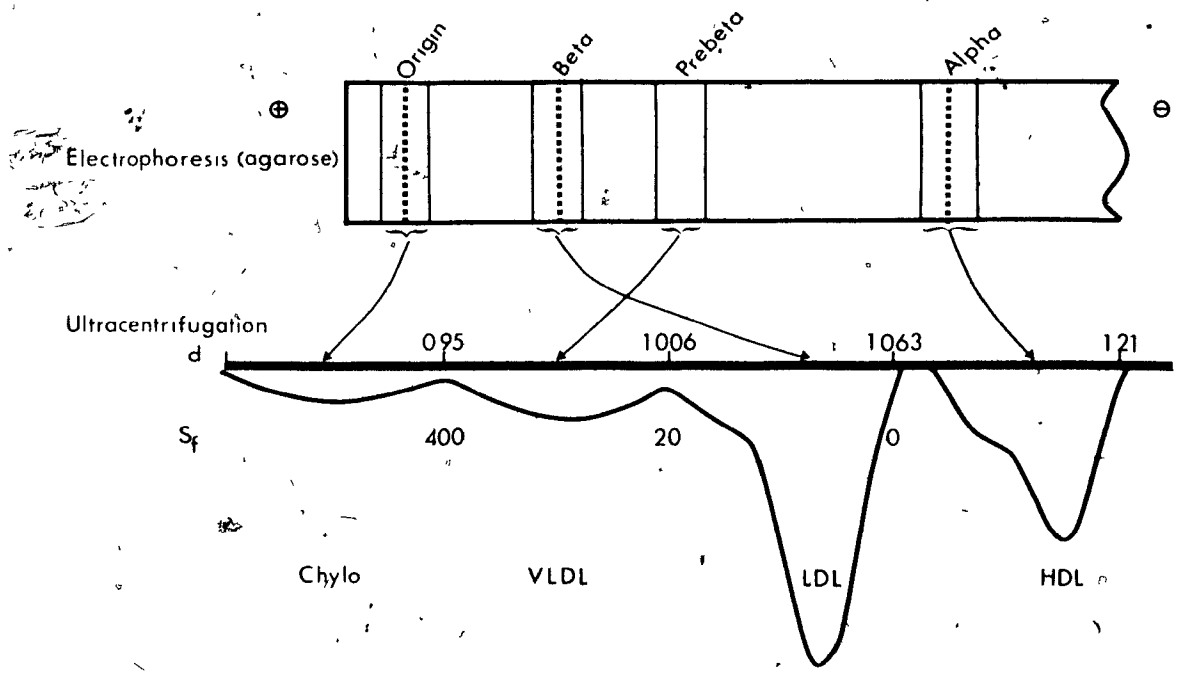
Over the past few years several other techniques have been developed and they will be briefly described.

Agarose gel filtration can be used to separate the major lipoprotein classes on the basis of size (41-44), although when serum is fractionated, considerable contamination of the smaller lipoproteins (LDL and HDL) by large serum proteins occurs. Several precipitation methods

FIGURE 1

DISTRIBUTION OF HUMAN LIPOPROTEINS IN THE SERUM

AND THEIR ELECTROPHORETIC MOBILITIES IN AGAROSE



are available using polyanionic polysaccharides such as heparin or dextran sulphate (45-47), or specific antibodies (48-50).

2. ApoTipoproteins

As will be discussed later, apoproteins play an important role not only in maintaining the structure of lipoproteins, but also in their catabolism. The existence of different apoproteins is a reflection of their functional diversity. Prior to the analysis of their physical and chemical properties as well as their quantitation (in metabolic studies), the apoproteins are usually dissociated from the lipid.

a) Isolation and Purification

Several methods of delipidation of lipoproteins are available. Organic solvents can be used alone or in mixtures of varying proportions to dissolve the lipid and precipitate the protein. Each particular system has some advantages and disadvantages. The use of chloroform/methanol (2:1, v/v), results in a complete delipidation, but the protein precipitate is refractive to solubilization in most aqueous solvents. The most commonly used solvent system is that of ethanol/ether in various proportions (usually 3:1, v/v), but some of the protein may be lost through solubility in ethanol (51,52). Increasing the proportion of ether minimizes the loss of protein, but a less complete delipidation may result. Tetramethylurea, a denaturing agent, has also been used (53). This agent directly solubilizes all but one of the apoproteins (apoLDL). A recently developed technique utilizes a variety of organic solvents, such as acetone or isooctane to selectively solubilize some of the apoproteins of VLDL (54). While this method may be excellent for preparative purposes, its suitability for analytical purposes remains to be established.

A variety of detergents have also been used to separate the lipid and protein moieties. Sodium dodecyl sulphate (SDS) and sodium deoxycholate remove lipids from LDL, avoiding the problem of the extreme insolubility of apoLDL following extraction with organic solvents (55). The lipid and the detergent can be subsequently removed from the protein by successive gel filtration steps.

The isolated protein moiety of the lipoproteins is usually solubilized in an aqueous buffer, containing denaturing agents such as urea, SDS, guanidine-HCl, or a mixture of these. When submitted to PAGE or isoelectric focusing, the protein moiety can be separated into several distinct bands, reflecting their extensive heterogeneity. For large-scale preparation of individual apoproteins, gel filtration, ion exchange chromatography, preparative PAGE, and isoelectric focusing can be used.

An introduction of the apoprotein nomenclatures used will facilitate further discussion of some of the known human and rat apoproteins.

b) Nomenclature

i. Carboxyl-Terminal Nomenclature

In this nomenclature, the C-terminal residue of a single polypeptide chain becomes its principal nametag (56). For example, a small polypeptide, found in both VLDL and HDL has a C-terminal residue alanine, and is named R-ala, apoprotein-ala, or if the source is to be indicated, apoVLDL-ala or apoHDL-ala. This nomenclature has some distinct disadvantages. It is not only cumbersome, but errors in the C-terminal residue can lead to confusion. The occurrence of more than one polypeptide with the same C-terminal residue can be solved by the use of numbers (ie. apoLp-aln-I and apoLp-aln-II), but this makes the system

even more difficult to remember. Furthermore, some apoproteins have proved to be refractive to C-terminal determination (eg. apoLDL). Many animal species have homologous apoproteins with those of the human, but do not necessarily share the C-terminal residue, making the comparison of the apoproteins difficult.

ii. ABC Nomenclature

An alternate nomenclature has been developed, in which the various apoproteins are assigned capital letters. Thus the major apoproteins of the α-migrating lipoproteins (HDL) have been designated as apoA, while that of the β-migrating lipoproteins (LDL) as apoB. Subsequently, further apoproteins were identified and named, in the order of their discovery, by additional letters. The low molecular weight apoproteins, found primarily but not exclusively in VLDL, were named apoC; the "thin-line" peptide, associated with HDL was named apoD; and the "arginine-rich" apoprotein, found in VLDL, HDL and other, abnormal lipoproteins associated with hypercholesteronemia, was designated as apoE. Recently two more apoproteins have been identified and isolated from human HDL and designated as apoF and apoG. Although the apoproteins in the different groups are immunologically distinct from those in any other group, many of these groups are composed of several immunologically related, but not identical polypeptides. These are distinguished from each other by a roman numeral placed after the letter. The apoA group is thus composed of apoA-I and apoA-II (which in the human was formerly refered to as apoLp-gln-I and apoLp-gln-II), and the apoC group consists of three polypeptides, apoC-I, apoC-II, and apoC-III. Polymorphism, encountered in several of the polypeptides, can further be designated by the addition of an arabic numeral. The apoC-III

apoprotein has for instance, several polymorphic forms: apoC-III-1, apoC-III-2, apoC-III-3, etc. Operationally this nomenclature is much simpler and easier to remember than the C-terminal one, and offers the advantage of a direct comparison between homologous apoproteins of different animal species, often differing in their C-terminal amino acid.

Because of these advantages and its wider acceptance, the ABC nomenclature will be used throughout this thesis. The relationship between the C-terminal and ABC nomenclature of some apoproteins is shown in Table I.

In the next section the known human and rat apoproteins are briefly discussed, using the ABC nomenclature.

3. Characteristics of Individual Apoproteins

a) Apoprotein A

Apoprotein A-I: ApoA-I is a well characterized polypeptide of 245 (57) or 243 (58) amino acid residues. The reasons for the differences in the reported amino acid sequences is unknown. Two polymorphic forms with similar molecular weights (27,000), amino acid composition, and immunological properties have been recognized: apoA-I-1 and apoA-I-2 (59). Since neither form contains detectable carbohydrate, the presence of an unidentified labile prosthetic group on apoA-I-1 has been proposed to account for the conversion of apoA-I-1 to apoA-I-2 on repeated ion exchange chromatography in urea (59). ApoA-I tends to self-associate in aqueous solutions (60,61). It does not bind significant amounts of phospholipid in the absence of apoA-II (62). It accounts for over 60% of the human HDL proteins and has been isolated from many other mammals, all having similar molecular weights and amino

TABLE I

COMPARISON OF THE CARBOXYL-TERMINAL AND ABC NOMENCLATURE OF SOME OF THE HUMAN APOPROTEINS

ABC NOMENCLATURE		CARBOXYL-TERMINAL NOMENCLATURE
Apoprotein A	ApoA-I	R - Gln-I
	ApoA-II	R - Gln-II
Apoprotein C	ApoC-I	R - Ser
	ApoC-II	R - Glu
	ApoC-III-1	R - Ala_1
	ApoC-III-2	R - Ala_2

acid composition to human apoA-I, suggesting a major structural or physiological role. It is thought to be an activator of LCAT (63), and may have an important function in regulating the contents of membrane lipids (64) and fluidity (65). As with all the well characterized apoproteins, apoA-I lacks long regions of hydrophobic and hydrophilic amino acid residues, so typical of most membrane proteins.

Apoprotein A-II: This is the second major protein of HDL and is a disulfide-linked dimer of identical polypeptide chains of 77 amino acid residues (66). It has a high α-helical content (40%), and readily combines with phospholipid (67,68). Reduction of the disulfide bond does not affect its lipid binding, suggesting that the dimer form may not be absolutely essential. Indeed, apo-A-II exists as a monomer in a number of animals, such as the rat (69), as well as dog, rabbit and cow, lacking the half-cystine in position 6.

Apoprotein A-III: This apoprotein isolated from human HDL (70), is referred to by most investigators as apoD and will be discussed as such.

Apoprotein A-IV: The amino acid composition and the molecular weight (46,000) of this polypeptide, isolated from rat HDL, was recently reported (71). Information about some of the other properties and possible physiological role of this apoprotein is not available.

b) Apoprotein B

Only one apoprotein belongs to this family to date, but it is so poorly characterized that the presence of more than one polypeptide has not been ruled out. The characterization of apoB has been hampered by technical problems. It is extremely insoluble in aqueous solutions and detergents; dissociating agents and chemical modification have been

necessary to obtain soluble preparations of lipid-free apoB. Molecular weight estimates range from 8,000 to 275,000 (72). A molecular weight of carboxymethylated apoB of 255,000 ± 5%, as determined by SDS electrophoresis, gel filtration and equilibrium ultracentrifugation has been reported (73).

c) Apoprotein C

There are three known polypeptides that belong to this group of low molecular weight apoproteins.

Apoprotein C-I: A single polypeptide chain of 57 amino acid residues (74), it represents less than 5% of the HDL protein, but along with apoC-II and apoC-III, 40-60% of apo VLDL. It tends to self-associate (59) and form aggregates in aqueous solutions (75). It was reported to activate LCAT (63), as well as LPL (76), but these observations remain to be confirmed.

Apoprotein C-II: This apoprotein has 78 amino acids of known sequence and a calculated molecular weight of 8,835 (77). It is a potent activator of LPL from both human and rat post-heparin plasma and bovine milk (78).

Apoprotein C-III: This is a single polypeptide chain of 79 amino acid residues with a carbohydrate moiety attached to threonine-74 by an O-glycosidic linkage. The polysaccharide contains one residue of galactose and galactosamine followed by 0 - 2 residues of sialic acid (79), thus accounting for the microheterogeneity observed. Its physiological function is not known, but an inhibition of LPL, not reversible by apoC-II was reported (80). The inhibition however, occurred at higher than physiological concentrations.

d) Apoprotein D

ApoD, so far the only member of this group, was isolated from human HDL (81). It appears to be a glycoprotein (18% carbohydrate) with a molecular weight of about 28,000. A similar "thin-line" peptide was isolated from human HDL and designated as apoA-III. This designation was based on the fact that it was found in all fractions of HDL, together with apoA-II. It was found to be a potent activator of LCAT (50), although this could not be confirmed later (63). A comparison of the amino acid analyses of apoA-III and apoD indicates several differences, including the absence of cystine in apoA-III. In addition, apoA-III contains serine as the C-terminal amino acid, while apoD is reported to have a blocked amino and carboxyl-terminal amino acid. If both apoA-III and apoD are homogeneous proteins, it appears that they must be distinctly different apoproteins. Further studies are needed to establish the similarities or differences between these two apoproteins and their physiological significance.

e) Apoprotein E

ApoE is the only member of this group, and in the past used to be referred to as the "arginine-rich" apoprotein. It was initially isolated from human VLDL (82), and further characterized (83). The molecular weight estimates range from 33,000 (83) to 39,000 (84). ApoE has also been found in rat HDL and its molecular weight estimated to be 35,000 (71). The presence of several polymorphic forms of apoE has been reported in both the human (85) and rat (86). The reason for, and the significance of this microheterogeneity is not known, but the absence of one of the polymorphs (apoE-3) has been reported in patients with familial hypercholesteronemia (85).

f) Apoprotein F

ApoF, one of the newest apoproteins identified, is an acidic apoprotein (pI = 3.7), having a molecular weight of 26,000 to 32,000 (87). It was found in human HDL, and the amino acid analysis revealed the absence of tryptophan. Immunologically, it does not cross-react with any other known apoproteins, and in basic PAGE it migrates to a position similar to that of apoD.

g) Apoprotein G

The latest addition to the collection of the known apoproteins, this apoprotein has been identified in and isolated from human HDL (88). Its reported molecular weight is 72,000 and it too, migrates to a position similar to that of apoD in basic PAGE. It contains appreciable amounts of glucosamine. Nothing is known about its possible physiological role as an apoprotein.

h) Other Apoproteins

In addition to the apoproteins that have been assigned to individual groups, several minor polypeptides of unknown physiological significance have been recently isolated. These include the proline-rich peptide isolated from human chylomicrons (89), and the low molecular weight glycine and serine-rich polypeptides isolated from human HDL (90).

4. Chemical Composition, Size and Distribution of Lipoproteins

Based on their densities and S_f values, the chemical composition of the various lipoprotein classes and their distribution in the serum are shown in Table II and Figure 1 respectively. A brief discussion of the physico-chemical properties of the lipoproteins follows in the next section.

TABLE II

CHEMICAL COMPOSITION OF SERUM LIPOPROTEINS (HUMAN)

	d (g/ml)	S_f	Protein (%)	Triglyceride (%)	Cholesterol (%)	Phospholipid (%)
Chylomicrons	< 0.95	> 400	2	85	5	8
VLDL	< 1.006	20 - 400	10	55	18	17
LDL	1.006 - 1.063	0 - 20	25	10	45	20
HDL	1.063 - 1.21	sediment	50	<5	18	30

The compositional data shown represent averages. The published values for each component may vary (124). Variations in the chemical composition of the different subfractions of each lipoprotein class also exist and are pointed out in the text.

The chemical composition of rat serum lipoproteins is similar but some differences exist, which are discussed in the text.

a) Chylomicrons

Chylomicrons, the principal carriers of dietary TG, are synthesized by the intestine and reach the circulation via the thoracic duct. However, certain compositional changes occur during the passage through the lymphatics. When isolated from lymph, chylomicrons have a diameter of 750-10,000 Å, a molecular weight of $4\text{-}30\times10^9$ daltons, and a flotation of S_f = 400-15,000 (38). While normally absent from fasting serum, their concentration rapidly increases after food ingestion, giving the serum a milky appearance. Triglyceride forms up to 86-90% of the total weight of the human lymph chylomicrons. Phospholipids make up about 9%, cholesterol between 0.5-3%, and the protein moiety about 1-2% of the total weight. Rat lymph chylomicrons have a very similar composition. Both human and rat serum chylomicrons contain a higher proportion of protein (up to 3%), cholesterol and phospholipid (91). Their TG content is proportionately decreased and the appearance of partial glycerides is observed (92). Since chylomicrons have an extremely short half-life in serum (10-15 min. in human and 2-5 min. in rats) (93-96), some of these compositional changes, accompanied by a general decrease in size, can be explained by the presence of partially catabolized particles.

While the protein moiety represents a very small portion of the molecule, the variety of apoproteins found in chylomicrons is unrivaled by any other lipoprotein species. The presence of all of the major apoproteins (A, B, and C) was suggested by Alaupovic et al. (97), who showed that human lymph chylomicrons gave a precipitin reaction with anti-VLDL, LDL, and HDL. More specifically, the apoprotein composition

of human lymph (98) or pleural fluid (99) chylomicrons, determined by gel densitometry and gravimetric procedures is as follows: 12-21% apoB, 7-9% apoA-I, 4.5% apoA-II, and 50-68% apoC. Small amounts of apoE are also found. It should be pointed out however, that scanning bands obtained by PAGE may not give true relative amounts of each apoprotein due to their different chromogenicities; and in light of recent work, it is questionable whether a 100 cm column of Sephadex G-100 will separate the apoproteins well enough for quantitation. In these studies, de novo synthesized apoproteins were not distinguished from the ones acquired after the entry into the lymphatics or the pleural fluid. A good deal of chylomicron apoproteins are acquired from lipoproteins already present in the lymph, but not necessarily synthesized by the gut. Indeed, iodinated apoproteins (apoA, B, and C), injected into the blood reach the lymph through the interstitial fluid (100). Studies where the incorporation of radioactive amino acids into chylomicron apoproteins was followed, indicate that the intestine does not synthesize apoC (101,102). Recent studies have shown that in addition to the acquisition of the apoC peptides in the lymph and serum from HDL (103), a large portion of the chylomicron apoA is rapidly lost to HDL (99). Recently, a proline-rich peptide (PRP) has also been found exclusively in chylomicrons (89). The physiological significance of this peptide remains obscure.

b) Very Low Density Lipoproteins.

Very low density lipoproteins are the main carriers of endogenous TG. Their average concentration in human plasma is 80 mg/dl for women and 150 mg/dl for men (104). In male rats the VLDL concentration was

calculated to be about 70 mg/dl (45). They are smaller in size than chylomicrons, having a diameter of 200-1000 Å, with the bulk of material being between 300-700 Å (105,106). They are usually isolated from fasting serum (to assure the absence of chylomicrons), at d < 1.006 g/ml, with a S_f value of 20-400. Several subclasses of VLDL of decreasing size can be isolated within these limits by density gradient ultracentrifugation. As with chylomicrons, particles of decreasing size contain a decreased proportion of lipid and an increased proportion of protein (107,108).

The chemical composition of VLDL is given in Table II. Protein represents about 8-10% of the VLDL mass, while the remaining 90-92% is lipid with trace amounts of carbohydrate. Of the lipid, TG is the major component, accounting for 55-60%; followed by phospholipid, 20%; and total cholesterol, 15-25%.

Significant sex differences in the lipid composition have been reported (109). Females have less VLDL-TG and cholesteryl ester and a higher proportion of phospholipid. Rat VLDL lipid composition is similar to that of human VLDL, containing an even higher proportion of TG (80%). Total cholesterol contributes about 5% and phospholipid 15% (105).

The elucidation of the protein composition of VLDL was difficult. Initial studies suggested that VLDL contained only one distinct protein, similar to that of LDL (110,111). It soon became apparent that this was an oversimplification. Today it is recognized that qualitatively, human VLDL contains several apoproteins, including apoB, apoC-I, apoC-II, and several polymorphic forms of apoC-III and apoE, as well as trace amounts of apoA (40,85,112-116). The reported apoprotein composition

of human VLDL is considerably variable: 40-60% apoB, 30-50% apoC, and 10-15% apoE, with trace amounts of apoA.

Over the years it became evident that rat apoproteins are very similar to human apoproteins structurally and functionally and thus direct comparisons can often be made. Recent reports on rat VLDL apoprotein composition established that it is qualitatively very similar to that of human VLDL (117-121), and contains 23-40% apoB, 30-60% apoC, and variable amounts (13-30%) of apoE.

The values obtained in the various studies undoubtedly depend on the particular technique used and the size distribution of the VLDL particles analyzed. Differences in size result not only in variations in the total protein content of VLDL, but also in its apoprotein composition. With decreasing particle size the proportion of apoB increases while that of apoC decreases (116). It should be noted that even in normal individuals the apoprotein composition can be altered by diet (such as cholesterol), and that some of the disorders of lipoprotein metabolism are related to abnormal apoprotein composition.

c) Low Density Lipoproteins

Serum LDL is operationally defined as the material isolated between d = 1.006 - 1.063 g/ml. In the past they were referred to as β-lipoproteins. Even though LDL, with respect to size and composition, appear to be much more homogeneous than VLDL, they can be resolved into two major components: IDL or LDL_1 (d = 1.006 - 1.019 g/ml, S_f 12-20), and a second fraction containing most of the material, called LDL_2 (d = 1.019 - 1.063 g/ml, S_f 0-12). The second fraction may also contain a minor HDL contaminant at the denser end of the spectrum (S_f 0-3). The IDL is intermediate between the VLDL and LDL_2 in chemical composition and size also.

As determined by electron microscopy, normal human LDL appears as a collection of almost spherical particles, 80% of which are between 210-250 Å in diameter (123). The average serum concentration of LDL, the most abundant lipoprotein in the human serum, is 400 mg/dl and 340 mg/dl in fasting adult males and females respectively (124).

The rat, like most other mammalian species, has very low levels of serum LDL, about 70 mg/dl (45). Low density lipoproteins carry the bulk of lipid in the human plasma. They contain approximately 75% lipid and 25% protein (124). The lipid composition is shown in Table II. While apoB represents about 90% of the total protein, small amounts of apoC, apoA and apoD are also found in LDL (115).

The contribution of the non-apoB proteins varies. In the fraction where over 90% of all the LDL apoB is found (d = 1.019 - 1.05 g/ml), the contribution of apoC and apoA is minimal. As expected, the significant portion of apoC in LDL is found in the IDL region, and that of apoA in the region overlapping with HDL (d = 1.05 - 1.063 g/ml) (115).

d) High Density Lipoproteins

High density lipoproteins are isolated from the serum at d = 1.063 - 1.21 g/ml. They are also referred to as α-lipoproteins, based on their electrophoretic mobility in paper or agarose. Smallest of the lipoproteins, they have diameters of 90-120 Å (125). Their concentration in normal human plasma is about 260 and 330 mg/dl in males and females respectively (126). A similar concentration is found in the rat (45), where HDL is the major cholesterol-carrying protein. High density lipoprotein levels are relatively high (in comparison with LDL), in animals that are resistant to artherosclerosis, such as dogs and rats, but are almost non-exisent in the guinea pig, which is quite susceptible to this disease.

About 50% of the HDL mass is protein, 30% phospholipid, and 20% cholesterol. The ratio of esterified to free cholesterol is about 3:1.

High density lipoproteins can be divided into two density classes: HDL_2 (d = 1.063 - 1.125 g/ml), and HDL_3 (d = 1.125 - 1.210 g/ml) (127). The latter contains a higher proportion of protein (55%), and the ratio of esterified to free cholesterol is also higher. It is now well accepted that these two fractions are not ultracentrifugal artifacts, but their physiological relationship and significance are unknown. Heterogeneity within HDL can also be shown by analytical and preparative gel electrofocusing (128,129) and ion exchange chromatography (130).

The protein moiety of HDL is composed of at least two groups of apoproteins: apoA and apoC. ApoA-I and apoA-II contribute about 90% of the total HDL protein, with a ratio of 3:1 in both HDL_2 and HDL_3. ApoA-III and/or apoD is a minor constituent of HDL (70,81). ApoC is present in the HDL fraction in small amounts, comprising 5-10% of HDL_2, and 1-2% of HDL_3 protein (131,132). All three of the apoC apoproteins are present. It should be noted that in absolute amounts, the apoC present in HDL accounts for more than 50% of the total apoC pool in normal fasting plasma. Small amounts of apoE are also found in HDL.

The apoprotein composition of rat HDL is similar to that of human. While apoA-I represents 50-60% of the protein moiety, only small amounts of the monomeric apoA-II are found. ApoC contributes 15-20%, while apoE and apoA-IV about 10-15% each, to the rat HDL apoproteins (71,120,121).

Ultracentrifugally isolated HDL from most individuals contain, in the d = 1.050 - 1.12 g/ml fraction, small but variable amounts of Lp(a) lipoprotein (133). In lipid composition it is similar to LDL, but it

contains more protein. The distinguishing feature of this lipoprotein is that it contains, in addition to large amounts of apoB and variable amounts of other apoproteins, a specific Lp(a) protein. This large glycoprotein exhibits high affinity for apoB and is also found in trace amounts in the other apoB-containing lipoproteins. It appears to be synthesized independently of the lipoprotein apoproteins, and its role is not known (134).

It should be pointed out that substantial amounts of apoB in the d = 1.060 - 1.21 g/ml, are found in the sera of patients with familial LCAT deficiency (135). An apoB containing lipoprotein has also been isolated from rat liver perfusates at d = 1.075 - 1.175 g/ml (136).

The finding of apoB-containing lipoproteins in the HDL density range illustrates particularly well the shortcomings of isolating the various lipoprotein species on the basis of their hydrated densities. It is likely that the distribution of apoB and other apoproteins in the density fractions of the serum is determined to a large extent by the amount and kind of lipid it is associated with. Ultracentrifugally isolated lipoproteins may thus contain different lipoprotein species of the same hydrated density.

e) Other Lipoproteins

As a result of improved lipoprotein detection techniques, additional lipoprotein species, often associated with abnormal conditions have been isolated from the sera of humans and some animal species.

i. Lipoprotein(a)

Even though this lipoprotein exhibits many of the properties of LDL, Lp(a) is considered a distinct lipoprotein species. It can be isolated from plasma at d = 1.05 - 1.120 g/ml, and exhibits a pre-β mobility on

agarose gel electrophoresis (134). Although its lipid composition is similar to LDL, its apoprotein composition is quite different: it contains 65% apoB; about 15% apoC, albumin and other, uncharacterized proteins; and 20% is a specific Lp(a) protein (137-139). In earlier studies, Lp(a) could be detected in only a fraction of the normal population; however recent studies, using more sensitive immunological techniques, have shown the presence of this lipoprotein in over 90% of the normal population. A polygenic inheritance was suggested to be responsible for its serum concentration, which did not correlate well with age, sex, lipid concentration, or occurrence of CHD (140-142).

ii. Lipoprotein(X)

This abnormal lipoprotein is found in patients with biliary obstruction and familial LCAT deficiency. The major protein components are albumin and apoC. Phospholipid and unesterified cholesterol are the predominant lipids (143-145). It may be isolated from LDL by zonal ultracentrifugation or hydroxyapatite chromatography (146), and quantitated by electrophoresis in agar (147). When examined by electron microscopy, it appears to have a discoidal shape (148).

iii. β-Very Low Density Lipoproteins and High Density Lipoproteins$_c$

These two abnormal lipoproteins have been isolated from a variety of hypercholesteronemic animals, such as rabbits, guinea-pigs, dogs, monkeys and rats (32,149-154). The hydrated density of HDL$_c$ depends on the amount of cholesterol, its major lipid component. The chief apoprotein component of HDL$_c$ are apoE and apoA-I.

β-very low density lipoproteins are found in the VLDL density range, but exhibit abnormal, β-mobility in agarose electrophoresis. Cholesterol and small amounts of TG make up the lipid moiety of β-VLDL, while the

major apoproteins are apoB and apoE, with small amounts of apoC. A similar lipoprotein has been isolated from humans with type III hyperlipoproteinemia, and it was suggested that they represent an accumulation of VLDL remnants (155). The precise relationship between the B-VLDL found in animals on a high cholesterol diet, and that of type III hyperlipoproteinemia in the human is uncertain, but the possibility that B-VLDL is also a VLDL remnant has been proposed (152).

A recent study of apoB and apoC metabolism in several normal and hyperlipoproteinemic subjects (156), confirmed the stepwise delipidation of VLDL into IDL, and ultimately to LDL, and suggested that in type III patients an increased synthesis (two-fold) of VLDL apoB, as well as some induced synthesis of IDL or LDL apoB occurs. The rate of VLDL catabolism was decreased and much of it was catabolized in a different manner, producing an LDL-like particle of VLDL density, β-VLDL, removed from the circulation without LDL formation.

In summary, perturbations in lipoprotein metabolism and the appearance of unusual lipoproteins can be caused by many factors, some of which may be: the synthesis of lipoproteins of abnormal composition that may or may not be catabolized at a slower rate: a decreased catabolic rate of lipoproteins due to a fault in their removal system (for instance, the absence of specific cell receptors), resulting in the accumulation of catabolic products; and a decreased rate of removal of a "faulty" degradation product, due to the absence of an additional enzyme(s) necessary for further degradation or alteration prior to removal from the circulation.

5. Structure of Lipoproteins

a) Triglyceride-Rich Lipoproteins

While ultracentrifugation studies have been particularly valuable in defining such physical parameters as flotation and sedimentation rates, hydrated densities, and molecular weights of each lipoprotein species, they are of limited use in morphological and structural investigations. Electron microscopy is useful in determining the size and some morphological properties of lipoproteins, but it does not provide any information about the organization of the lipid and protein moieties within the particle. Several spectroscopic techniques, such as Optical Rotary Dispersion, Circular Dichroism, Infra-Red Spectroscopy, as well as Nuclear Magnetic Resonance and small angle X-ray scattering, have been used in determining the structure of LDL and HDL. These techniques, however, have limited application in determining the structure of chylomicrons and VLDL because of heterogeneity in size and composition, as well as their significant turbidity in solution.

As early as 1962, a model was proposed for the TG-rich lipoproteins, consisting of a lipid core and surface film of protein and phospholipid, resembling a micelle-like structure (157). It was reasoned that one possible manner in which chylomicrons could become enveloped in a layer of protein and polar lipid, was a process where a piece of plasma membrane was "pinched-off" during the extrusion of the lipid particle from the intestinal cells into the extracellular spaces. If this mechanism operates in chylomicrons, one would expect to see "unit membranes" adhering to the oil droplets. Such membranes could not be visualized by electron microscopy. However, an electron-dense surface material, about 20 Å thick surrounding the chylomicrons was observed (158).

Repetitive freezing and thawing, or rotary evaporation and rehydration techniques were utilized to separate the surface material from the lipid core. Eighty percent of this material was lipid, 60-75% of which was phospholipid. Small amounts of TG, DG, fatty acids and cholesterol were also found, but no cholesteryl esters were detected. The lipid core on the other hand, contained TG and cholesteryl ester exclusively, without detectable phospholipid (159-161).

If one assumes that a surface layer of 20 Å is present on chylomicrons and VLDL, the portion of surface covered by protein is constant at about 20%, while the size varies. The increase in the surface-to-volume ratio as particle size decreases, is compensated by an increase in the weight percentage of the protein (38). This progressive increase of the polar components in general, with the decrease in particle size and molecular weight, is a phenomenon common to both chylomicrons and VLDL (162).

The concept that TG and cholesteryl ester occupy the inner core of the VLDL particle is now well accepted, and is supported by theoretical calculations (163). No distinction can be made between several structural arrangements of the protein within the surface lipid components, however. The protein moiety could orient itself in a monomolecular film completely within, or completely outside the interfacial lipids, and is only partially capable of surrounding the lipid core. Alternatively, it could form discrete units, or "islands" within the surface lipids.

The structure of VLDL is of particular interest, since there is good evidence that they are the precursors of LDL. Elucidating the structural relationship between these two lipoproteins would facilitate the understanding of the interconversion process.

b) Low Density Lipoproteins

While at low magnifications normal human LDL appears spherical, at higher magnifications the images reveal some structural detail of the particle surface. A three dimensional isodensity map constructed from high image enlargement, reveals an overall icosahedral symmetry consistent with a model in which the protein forms a network over the particle surface; leaving open, slightly twisted pentagonal faces, filled with phospholipids (164). Small angle X-ray scattering (165,166) and NMR (167) studies have been particularly useful. One model proposed a spherical phospholipid bilayer, with an average radius of 65 Å (166). The outer surface of the bilayer is covered by 60 protein subunits organized in an icosahedral symmetry. Free cholesterol and cholesteryl esters are equally distributed on both sides of the bilayer. In this model, the core of the particle, inside the bilayer, is protein. A similar model proposed a trilayer of lipid with the phospholipid polar groups localized on both sides, surrounding a protein core (167).

More recent small angle X-ray scattering studies led to a modified model, consisting of a hydrocarbon chain core with an outer shell sparsely occupied with protein molecules, emerging from the lipid core. The core lipids are organized in a micelle-like structure, with the steroid nuclei segregated in regions distinct from those occupied by the hydrocarbon chains (168,169).

The following organization of the LDL lipids was recently proposed: the core is occupied by cholesteryl esters, and a band of the apolar hydrocarbon chains is followed by a narrow band of the sterol rings. This structure is repeated once again, possibly with a reverse orientation of the cholesteryl esters. The core is then surrounded by a band of

protein (20 Å thick), polar groups of phospholipid, and free cholesterol. An ordered liquid crystalline structure of the core is observed at 4° C, but increasing temperature causes a thermotropic transition into a disordered state (170).

c) High Density Lipoproteins

Several models for HDL have been presented in the literature. Based on low angle X-ray scattering data, both HDL_2 and HDL_3 have two regions of different electron density; and thus a model with a central lipid core surrounded by a thin outer shell, containing phospholipid and protein is generally accepted (171). The assignment of the phospholipid and protein to the outer shell seems reasonable, as over 90% of the ϵ - NH_2 groups of lysine in HDL are susceptible to succinilation, and both the protein and phospholipid of HDL are accessible to proteolytic and lipolytic enzymes (172,173).

Several arrangements of the protein and phospholipid have been proposed. In one model, about half of the surface is covered by protein and the other half by the polar head groups of phospholipids and cholesterol, organized in a monolayer (174). In a different model, the HDL protein is depicted as an "iceberg" floating in a "sea of lipids", similar to the fluid mosaic model of the membrane. The amphipathic portions of the apoproteins may be oriented parallel to the fatty acid chains of the phospholipids, thus extending well into the neutral lipid core (175); or perpendicular to the fatty acid chains of the phospholipids (68). In this case the protein would not extend far into the core, which would be more consistent with the low electron density observed.

6. Lipoprotein Families

While the A, B, C nomemclature and the concept of apoprotein families was described earlier in this chapter, it can be further expanded and applied to lipoproteins. It was Oncley in 1963 (176) who suggested that,

> "...there may be present among serum proteins a limited number of apolipoproteins with highly developed binding capacities for lipid, giving rise to lipoprotein 'families' - each completely specific insofar as their protein moiety but exhibiting considerable heterogeneity in regard to lipid content, lipoprotein density and size."

The fact that the apoprotein moiety may be the only specific and distinguishing mark of lipoproteins was also recognized by Alaupovic. An attempt to integrate this concept with the observed presence of several apoproteins in any one density region, has resulted in a definition of lipoprotein families as polydisperse systems of lipid - apoprotein associations, characterized by the presence of a single, distinct apoprotein or its constitutive polypeptides. Furthermore, depending on the composition and amount of lipid complement, each lipoprotein family may be found at various density regions and thus occur simultaneously in several segments of the density spectrum (177). For example, while HDL is normally thought of as a collection of particles in the density range of 1.063 - 1.21 g/ml, each having a certain heterogeneous apoprotein and lipid composition, one can also visualize it as a collection of several distinct kinds of lipoprotein particles, each composed of a specific apoprotein or apoprotein family with the appropriate amount of lipid for this density.

This concept further envisages the presence of free, primary lipoprotein particles (such as LpA, LpB, LpC, LpD, and LpE) at a $d > 1.030$ g/ml; and secondary, associated forms, such as LpB,C,E, (VLDL) in the

d < 1.030 g/ml region of the serum (115). Clearly then, the concept hinges on the demonstration of primary particles in the serum. Alaupovic and coworkers drew heavily on immunological techniques for this purpose. The identification of the two primary particles, LpA and LpB, was relatively easy due to clear nonidentity of these particles by immunodiffusion and immunoelectrophoresis, as well as to the ready separation of the two particles by ultracentrifugation (LpB is confined to d < 1.070 g/ml and LpA is found predominantly in d > 1.070 g/ml). Recently, other primary lipoprotein particles have been isolated from the HDL density range (81,178). Similarly, the presence of primary and secondary (free and associated) lipoproteins in the rat serum has also been suggested (179).

Undoubtedly, a broader acceptance of this concept will depend on demonstrating the presence and physiological function of the primary particles in the course of lipoprotein metabolism under physiological conditions.

In the studies of VLDL catabolism reported in this thesis the concept of lipoprotein families will occasionally be useful in interpreting the results. The system utilized to study VLDL catabolism in the reported experiments is devoid of other lipoproteins capable of acting as acceptors for some of the apoproteins. If, as a result of VLDL catabolism in the absence of an acceptor, some of the apoproteins are removed from the VLDL, but retain enough lipid to have a d < 1.21 g/ml, they could be thought of as primary lipoproteins.

C. LIPOPROTEIN METABOLISM

1. Synthesis of Triglyceride-Rich Lipoproteins

It is believed that the intestine and liver are the two organs involved in the biosynthesis of lipoproteins. The synthesis of lipoproteins by the perfused liver was demonstrated as early as 1955 (180). Since that time numerous investigators have shown that the perfused liver (48,101,181-186), liver slices (187-189), and isolated hepatocytes (190,191) are capable of synthesizing VLDL and HDL. Isolated rat liver ribosomes can also synthesize lipoprotein apoproteins (192). The importance of the intestine in the synthesis of chylomicrons, VLDL and HDL was established by Roheim et al. (193) and confirmed by the use of perfused intestine (101,102).

The liver develops as an outgrowth of the primitive foregut, and the hepatic parenchymal cells share many morphological features with the absorptive cells of the small intestine (194). Thus the subcellular pathways of chylomicron and VLDL assembly and secretion share many features which will be discussed together.

In the intestinal cells, TG is resynthesized from the absorbed MG and free fatty acids from the intestinal lumen, and some fatty acids from the portal circulation. There are two pathways for the TG resynthesis in the mucosal cells. The MG pathway, peculiar to the intestinal mucosa and the adipose tissue (195), involves the direct acylation of the absorbed MG with activated free fatty acids. The three enzymes necessary for this pathway are located in the microsomal fraction in the form of a multienzyme system, the TG synthetase (196). The α-glycero-phosphate pathway, present in most tissues, including the liver, involves the acylation of the glycerophosphate to phosphatidic acid, dephosphorilation

of phosphatidic acid to form DG, and further acylation to form TG.

While the majority of the TG synthesis by the intestinal cells occurs via the MG pathway, the α-glycero-phosphate pathway, normally inhibited by the MG present (195), may contribute significantly, especially when an excess of free fatty acids is presented to the cell and the amounts of MG are diminished. In any case, fatty acid utilization for TG resynthesis requires its activation by the formation of a CoA derivative of the fatty acid. This energy-requiring reaction is catalyzed by an enzyme, fatty acid:CoA ligase which has a marked specificity for long-chain fatty acids (195). Thus long-chain fatty acids appear in the thoracic duct TG while short and medium-chain fatty acids are transported, bound to albumin, in the portal circulation.

The biochemical events in the liver differ from those in the intestine. During feeding, glucose, amino acids, and short and medium-chain fatty acids rise in the portal circulation. Glycogen is synthesized from glucose-6-phosphate until the hepatic storage capacity is filled. If portal input of nutrients persists, glucose is converted into fatty acids via acetyl CoA. Excess amino acids are deaminated and also contribute to the acetyl CoA and pyruvate pool. Part of the acetyl CoA undergoes further oxidation via the TCA cycle for energy production, but most of it is used for de novo synthesis of fatty acids or elongation of the short and medium-chain fatty acids. Another source of fatty acids and primarily dietary cholesterol are the partially catabolized chylomicrons (remnants), rapidly removed from the circulation by the liver (7,8,9).

During fasting, the primary source of fatty acids for the liver are those released from the adipose tissue by the action of a hormone-sensitive lipase (197). Fatty acids released from the adipose tissue are removed

by the liver in proportion to their concentration in the blood and serve, through their oxidation to ketone bodies, as an important source of energy for gluconeogenesis (198). Amino acids mobilized from the muscle tissue can also be used for glucose production. Because the liver is primarily involved in maintenance of blood glucose levels necessary for central nervous system functioning during fasting, TG synthesis is diminished markedly (198).

Before the secretion of TG by the intestinal and liver cells can occur, it must be assembled together with cholesterol, phospholipid, and the specific apoproteins, into chylomicrons or VLDL. The point at which the newly synthesized lipids and apoproteins associate into lipoproteins during this assembly is uncertain. Large pools of apoB and apoA-I, possibly in association with lipid, in the apical portions of the mucosal cells, followed by a further increase throughout the cell during absorption, indicate the presence of presynthesized apoproteins in the intestinal cells and suggest an early association with the lipid in chylomicron formation (199,200). As apoB appears to be essential for TG secretion (in abetalipoproteinemia the absence of apoB synthesis is accompanied by a complete absence of the usual TG-rich lipoproteins in the serum), and large pools of the slowly turning over apoB are present in the intestinal cells; inhibition of protein synthesis markedly diminishes the rapidly utilized apoA, but does not have a profound effect on chylomicron or apoB synthesis (102).

The presence of large pools of presynthesized apoproteins in the liver cells is uncertain, since puromycin causes total inhibition of VLDL secretion (201,202). However, a lag period between the administration of puromycin and the cessation of VLDL secretion indicates an early

association of the protein and lipid moities in VLDL assembly as well. The lag period in amino acid incorporation into VLDL apoproteins, but not their secretion, also indicates early protein and lipid association (182).

The biochemical aspects of lipid and apoprotein synthesis in chylomicron and VLDL production were extensively investigated but the ultrastructural and morphological aspects of their formation and transport within the cell and their extrusion has only become evident in the past few years. The endoplasmic reticulum represents a complex intracellular system of tubular structures, which channel biosynthetic products to other organelles, while providing an environment that facilitates biochemical transformation of the transported substances (203). The rough endoplasmic reticulum is the site of apoprotein synthesis. Recent evidence suggests that phospholipids are also synthesized here, while the smooth endoplasmic reticulum is the site of TG synthesis (204). The apoproteins, together with the newly synthesized phospholipids move along from the rough endoplasmic reticulum to the cisternea of the smooth endoplasmic reticulum, where the synthesis of TG and possibly cholesteryl esters occurs. Indeed, immediately after fat ingestion, osmiophilic droplets in great quantities occupy the cisternea of the smooth endoplasmic reticulum (205,206). This process results in the formation of an almost complete secretory product. The nascent chylomicrons or VLDL are then transported within the channels of the endoplasmic reticulum, to the Golgi apparatus in the preparation for the final assembly and secretion (205). It is evident from many investigations that the Golgi serve an essential function in the final assembly of cellular secretory products (203). In the case of lipoproteins, final assembly would involve the addition of the sugar

moieties to the apoproteins.

The importance of the Golgi apparatus, specifically in glycosylation of glycoproteins has been demonstrated (207-210). The preferential incorporation of glucosamine into chylomicrons by isolated Golgi fractions *in vitro* (211), and increasing glycosylation of hepatic VLDL during the passage through the Golgi *in vivo* (212), support this hypothesis. It is of interest that orotic acid, known to cause decreased hepatic levels of adenine and cytidine nucleotides (213), necessary for adequate sialylation of proteins, causes massive accumulation of VLDL in the liver cells (214); first within the Golgi and later throughout the cell (215). Why orotic acid has little effect on the secretion of intestinal lipoproteins is not known.

The mechanism of the translocation of the secretory vesicles to the plasma membrane is not clear. Recent evidence suggests that the microtubular filaments, in addition to functioning during cell mitosis and having important cytoskeletal function in cell shape maintenance, may also be important in the directed movement of the secretory vesicles towards the cell membrane (216). This concept is supported in the case of chylomicrons and VLDL in studies using colchicine, a known inhibitor of microtubular polymerization. This agent causes an accumulation of chylomicrons and VLDL of normal apoprotein composition within the Golgi apparatus (217,218), and their excretion is greatly delayed.

Recent electron microscopic studies (205) demonstrated the occurrence of microfilaments in close proximity to secretory vesicles filled with chylomicrons, but they were observed infrequently and not in a specific relationship to them. Additional work is required to assess the importance of these filaments in lipoprotein secretion. Fusion of the

secretory vesicle with the plasma membrane, followed by exocytosis of chylomicrons has been observed (205), and it is reasonable to assume that a similar process is responsible for VLDL secretion.

The appearance of chylomicrons in the intercellular space is followed by their passage into the lamina propria through gaps or discontinuities in the basement membrane, and into the lymphatics through gaps between the overlying processes of adjacent endothelial cells (205). Hepatic VLDL are first secreted into the space of Disse from which they reach the liver sinusoids and the circulation (194).

Although direct demonstration of a precursor-product relationship between VLDL apoproteins in the liver Golgi and the serum was recently demonstrated, Golgi VLDL contain only trace amounts of apoC (218). Since substantial amounts of this apoprotein are present in the liver perfusate VLDL (182), it must be acquired at a later stage of secretion, or even in the space of Disse. Because the intestinal cells are not capable of synthesizing apoC at all (101), nascent chylomicrons must acquire this apoprotein while in circulation, from the large pool present in HDL (103). In addition, most of the apoA present in nascent chylomicrons is rapidly lost to HDL in the circulation (99).

2. Catabolism of Triglyceride-Rich Lipoproteins

The catabolism of the TG-rich lipoproteins is complex due to the presence of several protein and lipid components. These are catabolized by different tissues, mechanisms, and at different rates. In addition, there is a dynamic equilibrium of some of the protein moieties between the different lipoprotein classes. For instance, an *in vitro* exchange of the apoC in association with phospholipid has been observed between VLDL and HDL (19). Low density lipoprotein does not participate in this

process. Besides the rapid exchange of the apoC, there also exists a transfer of these apoproteins from HDL to the newly synthesized TG-rich lipoproteins (103). The magnitude of this transfer is determined by the plasma TG concentration.

a) Remnant Formation

The first step in the catabolism of the chylomicrons and VLDL involves the action of LPL. Inhibition of the enzyme with specific antibodies prevents the catabolism of VLDL (219). Under normal physiological conditions, the serum contains only traces of lipolytic enzymes. Injection of heparin releases high amounts of LPL; and the effect is particularly dramatic when heparin is injected into subjects during alimentary lipemia, which is characterized by a milky appearance of the plasma. Plasma clears within minutes, and thus the enzymes released by heparin were originally referred to as the heparin-induced "clearing factor lipase" (220). The release of LPL from the endothelial cells of the capillaries can be inhibited by concanavaline A and colchicine (221).

The enzyme has been extracted and purified from a variety of sources, including human and rat adipose tissue (20,222,223), heart (21,224), pig adipose tissue (225), and bovine milk (226). The solubilization of the rat heart LPL by heparin perfusion does not alter the kinetic properties of the enzyme (22). The reported molecular weight varies from 60,000 to 70,000. Rat post-heparin plasma contains two LPL: one is a low molecular weight (37,000), high affinity LPL, released from the heart (227); and the other is a low affinity LPL, released from the adipose tissue (228). The low affinity LPL has a high molecular weight (69,000), and different amino acid and hexosamine composition than the purified heart LPL. Both are inhibited by 1 M NaCl and protamine sulphate,

and require an activating factor in VLDL (228). The activating factor necessary for all LPL is now known to be apoC-II (78), while apoC-III is believed to inhibit the enzyme at higher than physiological concentrations (82). TG emulsions (229) or even purified TG (230) can be hydrolysed by the LPL only in the presence of serum.

It seems likely that the activity of LPL corresponds to the physiological requirements of the various tissues and it may be ultimately shown that there are a number of LPL isozymes, related to the function of different tissues. For example, the low affinity LPL associated with the adipose tissue may function when the supply of chylomicrons and VLDL is high and fatty acids are stored, whereas the high affinity enzyme of the heart functions when the supply of the circulating lipoproteins is low and energy is required. Thus the heart LPL activity is increased during starvation, and the adipose tissue LPL is increased in the post-prandial state (231,232). During suckling, the mammary gland is most efficient in clearing chylomicron lipids, whereas in the non-suckling state, adipose tissue has the highest LPL activity (233).

The post-heparin plasma contains two lipases, one having the characteristics of LPL described above, and a second one which does not require activation by a serum factor and is activated, rather than inhibited, by 1 M NaCl (234). The second enzyme has been isolated from the liver, and is not found in the post-heparin plasma of hepatectomized pigs (235). It is usually referred to as the hepatic lipase. In addition to the LPL and hepatic lipase already described, the presence of another lipase in post-heparin plasma has been reported (76). This enzyme is activated by apoC-I and is not present in the adipose tissue or post-heparin plasma

of type I hyperlipoproteinemic patients.

A number of observations suggest that the LPL is located on the luminal surface of the endothelial cells of the capillaries. Among them are: histo-chemical evidence of lipolysis in the capillary lumen, electron micrographs of partially digested adherent chylomicrons, and the rapid appearance of this enzyme in heparin-containing perfusates (236,237). Relatively little is known about the mode of interaction of the TG-rich lipoproteins with the membrane-bound LPL. Based on electron microscopy and biochemical studies, it has been proposed that the initial TG hydrolysis occurs within 1-2 minutes after attachment of the chylomicrons to the capillary endothelial cells, producing fatty acids, MG and DG. Some of the fatty acids are released into the circulation. The mechanism by which the remaining hydrolytic products cross the endothelial membrane and eventually reach the surrounding tissue is not clear. It has been proposed that they move from the chylomicrons to the endothelial cells by lateral diffusion in a continuum created by the fusion of the chylomicron surface film and the external portion of the plasma and intracellular membranes of the cell (238). It is possible that the hydrolytic products are then carried within the cellular membranes to other tissues surrounding the endothelial cells, if a concentration gradient is present. It should be pointed out that little experimental evidence is available to date to support this hypothesis.

The action of LPL on chylomicrons and VLDL results in a production of remnant particles of considerably altered chemical and physical properties. Post-heparin plasma (239,240), supradiaphragmatic (105) and hepatectomized rats (8,241,242) and perfused rat hearts (9), have been used as a source of LPL to evaluate the characteristics of the rat TG-

rich lipoprotein remnants. Rat chylomicron and VLDL remnants show a considerable decrease in size and TG and apoC content (9,105,240). Although the proportions of cholesterol, cholesteryl ester and phospholipid increase, a net loss of these substances occurs during lipolysis (9,105,240). Concomitant with these changes, the mass contribution of apoB in the remnant particle increases, but the absolute amount does not change, suggesting that the apoB remains with the particle during the delipidation stages (243).

No information is available about the composition of human VLDL and chylomicron remnants, but the analysis of increasing density fractions of VLDL isolated from normal serum also indicate a gradual decrease in TG and apoC content, and increased proportions of cholesteryl esters and apoB as the particle size decreases (243). The distinction between remnants and smaller, intact VLDL in the human serum is difficult to make since no information is available about the variations in chemical composition of newly secreted VLDL of decreasing size. A precursor-product relationship has been observed for the in vivo transformation of large VLDL particles (S_f > 100), into smaller ones (S_f 20-100) as a result of LPL action (244). A stepwise delipidation scheme was implied: the VLDL particles lose some of their TG at the delipidation site, and reappear in the plasma as VLDL particles of higher density and smaller size.

It is of interest that in about 50% of individuals, VLDL particles of remnant-like characteristics can be isolated electrophoretically (a slow pre-β mobility), containing a higher proportion of apoB and apoE with reduced amounts of apoC and much increased cholesteryl ester: TG ratio. While this slower migrating pre-β VLDL resembles remnant VLDL, it is not unlike the abnormal, β-migrating VLDL present in large concen-

trations in patients with type III hyperlipoproteinemia (245). These observations led to the hypothesis that in these patients, the abnormal β-migrating VLDL represents an accumulation of VLDL remnants. Indeed, recent studies of apoB decay in the circulation of type III patients suggest the presence of an alternate pathway for VLDL degradation, producing β-VLDL (156,246). A precursor-product relationship between the normal and β-VLDL TG (247) and apoB (246,248) has been shown. Interestingly, none of the injected ^{125}I - VLDL apoE transfered to β-VLDL in type III patients, in spite of the fact that apoE is a major constituent of β-VLDL (248). Thus, direct hepatic synthesis of β-VLDL cannot be ruled out at this time.

A sizable reduction of the TG-rich lipoprotein core during lipolysis must be accompanied by a reduction of the surface components as well, if the spherical shape is to be preserved. While the loss of apoC, phospholipid and cholesterol may account for some of the reduction, the possibility also exists that the action of a second enzyme, the LCAT, which is normally present in the serum and responsible for cholesterol esterification, is necessary (249). Support for this hypothesis comes from observations of abnormal LDL particles with seemingly excessive surface coat in patients with familial LCAT deficiency. Upon incubation of the patients' serum with LCAT, much of the excessive coat disappeared. This was accompanied by a net transfer of cholesteryl ester to LDL and VLDL (135). Since LCAT does not react directly with VLDL (250), the increment in cholesteryl ester content of VLDL may occur through a transfer of cholesteryl esters from HDL, possibly mediated by apoE. The apoE content of VLDL of the LCAT-deficient patients increased on incubation with LCAT, at the expense of apoB and apoC (135). It is possible that the apoE-choles-

teryl ester complex displaces the apoC-phospholipid complex, resulting in a significant decrease in the surface to coat ratio.

The catabolism of the TG-rich lipoproteins thus appear to follow a common initial pathway of TG depletion. This is accompanied by some significant changes in lipid and apoprotein composition as well as a decrease in the mean particle size. At this point however, the fate of chylomicron and VLDL remnants diverge and will be discussed separately.

b) Fate of the Chylomicron Remnants

Rat chylomicron remnants produced by the action of the extrahepatic LPL are rapidly removed from the circulation by the liver. Chylomicron remnants prepared by incubation with post-heparin plasma, perfusion through rat hearts, or isolation from hepatectomized rats are rapidly removed by the liver of the intact rat, isolated perfused liver, or hepatocyte suspensions (8,9,239,241,242). The uptake of chylomicron remnants by the perfused liver or hepatocytes is significantly greater than that of intact chylomicrons (9,239,241,242).

The reason for the preferential uptake of the remnants and the nature of this process has been investigated. It was found that neither size nor gross lipid composition alone, at least as manifest by the total lipid to cholesterol ratio, was of major importance in determining the rate of hepatic uptake of the particle (251). Furthermore, two types of hepatic uptake of chylomicron particles were found. The first of these was a low velocity transport system capable of clearing intact chylomicrons from the perfusate. The rate of uptake was linear with time, and independent of the size of the chylomicrons. This process presumably reflects nonspecific endocytosis of unmetabolized chylomicrons by the liver, and is of such low velocity that it plays a small physiological

role. The second transport process involves the uptake of remnant particles. The uptake was linear and showed apparent saturation kinetics with respect to concentration. The particles appeared to be taken up intact by a process having a very high activation energy. This suggests an attachment of the particles to a finite number of receptor sites on the sinusoidal membrane, followed by a translocation by a process such as endocytosis (251). Indeed, the removal of the whole remnant particle has been suggested by other investigators (9, 239). The remnant-derived cholesterol causes a significant suppression of de novo cholesterol synthesis within the liver cells (252).

Although the outlined pattern of chylomicron catabolism has been observed in the rat, it has not been directly demonstrated in the human. Recent work by Schaeffer et al. (99) represents an initial attempt to follow the fate of the chylomicron apoproteins in the human. Using ^{125}I-labelled chylomicrons injected directly into the circulation and correcting for the initial rapid equilibration of some of the apoproteins, they found that the apoA-I radioactivity was rapidly removed from the chylomicron fraction and recovered in the HDL. The decline in the chylomicron apoC activity was significantly slower than that of apoB, reflecting the continuous reassociation of the apoC with the newly secreted chylomicrons. While only a small proportion (18%), of the chylomicron apoB was recovered in the LDL density range, no attempt was made to explain similar recoveries of this apoprotein in the VLDL and IDL fraction. If human VLDL catabolism results in LDL production, presumably through IDL (10-13), then in fact the chylomicron apoB found in VLDL and IDL may also be converted into LDL apoB. It is interesting that the decay of chylomicron apoB from the VLDL density range appears to be

slower than that reported for VLDL apoB (156,246). It is possible that the chylomicron particle is metabolized in the human in a more complex manner. While the labelled lipid is rapidly removed from the circulation, a significant portion of the chylomicron apoB remains in the circulation for prolonged periods. The posibility that a significant portion of the chylomicron apoB is catabolized to LDL or remnants of densities similar to VLDL, IDL, or even LDL cannot be excluded. Obviously, additional experimental data are required to establish the fate of human chylomicron apoproteins in the circulation.

c) Fate of the Very Low Density Lipoprotein Remnants

On the basis of the experimental evidence presented in the previous section, the catabolism of chylomicrons, at least in the rat, can be divided into two distinct stages: one of extrahepatic hydrolysis of the TG, followed by the hepatic uptake of the resulting remnant.

Very low density lipoproteins share with chylomicrons the first stage of the catabolism ; the extrahepatic removal of much of their TG and considerable amounts of cholesterol and phospholipids. Significant changes in the apoprotein composition, already described, also occur. Since the exposure of both chylomicrons and VLDL to LPL results in qualitatively similar compositional changes in the particle, the term remnant has been applied to both of them.

Although the remnant designation for VLDL may be arbitrary, it is based on several observations. Very low density lipoprotein particles of decreased size and flotation rates (300-400 Å, $S_f \simeq 30$), accumulate in hepatectomized rats and incubation media containing LPL, without further significant TG hydrolysis (105,240). A rapid <u>in vivo</u> transformation of VLDL apoB from the S_f 100-400, to the S_f 20-60 fraction

was observed in humans after heparin injection (13). Recent *in vivo* kinetic studies also indicate that the formation of VLDL particles of S_f 12-60 from S_f 60-400, is relatively rapid when compared to the further degradation into higher density regions (246).

The experimental approach used to study VLDL catabolism has so far been quite different from that used for chylomicrons. The physical and chemical properties of VLDL remnants have already been described, but little is known about the fate of the remnant particle. Most investigators have concentrated on the fate of the VLDL apoproteins after injection of radio-labelled VLDL into humans or intact animals. Although these methods give ample information about the temporal movement of the various apoproteins of VLDL within the different density fractions, little insight is obtained about the actual mechanism of these transformations and the relative importance of the various serum and tissue components in this process. With this experimental design, the individual catabolic steps and the immediate products formed, are impossible to isolate. The interpretation of the results is complicated by the exchange of the various apoproteins and lipids of VLDL with those present in the other lipoproteins of the serum.

Recent *in vivo* work, utilizing ^{125}I-VLDL injected into the circulation (11-13), confirm and expand the early observations of a precursor-product relationship between VLDL and LDL apoproteins in the human (10). Injection of ^{125}I-VLDL into humans was followed by an immediate rise of radioactivity in HDL accompanied by a rapid decrease in the VLDL. This phenomenon can be attributed to a rapid equilibration of apoC, freely exchangeable between VLDL and HDL (19). The initial and very rapid loss of VLDL radioactivity recovered in HDL, was followed by a gradual

transfer of radioactivity, first to IDL and then to LDL. During this second, slow phase of VLDL radioactivity decay, the decline of the apoB portion of the VLDL far exceeded that of the apoC (11). Though in this study no specific activity data were obtained, the time sequence of the transfer of the radioactivity from VLDL to LDL suggested a precursor-product relationship between them, presumably through the IDL. Indeed, there is ample evidence today that the rate of removal of apoB far exceeds that of apoC (156,248). This seemingly paradoxical observation can be explained as follows: as VLDL moves through the progressive stages of delipidations, apoC is transferred to HDL, while the apoB is retained. Thus by the time VLDL becomes IDL, most of its apoC is lost and it is greatly enriched in apoB. The apoC however, transfers again from HDL to the newly systhesized VLDL. Thus while the overall specific activity of apoB in the VLDL fraction is declining at a fairly rapid rate, the constant reassociation of labelled apoC from HDL, with the newly systhesized VLDL, results in a slower decline of the specific activity of VLDL apoC. In the human, under normal conditions, LDL is the final product of VLDL catabolism, and all of the apoB it contains is derived from VLDL (156,246,253).

The transformation of large VLDL into smaller ones as a result of lipoprotein lipase action has been described (13,244), but the mechanism of conversion of the small VLDL particles into IDL and ultimately into LDL is not known. It has been observed that when the VLDL of patients with LCAT deficiency is incubated with LCAT, some of the apoB-containing material is lost from VLDL and recovered in the IDL and LDL region (250). The LCAT reaction may contribute to the formation of LDL <u>in vivo</u>, but clearly, other mechanisms of greater significance must be involved, since

even in patients with LCAT deficiency, LDL of almost normal composition is found.

In spite of the observed low levels of circulating LDL in the rat, conversion of some VLDL into LDL has been reported. Injection of ^{125}I-VLDL caused an immediate rise in HDL radioactivity and a slow increase in the IDL range, followed by a slow (30 - 60 minutes) increase in the LDL radioactivity (254). In another study, the disappearance curve of injected ^{125}I-VLDL apoprotein radioactivity in the rat plasma was triphasic: an initial rapid clearance of 20-30% was followed by a second phase (5 - 120 minutes), where up to 85% of the remaining radioactivity was removed. The remaining 15% of the radioactivity had a slower rate of disappearance. The peak of apoprotein activity in LDL occurred at 15 - 20 minutes after injection, and did not exceed 2.5% of the injected dose (14); in direct contrast to human VLDL metabolism, in which a much larger proportion of the initial VLDL radioactivity is recovered in LDL (13).

Similar results were reported using <u>in vivo</u> labelled VLDL (17). Injection of ^{3}H-lysine into rats resulted in maximal apoB specific activity in VLDL and LDL, 1 and 1.5 hours later respectively, in a manner consistent with a precursor-product relationship. When such labelled VLDL was reinjected into rats, the relationship of the specific activities again indicated that the apoB of the VLDL may be the sole precursor of LDL apoB. Nevertheless, less than 10% of labelled apoB in VLDL was reported to have been recovered in LDL, whereas the uptake of label in the liver was substantial (17).

The extensive hepatic uptake of both VLDL cholesteryl esters (16) and apoB (17), may thus explain the characteristically low concentra-

tions of plasma LDL in the rat. On the basis of these observations it would appear that in the rat, the VLDL is only partially depleted of TG before removal by the liver, or possibly other tissues (17,255,256). The extent of the VLDL TG hydrolysis necessary for the removal of the remnant from the circulation is not known.

Very little is known about the fate of the apoE of both human and rat VLDL. The study of the apoE removal from the circulation has been hampered by the relatively small incorporation of ^{125}I into this apoprotein. The specific activity of VLDL apoE following injection of ^{125}I-VLDL into humans, closely resembles that of the apoC; having an irregular, slow decay with intermittent rises (248). This behavior would indicate that the apoE was sequestered from the VLDL pool and later reassociated with it. The nature or even the necessity of the temporary acceptor is uncertain. While the disappearance curves of apoE and apoC were similar, they were out of phase, indicating that the apoE does not leave and reenter the particle as a unit with apoC. This work was carried out with one patient only, and the results should be interpreted with caution.

3. Low Density Lipoproteins

a) Synthesis

In vivo kinetic studies have established that in the human all of the LDL is derived from VLDL catabolism (156,246,253). Catabolism of rat VLDL on the other hand, results in the production of only minimal amounts of LDL (14,17). This finding may explain the low levels of circulating LDL in this animal. Direct hepatic synthesis of some LDL in the rat and other animal species has not been ruled out, however.

Indeed, the appearance of some material in the LDL density range has been consistently reported in rat (182,184) and pig (185) liver perfusates. The small amounts found in this density range precluded their full chemical characterization, and the possibility that this material may represent a VLDL contamination produced during the ultracentrifugation has been pointed out (184). In vivo experiments in rats suggest that the LDL fraction of S_f 0 - 5, representing the bulk of LDL in this animal may be produced independently of VLDL catabolism (257).

b) Catabolism

While in the rat VLDL remnants are removed by the liver and possibly other tissues without significant formation of LDL (14,17), in the human the TG-rich VLDL becomes the cholesteryl ester-rich LDL. Since LDL is associated with atherosclerosis, there is considerable interest in the catabolism of this most abundant lipoprotein in the human serum.

In spite of the earlier observation that the perfused rat liver is capable of removing a significant portion of LDL (258) (the LDL fraction investigated contained a significant amount of VLDL remnants, probably accounting for the apparent uptake of LDL), there is increasing evidence that the liver does not play a major role in LDL catabolism. Among these is the observation that the catabolic rate of LDL is not decreased in hepatectomized animals (259). Similarily, minimal hepatic contribution to rat LDL catabolism was recently shown in studies of LDL degradation by the perfused rat liver and the intact animal (260). Instead, the catabolism of LDL is now thought to occur in the extrahepatic tissues, as indicated by the presence of specific binding sites on the surface of a variety of cultured cells.

The difference between cell cultures and in vivo conditions must be

kept in mind when evaluating the data presented. Cultured cells are not organized into specialized tissues and cell density is markedly lower than in the intact animal. Even though plasma is the source of nutrients and growth factors *in vivo*, serum is the source used almost universally in culture media. Moreover the concentration of nutrients in tissue culture may be quite different from that present *in vivo*, because of cell to cell interactions. For example, the effects of the arterial endothelial monolayer on the composition and concentration of plasma components, mainly lipoproteins, reaching the underlying smooth muscle cells in the aorta are unknown but certainly important. Despite these complications and limitations, the investigation of the effects of lipoproteins in tissue culture has produced important concepts and insights into the regulation of lipid metabolism in general, and LDL catabolism in particular.

The finding of high affinity receptors on human skin fibroblasts has led to the development of a model for the extrahepatic catabolism of LDL (261). While the model is described using fibroblasts, the most extensively studied cells, a similar sequence of events has been described for lymphocytes, arterial smooth muscle cells, and endothelial cells (262-264). The following events are suggested to take place during the uptake of LDL: binding of LDL to a specific receptor is followed by its incorporation into an endocytotic vesicle, and uptake by the cell. The LDL-containing vesicle fuses with lysozomes, the cholesteryl ester and protein of the LDL are hydrolyzed, and the free cholesterol is transferred to the cell membrane. The accumulating cholesterol inhibits the HMG-CoA reductase, a rate-limiting enzyme for the biosynthesis of cholesterol by the cell. A concomitant activation of cholesteryl ester formation by the membrane-bound acyl-CoA cholesterol acyltransferase

occurs. As the cholesteryl ester accumulates, synthesis of the LDL receptor sites is suppressed and additional LDL uptake is inhibited, resulting in an effective control of the cell's cholesterol content. The suppression can be overcome by incubating the fibroblasts in a lipoprotein-free medium, but the restoration can be prevented by the presence of an inhibitor of protein synthesis or transport, such as cyclohexamide or colchicine. Treatment of the cells with pronase also prevents LDL binding (265).

Studies of LDL catabolism by fibroblasts obtained from patients with familial hypercholesteronemia have produced a number of interesting observations and some insight into the genetic aspect of this disease. Fibroblasts derived from heterozygous hypercholesteronemics have been shown to express about one half of the normal number of the LDL receptors under conditions that should elicit a maximal rate of receptor synthesis (266). Examinations of the fibroblasts obtained from homozygous hypercholesteronemics indicate at least three mutant classes. One class of mutant fibroblasts, termed receptor negative, completely lacks the cell surface receptors (267). The second class, termed receptor defective, exhibits a detectable ability to bind LDL, but the apparent number of functional receptors is greatly reduced (267). Since in both of these types of mutant cells LDL does not bind in normal amounts to the cell surface, the subsequent internalization of the lipoprotein cannot occur at normal rates, and defective regulation of cholesterol metabolism ensues (268). Recently, a third class of mutant fibroblasts has been detected. This mutant appears to have the usual number of functional LDL receptors, but the receptor-bound LDL cannot be internalized, possibly due to a mutation in a protein mediating this process (269,270).

The existence of at least three types of mutations that can produce the clinical phenotype of homozygous familial hypercholesteronemia, raises the possibility of extensive genetic heterogeneity among phenotypic homozygotes, as well as heterozygotes with this syndrome.

The nature of the receptor specificity is uncertain at this time. The concept that the receptor is specific for the apoB of LDL cannot explain the inability of the fibroblasts to degrade normal VLDL. Indeed, recent studies with lipoproteins from cholesterol-fed dogs and pigs, indicate that not only apoB, but also the apoE might be involved in the interaction of lipoproteins with fibroblasts and arterial smooth muscle cells. The cholesterol-induced HDL_c of the dog, which contains only apoE, or the HDL_c of the pig, which contains apoE and apoA-I, are as actively taken up as LDL. Selective modification of the arginyl residues of LDL or HDL_c with cyclohexanedione abolishes their uptake by the cells (271). Removal of cyclohaxanedione regenerates the original activity of these lipoproteins. It thus appears that the specificity for binding of plasma lipoproteins to the cell surface and their uptake, resides with the apoproteins and that both apoB and apoE are capable of reacting with the receptor. It also appears that the arginyl residues are a functionally significant part of the recognition site. The importance of a particular protein-lipid configuration on the surface of these lipoproteins has not been ruled out.

Studies to date indicate that the receptors may exhibit specificity towards certain apoproteins, such as apoB and apoE, but they appear to have little or no species specificity. The presence of receptors on the cell types investigated appears to be universal. Significantly however, cultured rat hepatocytes do not have a high affinity receptor for LDL;

whereas VLDL and HDL increase the activity of hepatic HMG-CoA reductase, LDL has no effect on this enzyme. On the other hand, lipoproteins rich in apoE from the hypercholesteronemic rat inhibited the hepatic HMG-CoA reductase (272). Thus it appears that rat hepatocytes have a receptor for cholesteryl ester and apoE-rich lipoproteins, which would include VLDL remnants. The precise mechanism of the lipoprotein-receptor interaction awaits further characterization.

The significance of extrahepatic degradation of LDL as well as its real role in the intact organism is a matter of speculation at this time, but the observations presented are consistent with the view that LDL serve to transport cholesterol to the extrahepatic tissues, and play an important role in their regulation of cholesterol synthesis.

4. High Density Lipoproteins

a) Synthesis

The synthesis and secretion of HDL by the intestine and the liver has been demonstrated (101,182-184,193), but the relative contribution of these organs to total serum HDL levels is not known. While the presence of HDL apoproteins has been suggested in sonicated Golgi fractions (194), the subcellular pathway for HDL assembly and secretion is not clear. For lack of more evidence, it is presumed to be similar to that of the TG-rich lipoproteins, but many observations are inconsistent with this assumption. Thus, although orotic acid, colchicine, and protein synthesis inhibitors have a profound effect on VLDL assembly and secretion, they effect HDL secretion to a lesser extent (273-275).

The nature of the assembly of the HDL apoproteins into the nascent particle is far less defined than that of the VLDL particle. While

HDL particles appear in liver perfusates and intestinal lymph, they have not been visualized inside the liver or intestinal cells. The possibility that the HDL apoproteins are synthesized individually and each secreted with its complement of lipid, as LpA, LpE and LpC has not been excluded.

Secretion of apoC may be a good case in point. As indicated in the section dealing with VLDL synthesis, nascent VLDL isolated from the Golgi contained only minimal amounts of apoC (218). Furthermore, it was pointed out that the association of apoC with the VLDL particle probably takes place at very late stages of secretion, or even in the space of Disse. As both perfusate VLDL, and to a lesser extent HDL (182) contain apoC, several possibilities exist which at this point are difficult to distinguish. Apoprotein C may be attached to VLDL and HDL independently, just before secretion. It also may be secreted exclusively with one of these lipoproteins, but rapidly redistributed between VLDL and HDL after excretion, in the space of Disse. A possible alternative is the direct and independent synthesis and secretion of C protein with lipid (LpC) or without (apoC), into the space of Disse where it rapidly associates with the nascent VLDL or HDL depending on its relative affinity for each of these lipoproteins. Liver perfusion studies suggest VLDL as the main carrier of apoC into the circulation (182), but a recent in vivo kinetic study in humans was in favour of HDL as the major route of apoC entry (156). It is of interest however, that in patients with Tangiers disease, almost completely lacking HDL, normal or slightly less than normal levels of circulating apoC are found (276).

Regardless of the form in which the major HDL apoproteins are secreted into the space of Disse, distinct HDL particles appear in the liver perfusate. To study the nature and composition of this particle in the

liver perfusate or intestinal lymph, it was necessary to inhibit LCAT, an enzyme which rapidly alters nascent HDL. In the presence of the LCAT inhibitor, DTNB, nascent HDL particles originating from the liver or intestine appeared to be disc-shaped, with dimensions of 50 × 190 Å. While the major apoprotein of hepatic HDL was apoE, apoA-I was the major component of intestinal HDL (186,277). The relative amount of apoE and apoA-I in the hepatic HDL was a function of the extent of LCAT inhibition (278). The proportion of the cholesteryl ester, phospholipid and apoA-I in HDL, in the absence of DTNB were significantly higher than in the HDL secreted in the presence of the inhibitor. It is interesting that while the presence of DTNB had no effect on the total apoA-I secreted by the perfused liver, a larger amount of this apoprotein was found in the d > 1.21 g/ml fraction of the perfusate. The inhibition of LCAT on the other hand, decreased the total amount of apoE secreted. Virtually nothing is known about the secretion of the other apoprotein components of serum HDL, such as apoA-II and apoA-IV.

The appearance of HDL particles of normal spherical shape is observed if the LCAT, synthesized by the liver (279), is not inhibited (186). It appears that the LCAT acts on the nascent HDL particles, its preferred substrate, without delay. This enzyme catalyzes the transfer of the C-2' fatty acid from lecithin to cholesterol and is responsible for the formation of most of the cholesteryl ester in the circulation. For full activation the enzyme requires small amounts of apoA-I (63), already present in the nascent HDL. In the course of the reaction, HDL cholesterol is esterified and moves into the center of the bilayer structure. As more cholesteryl ester is formed, the HDL molecule expands until it becomes completely spherical, as found in the normal serum (186).

b) Catabolism

The in vivo study of HDL catabolism is extremely complicated by the exchange of many of its protein and lipid constituents with those of the other lipoproteins or cell membranes. The half-life of HDL apoproteins in the normal human was estimated to be 4 days (10), and was the same if injected as whole HDL or purified apoproteins (280). In contrast, whole rat HDL was shown to have a shorter half-life, about 11 hours (281). From the limited observations available, it appears that all of the HDL apoproteins in the rat are removed from the circulation at similar rates, 8 - 12 hours (282). In the human, the apoA-I and apoA-II also decay from the circulation at similar rates (133). The fate of HDL apoprotein has been studied in the rat, and the liver was found to be the major site of removal (281). In studies combining electron microscopy and radioautography, it was found that 6 hours after the injection of ^{125}I-HDL, the label was located predominantly in hepatocytes, mainly over secondary lysozomes. Over 80% of the protein had already been degraded, as determined by anti-HDL serum (283). Recent studies indicate that non-parenchymal (Kupffer) cells possess a considerably higher capacity to degrade HDL per mg of cell protein, but their numbers are limited (284).

Increasing evidence about the protective role of HDL in CHD has led many investigators to study the interactions of HDL with the extrahepatic tissues. The role of HDL to transport cholesterol from the extrahepatic tissues back to the liver was proposed years ago (285). In accordance with this concept, the release of cholesterol from cultured human skin fibroblasts is markedly enhanced by the presence of whole HDL or even an apoHDL-sphingomyelin or lecithin mixture in the incubation medium (286).

A recent study reported that prolonged incubation of human skin fibroblasts with HDL produced an enhanced uptake of LDL. The effect was due to the induced synthesis of LDL recpetors by promoting the efflux of cholesterol from the cells (287).

The nature of the HDL interaction with the extrahepatic tissues and its precise physiological role in the organism remains to be established. Nevertheless, a few interesting observations, with respect to its possible role in atherogenesis are presented. As previously mentioned, high plasma concentrations of LDL are correlated with high risk of CHD, and low HDL levels seem to be an additional and independent risk factor. Plasma HDL levels are reduced in several conditions associated with increased risk of CHD, namely hypercholesteronemia, hypertriglyceridemia, diabetes mellitus, obesity, physical inactivity, and male sex. The prevalence of CHD in middle-aged and elderly people is inversely related to plasma HDL cholesterol concentrations. Evidence is also accumulating for the protective effect of high plasma HDL levels against atherosclerosis. The relative freedom of pre-menopausal women from this disease would be in accordance with this hypothesis. It is also known that people with familial hyperα-lipoproteinemia have an above average life expectancy. Eskimos in rural Greenland have higher HDL levels and lower CHD mortality than men in Denmark. Genetic factors may be important, as suggested by a recent follow-up on the Framingham study, showing that children of CHD patients have lower plasma HDL cholesterol concentrations than children of healthy parents (288-291).

CHAPTER II

METHODS AND MATERIALS

A. METHODS

1. Organ Perfusions

a) Heart Perfusion

Hearts, obtained from male hooded rats weighing 200 - 250 g and fasted overnight, were perfused by the method of Bleehen and Fisher (292) in a modification of the apparatus of Miller et al. (180), with a Silastic tubing oxygenator (293). The recirculating perfusate consisted of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 0.22 M Ca^{++} and 0.1% glucose. The gas phase was 95% oxygen and 5% carbon dioxide.

The hearts were cannulated through the aorta and perfused with the buffer to remove the residual blood prior to their installation in the apparatus. The substrate (chylomicrons or VLDL), was added to the perfusate in the apparatus. The hearts were changed every 40 minutes and beat steadily (> 175 beats/minute). Albumin was omitted from the perfusate, producing a more rapid and regular heart beat. The omission of albumin had no effect on the extent of TG hydrolysis, with the exception that the free fatty acids accumulated in the perfusate in the presence of albumin. After the perfusion, the hearts were flushed with 30 ml of the perfusate buffer alone, under pressure, to remove any lipoproteins trapped in the vascular system.

The control experiments consisted of circulating the perfusate, containing the substrate, through the apparatus in the absence of hearts.

b) Liver Perfusion

Livers, obtained from male hooded rats weighing 200 - 250 g and fasted overnight, were perfused by the method of Miller et al. (180), using a Silastic tubing oxygenator (293). The gas phase was 95% oxygen and 5% carbon dioxide. The liver perfusate consisted of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 20% (v/v) washed (four times) human erythrocytes, 3% albumin, 0.1% glucose and a 1% mixture of essential amino acids (294). Ca^{++} was omitted from the perfusate.

The perfusate entered the liver through the cannulated portal vein and left via the superior vena cava, cannulated above the diaphragm. Residual blood was removed from the liver by preperfusion with the buffer alone and the substrate was added to the recirculating perfusate in the apparatus only when it was visually apparent that the liver preparation was viable. In some experiments the viability of the liver at the end of the perfusion (1 hour) was monitored by measuring the oxygen and carbon dioxide content of the perfusate entering and exiting the liver. The pO_2 of the perfusate significantly decreased and the pCO_2 slightly increased during passage through the liver, indicating active metabolism.

All glassware used in both the heart and liver experiments were siliconized prior to use to prevent the substrate from binding to the glass surface (295).

2. Lipoprotein Isolation

All initial lipoprotein isolations by ultracentrifugation were done in Beckman L5-50 or L3-50 ultracentrifuges, using the SW-41 or SW-27 rotors at 33,000 and 27,000 rev/min respectively. For large amounts of plasma, the Ti-50.2 fixed-angle rotor was used and the lipoproteins were removed by tube slicing. Reisolation of the lipoproteins prior to or

after the experiments was done in the SW-41 rotor and the lipoproteins were removed by Pasteur pipette or by tube slicing. Both methods gave >95% recovery of fresh, ^{125}I-labelled VLDL.

a) Chylomicrons

Rat chylomicrons were obtained from male hooded rats from cannulated thoracic ducts following intubation with corn oil. During collection of the lymph the rats were kept in restraining cages and fed a standard diet saturated with corn oil, ad libitum. Lymph was collected overnight on ice, in tubes containing 10 mg/dl disodium EDTA. Chylomicrons were also obtained from fresh citrated rat or pooled human blood. The fresh plasma was always adjusted to 0.01% disodium EDTA, pH 7.2 .

The chylomicrons were isolated from rat lymph or plasma, or human plasma by a 1 hour ultracentrifugation at 33,000 rev/min at 5 °C. The isolated chylomicrons were washed twice by an identical procedure, by isolating them through large volumes of layered NaCl solution, d = 1.006 g/ml, containing 0.01% disodium EDTA, pH 7.2 . After the iodination of washed chylomicrons, the particles of S_f > 3,200 were isolated (38) and used for the perfusion experiments. At the end of the heart perfusion a 0.1% disodium EDTA solution in 0.15 M NaCl, pH 7.2 was added to the perfusate to obtain a final concentration of 0.01% EDTA. The chylomicron remnants (S_f > 400) were then isolated in saline (d = 1.006 g/ml) by a single, 1 hour ultracentrifugation at 33,000 rev/ min.

b) Very Low Density Lipoproteins and Other Perfusate Lipoproteins

Citrated plasma was obtained from male hooded rats by aortic or heart puncture, or from fresh, pooled and citrated human blood. Chylomicrons were removed as described above, and VLDL were isolated by a modification (19) of the method of Havel et al. (36), at d < 1.006

g/ml, at 33,000 rev/min for 18 hours. During the isolation procedure, VLDL, as all other lipoproteins, were always kept in solutions containing 0.01% disodium EDTA, pH 7.2, removed just prior to the perfusion experiments by dialysis. Isolated and washed VLDL were iodinated and ultracentrifugally reisolated for the perfusion experiments. At the end of the perfusion, after adjusting the perfusate to 0.01% disodium EDTA, pH 7.2, VLDL remnants were initially reisolated at d < 1.006 g/ml, and later at d < 1.019 g/ml, at 33,000 rev/min for 18 hours; as the proportion of the d = 1.006 - 1.019 g/ml fraction did not increase during the heart perfusion.

Higher density lipoproteins were isolated from the perfusate by ultracentrifugation after the addition of solid NaCl (d = 1.063 g/ml, at 33,000 rev/min for 24 hours) or solid NaBr (d = 1.21 g/ml, at 33,000 rev/min for 48 hours). In each case the adjusted perfusate was layered with a solution of appropriate density and the reisolated lipoproteins in the top 1 ml fraction were removed by a Pasteur pipette. For further chemical analysis, each density fraction was exhaustively dialyzed against 0.15 M NaCl, containing 0.01% disodium EDTA, pH 7.2 .

The lipoproteins found in the perfusate following the heart perfusion with VLDL were also separated by agarose gel filtration using two different columns: a 2.5 × 60 cm column, containing Bio-Gel A-150 m in the bottom 25 cm, Bio-Gel A-50 m in the next 25 cm, and 10 cm of Bio-Gel P-10 polyacrylamide gel at the top to retard any free iodine entering the agarose. For better resolution and larger scale preparation, a 4 × 100 cm column, packed with Bio-Gel A-150 m and Bio-Gel A-50 m in similar proportions to those in the smaller columns were used. In this column however, the top 20 cm was made up with Bio-Gel A-0.5 m. The columns were packed and equilibrated in 0.18 M Tris buffer, pH 8.5 or

0.15 M NaCl, pH 7.4 solution, both containing 0.01% disodium EDTA and 0.02% sodium azide as a preservative. The different composition and pH of the two buffers used did not have any effect on the elution profile of the lipoproteins. The latter buffer was used in the majority of the experiments because of its more physiological pH.

Two ml fractions (or 8 ml for the large column) were collected and their radioactivity monitored. In some experiments the lipoproteins from an aliquot of each fraction were applied to filter paper discs, precipitated in 10% trichloroacetic acid and delipidated in ethanol/ether (3:1, v/v), and the protein-bound radioactivity counted. The shape of the elution pattern obtained in this way was similar to the one obtained by monitoring the total radioactivity in each fraction with the exception that the small peak of free ^{125}I eluting at the bed volume of the column was removed.

3. Iodination of the Lipoproteins

Rat thoracic duct and rat and human serum chylomicrons were iodinated using carrier-free $Na^{125}I$, by a modification of McFarlane's method (296) as previously described (9). The iodination of VLDL, and in some experiments of LDL, d = 1.019 - 1.050 g/ml, was carried out as above except that the ratio of nmoles of ICl / nmoles of protein was changed to 8, in order to maintain an I/P ratio of ≃1. To remove free iodine, the iodinated lipoproteins were filtered through a 1.5 × 25 cm column of Sephadex G-50 and dialyzed overnight. The small amounts of iodinated lipoproteins were combined with the remainder of the equivalent unlabelled lipoproteins and ultracentrifugally reisolated at the appropriate densities prior to the perfusion experiments.

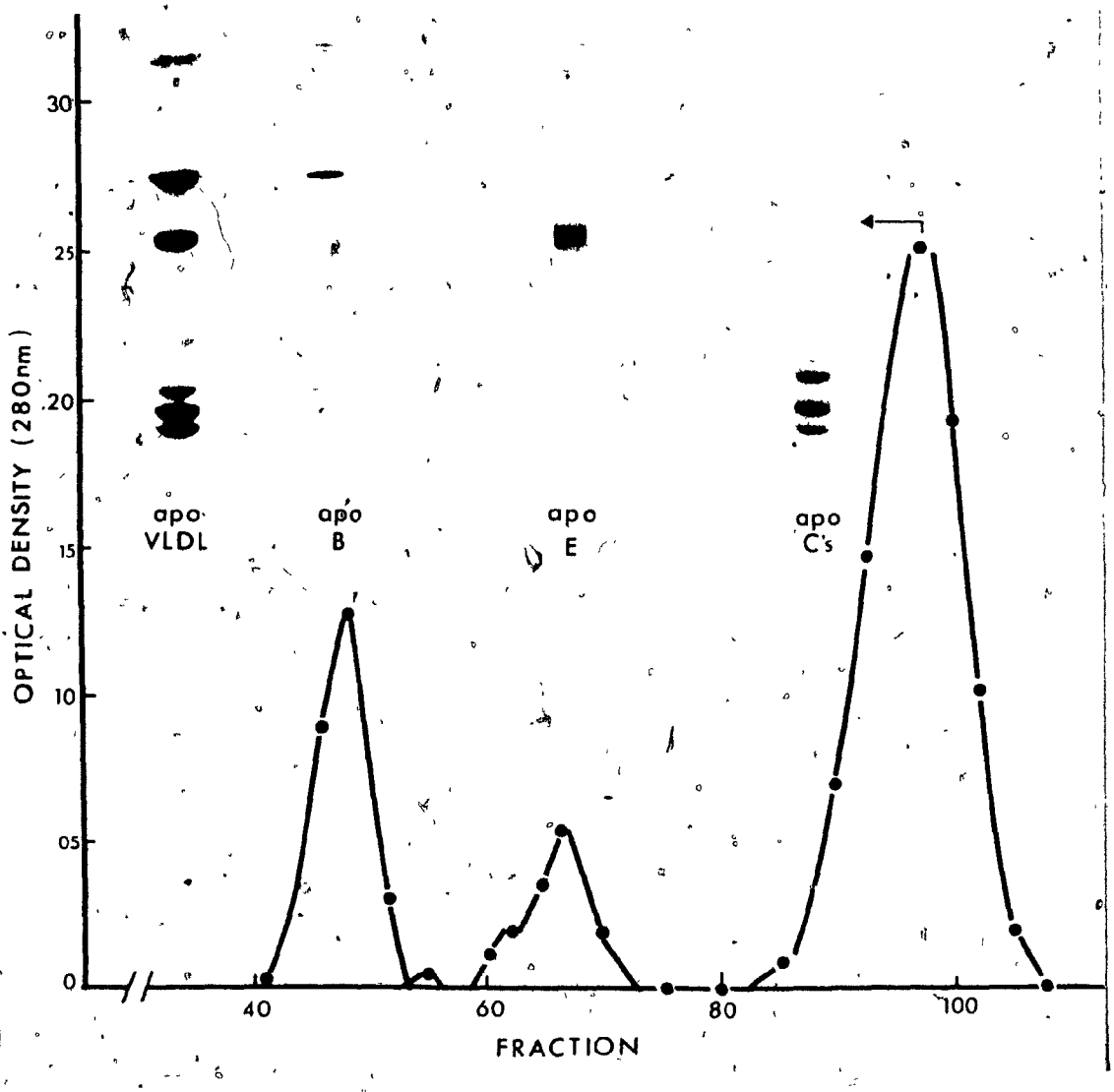
4. Purification of the Apoproteins for Production of Antibodies

Very low density lipoproteins were isolated from pooled fresh human plasma, washed twice, dialyzed against a solution containing 0.01% disodium EDTA, pH 7.2, and lyophilized. The dried lipoprotein was delipidated with ethanol/ether (3:1, v/v) in large volumes. The precipitated proteins were washed in cold ether and thoroughly dried. They were then dissolved in a 0.2 M Tris buffer, pH 8.2, containing 4 M guanidine-HCl, 0.1% azide, and 5 mM dithiothreitol and separated on two joined 2.5 × 100 cm columns packed with Bio-Gel A-1.5 agarose. The columns were prewashed and the apoproteins were separated with the same buffer. A typical separation is shown in Figure 2. The fractions containing the apoB and apoC were collected and pooled separately, dialyzed against 5 mM NH_4HCO_3 and lyophilized. Both the apoB and apoC fraction appeared to be free of any other apoproteins when analyzed by PAGE (see inset, Figure 2).

The antisera to purified apoB or apoC were prepared by injecting the apoproteins, emulsified in Freund's complete adjuvant into white New Zealand rabbits. The antigen was injected at 50 µg/kg rabbit weight in a less than 1 ml volume intradermally in several spots on the upper back. The injections were repeated three more times, 14 days apart, using Freund's incomplete adjuvant. One week after the final injection, the rabbits were bled by an ear vein puncture. The γ-globulins were isolated by $(NH_4)_2SO_4$ precipitation (297), and the specificity of the antisera was checked by double immunodiffusion and immunoelectrophoresis (178). The antisera were dissolved in 0.15 M NaCl, containing 0.02% azide, and frozen at -20°C in small aliquots.

FIGURE 2

SEPARATION OF HUMAN VERY LOW DENSITY LIPOPROTEIN APOPROTEINS BY COLUMN CHROMATOGRAPHY



Human apoVLDL dissolved in 4 M guanidine-HCl was separated on two joined 2.5 × 100 cm columns of Bio-Gel A-1.5 m agarose. Insets show 7 M urea PAGE of the material applied to the column (apoVLDL), and the apoprotein present in fractions 41 - 53 (apoB), 65 - 73 (apoE), and in 85 - 108 (apoC).

5. Analytical Procedures

The radioactivity of the perfusate or each lipoprotein fraction was determined by applying an aliquot (50 - 100 µl) to a 2 × 2 cm Whatman # 3 filter paper disc. The paper discs were air dried and counted in a Packard model 3002 gamma counter. The radioactivity of the protein moiety of each aliquot was determined by precipitating the lipoproteins on the paper discs in 10% trichloroacetic acid and delipidating them overnight in large volumes of ethanol/ether (3:1, v/v) at -10°C. The discs were then washed in cold ether for two hours, thoroughly dried and counted.

To analyze the separated apoproteins by PAGE, aliquots of the lipoprotein fractions were first delipidated in at least 50 volumes of ethanol/ether (3:1, v/v), shaking overnight at -10°C. The precipitated apoproteins were washed in cold ether, dried, and dissolved in 0.04 M Tris, 0.05 M glycine buffer, pH 8.9, containing 7 M urea and 1% SDS. No SDS was present in the polyacrylamide gel or the running buffer. The apoproteins were separated by electrophoresis in a 7 M urea on a 10% polyacrylamide gel with a 3% stacking gel, as previously described (218). The gels were stained with 1% amido black in 7% acetic acid for 15 minutes, and destained overnight in 7% acetic acid. Some of the stained gels were scanned in a Beckman Scanning spectrophotometer at 650 nm. The protein bands were then cut out, and the radioactivity of the bands and the intervening areas counted in the Packard gamma counter.

The ^{125}I-labelled protein in the cardiac tissue was estimated by homogenizing all the hearts used in a single perfusion experiment, in 10% trichloroacetic acid. The precipitated tissue was washed three times in 10% trichloroacetic acid and delipidated three times with chloroform/methanol (2:1, v/v). The residue was then dried, and the radioactivity

of a weighed aliquot determined. A second aliquot was dissolved in 1 M NaOH, the dissolved protein applied to a filter paper disc, which was then dried and counted. These two procedures gave similar results.

Protein was determined by the method of Lowry et al. (298). When intact lipoproteins were assayed, 5% deoxycholate was added to augment the delipidation of the protein during the assay. When large amounts of lipid were present, as with chylomicrons, the assay medium was extracted with a small volume of ether prior to the optical density determination to remove the cloudy appearance. Lipids were extracted for analysis by the method of Folch et al. (299), and TG was determined by the method of VanHandel (300). Total cholesterol was determined by the method of Zlatkis et al. (301).

Lipid classes were separated by glass paper chromatography and visualized by sulphuric acid char, using a modification of the method of Pocock et al. (302). The chromatograms were developed for 14 cm in isooctane/benzene/glacial acetic acid/acetone (100:30:0.1:0.6, v/v), air dried, and again developed for 16 cm in 100% isooctane, with the result that the cholesteryl esters remained behind the second solvent front.

Levels of apoB and apoC were determined by a modification of the "rocket" immunoelectrophoretic method of Laurell (303). The following conditions were used: for apoC, a 1.3% agarose gel was prepared in a barbital buffer, pH 8.6, containing 5% dextran T-10 and 2 mM sodium lactate; for apoB, a 1% agarose gel in the same buffer was prepared, but the lactate was omitted. In both cases the immunoelectrophoresis was run at 2V/cm for 18 hours. The plates were soaked in 0.15 M NaCl and then in distilled water for 15 minutes each, covered with filter paper,

and dried at 60°C. The rockets were stained in 0.5% Coomasie Brilliant Blue (R-250), in ethanol/water/glacial acetic acid (45:45:10, v/v), and destained in the same solution without the dye.

Purified apoC, whose protein content had been determined by the method of Lowry et al. (298), were used for the initial standard curve, and to determine the apoC content of a standard VLDL solution. The apoB content of the same VLDL solution was determined by preparing a standard curve using a purified LDL solution (d = 1.019 - 1.05 g/ml), of known protein content. Judging from the analysis of apoLDL by PAGE, apoB accounted for over 90% of the total protein applied to the gel. It was assumed that the immunoreactivity of apoB in LDL was the same as that of apoB in VLDL. These steps were necessary, as purified apoB obtained from column chromatography was impossible to quantitatively dissolve in the barbital buffer. The standard VLDL solution, containing 0.02% azide was kept at 4°C. The standard solution was present in each plate, and a sample standard curve for VLDL against anti-apoC and anti-apoB is shown in Figure 3.

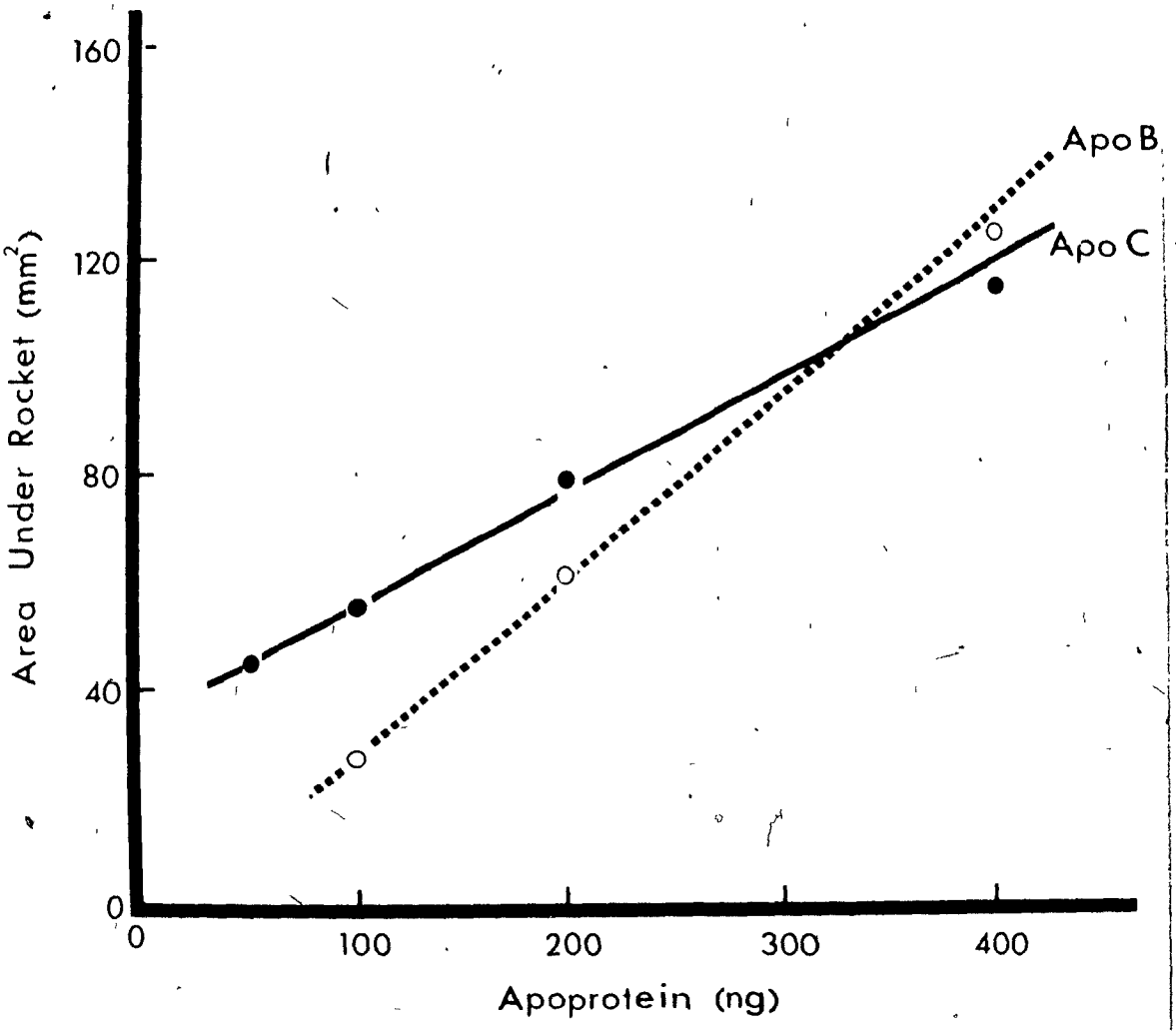
To visualize the lipoproteins by electron microscopy, small aliquots were applied to copper grids coated with a 1% solution of bovine serum albumin. The excess was removed by a piece of filter paper, and the lipoproteins were stained with phosphotungstic acid, pH 7.2 for 5 minutes. The lipoprotein-coated grids were examined under the electron microscope (Philips), within 30 minutes.

B. MATERIALS

The male hooded rats and the New Zealand rabbits were obtained from Canadian Breeding Farms, St. Constant, Quebec. Fresh human blood was

FIGURE 3

SAMPLE STANDARD CURVES FOR THE "ROCKET" IMMUNOELECTROPHORETIC DETERMINATION OF HUMAN APOPROTEINS B AND C



The primary standard curves to determine the apoB and apoC content of the standard VLDL solution used to obtain the above curves, was obtained by using purified apoC on a narrow density cut of human LDL (for apoB), as described in the text.

supplied by the Canadian Red Cross, Montreal, Quebec.

The $Na^{125}I$, (carrier-free), was obtained from Charles Frosst and Co., Montreal, Quebec. Agarose and polyacrylamide for gel filtration were purchased from Bio-Rad Laboratories, Toronto, Ont., and acrylamide and bis-acrylamide for PAGE from Eastman Kodak Co., Rochester, N.Y. Both were recrystallized prior to use from chloroform. Urea, purchased from Fisher Scientific Co., Montreal, Quebec, was recrystallized from methanol before use to remove the carbamylating impurities. Guanidine-HCl was purchased from Sigma Chemical Co., St. Louis, Mo., and recrystallized from methanol.

Silastic tubing was supplied by Dow-Corning Co., Midland, Mich. Tygon tubing and the Intramedic polyethylene tubing (PE- 90,190 and 205), used in the perfusion apparatus and cannulations were obtained from Fisher Scientific Co., Montreal, Quebec.

The agarose powder for immunoelectrophoresis (Seakem LE), was purchased from Marine Colloids Inc., Rockland, Maine. Glass fiber paper (ITLC-SG) was obtained from Gelman Instrument Co., Ann Arbour, Mich.

Standard cholesteryl palmitate and monopalmitoylglycerol were purchased from the Sigma Chemical Co., St. Louis, Mo.; tripalmitoyl- and dipalmitoylglycerol, from the Hormel Institute, Austin, Minn.; cholesterol from ICN Biochemicals, Cleveland, Ohio; and phosphatidylcholine from Supelco Inc., Bellefonte, Pa. Mixed fatty acids and TG were prepared from corn oil. Pesticide grade solvents, and all other chemicals were obtained from Fisher Scientific Co., Montreal, Quebec, or from Canadian Laboratory Supplies, Montreal, Quebec.

CHAPTER III

RESULTS

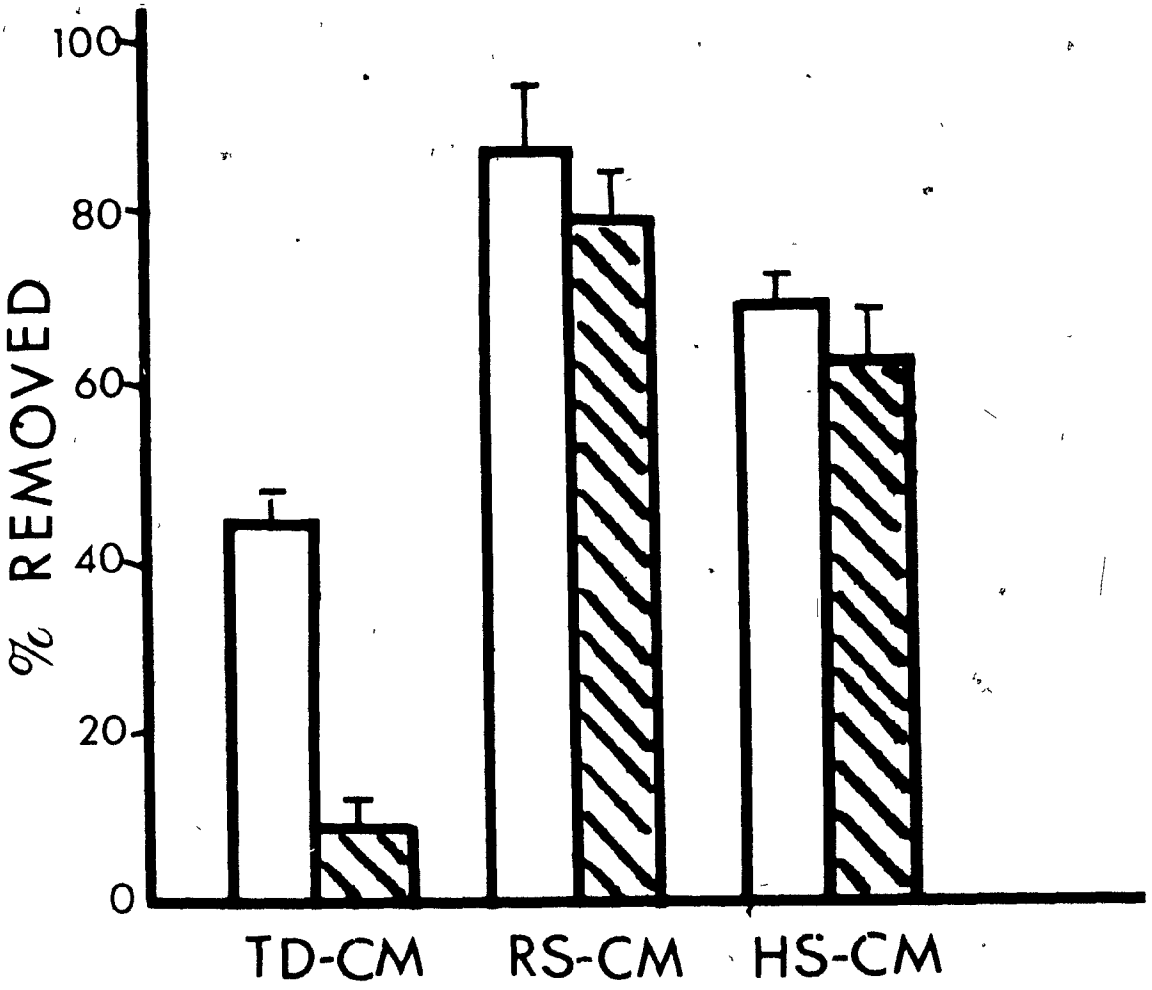
A. FORMATION OF CHYLOMICRON REMNANTS

Our laboratory has developed a two-stage in vitro model, consisting of perfused rat hearts and livers, to study the catabolism of rat thoracic duct chylomicrons (9). Although the main objective of this study was to examine the suitability of this model for the investigation of the products formed in the course of serum VLDL catabolism, it was also of interest to see if serum chylomicrons from both the human and the rat behave in a fashion similar to that established for rat thoracic duct chylomicrons. To this effect, chylomicrons obtained from rat thoracic duct and rat and human serum were isolated and iodinated. Only particles of $S_f > 3,200$ were used for the perfusion experiments. Differences in the distribution of the ^{125}I-label between the lipid and the protein moieties of the particle were noted. While only 13% of the iodine was bound to the protein moiety of the thoracic duct chylomicrons, protein labelling increased to 53% in rat serum chylomicrons and to 77% in human serum chylomicrons.

In the course of the heart perfusion, most of the chylomicron TG hydrolysis occurred in the first 60 - 90 minutes (not shown), in spite of the insertion of another fresh heart at 80 minutes. The removal of chylomicron TG was accompanied by the removal of chylomicron protein (measured as protein-bound ^{125}I). As shown in Figure 4, the relative removal of TG and protein of both human and rat serum chylomicrons exceeded that of the rat thoracic duct chylomicrons significantly. Especially striking

FIGURE 4

REMOVAL OF TRIGLYCERIDE AND PROTEIN FROM CHYLOMICRONS (S_f > 400) DURING HEART PERFUSION



Legend: □ - TG
▨ - Protein
TDCM - Rat Thoracic Duct Chylomicrons (n = 3)
RSCM - Rat Serum Chylomicrons (n = 2)
HSCM - Human Serum Chylomicrons (n = 3)

Heart perfusions were carried out for 120 minutes. The loss of TG was determined chemically and that of apoproteins by the decrease in the ^{125}I-labelled apoprotein.

is the difference in the ratio of percent TG / percent protein removed between the serum and thoracic duct chylomicrons: for the serum chylomicrons the ratio is close to unity (1.1), but it is significantly greater for the thoracic duct chylomicrons (4.7).

The correlation between the actual amount of TG removed from the perfusate (in mg) and the initial perfusate chylomicron TG concentration (in mg/ml perfusate) is shown in Figure 5. The data show that in the concentration range investigated (0.16 - 1.0 mg/ml), increased initial concentrations of both human and rat serum chylomicrons resulted in increased amounts of TG removed from the perfusate, suggesting an unsaturated system. In contrast, in the range of 0.7 - 2.2 mg/ml initial TG concentration, the amount of TG removed from rat thoracic duct chylomicrons is constant (40 - 50 mg of TG removed), and independent of the initial TG concentration, indicating a saturated system. Rat heart LPL may have a higher affinity for the rat chylomicrons, but not enough points are available to establish this.

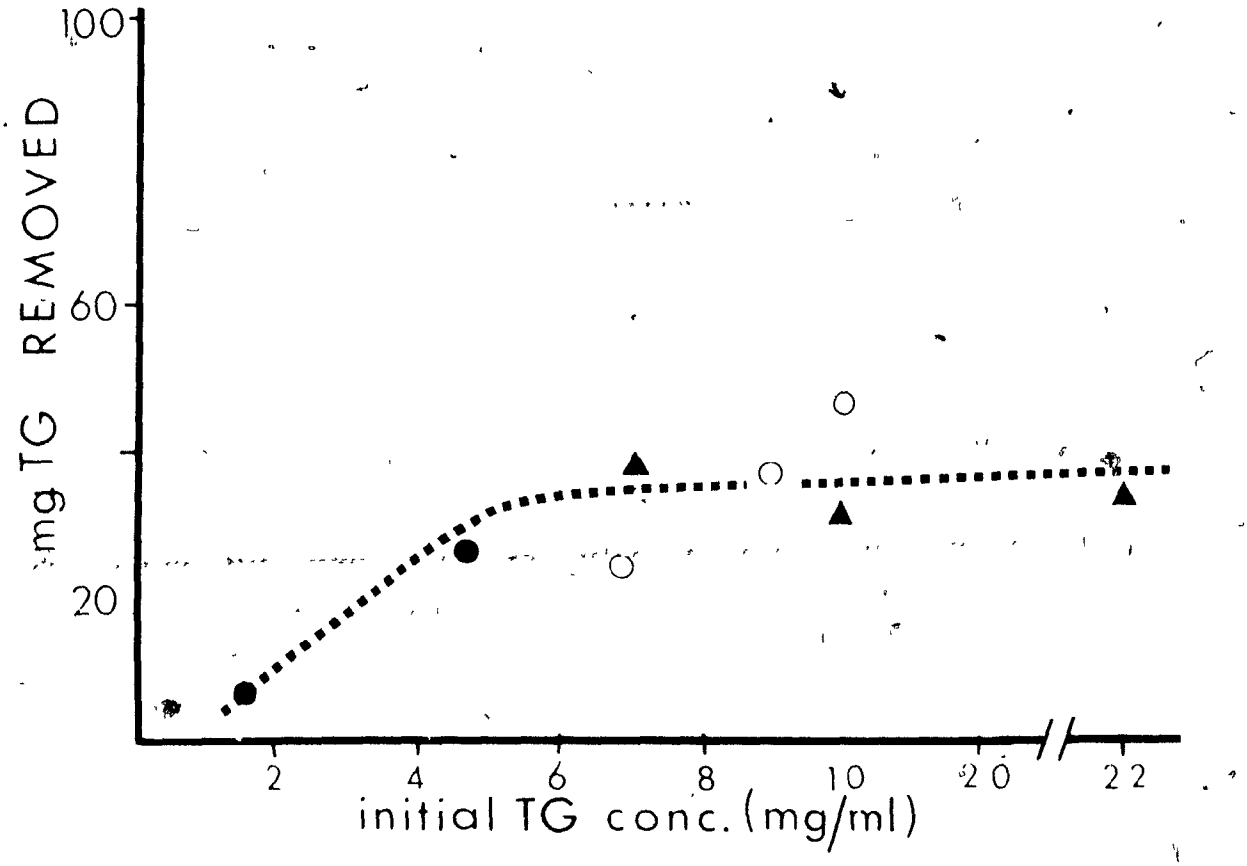
Since the perfused rat heart is capable of hydrolyzing human chylomicrons in addition to rat thoracic and serum chylomicrons, this system was applied to the study of the hydrolysis of human and rat serum VLDL and higher density products formed in the process.

B. FORMATION OF VERY LOW DENSITY LIPOPROTEIN REMNANTS AND HIGHER DENSITY PRODUCTS

The removal of TG and protein from the ^{125}I-labelled human VLDL during heart perfusion, shown in Figure 6, continued for approximately 90 minutes, after which there was little activity, in spite of another fresh heart insertion after 80 minutes. Nevertheless, to insure maximal hydrolysis

FIGURE 5

RELATIONSHIP BETWEEN THE LOSS OF TRIGLYCERIDE FROM THE PERFUSATE AND THE INITIAL PERFUSATE CHYLOMICRON CONCENTRATION



Legend: ● - Rat Serum Chylomicrons
○ - Human Serum Chylomicrons
▲ - Rat Thoracic Duct Chylomicrons

All perfusions were carried out for 120 minutes and each point represents a separate experiment using a different pool of chylomicrons.

by the LPL, subsequent experiments were carried out for 120 minutes. The data in Figure 6 were accumulated from experiments with initial TG concentrations ranging from 0.2 to 1.0 mg/ml perfusate. Due to this large range, the significance of the differences between the losses of TG and protein were calculated by paired differences. The differences at the 90 and 120 minute points were significant at $p < 0.0005$ and $p < 0.025$ level respectively. The loss of TG was thus significantly greater than that of protein, suggesting the formation of a remnant relatively poor in TG. Removal of TG and protein from the VLDL fraction was also accompanied by a significant removal of cholesterol (not shown). The pattern of TG and protein removal from rat VLDL remnants was similar to that for human VLDL.

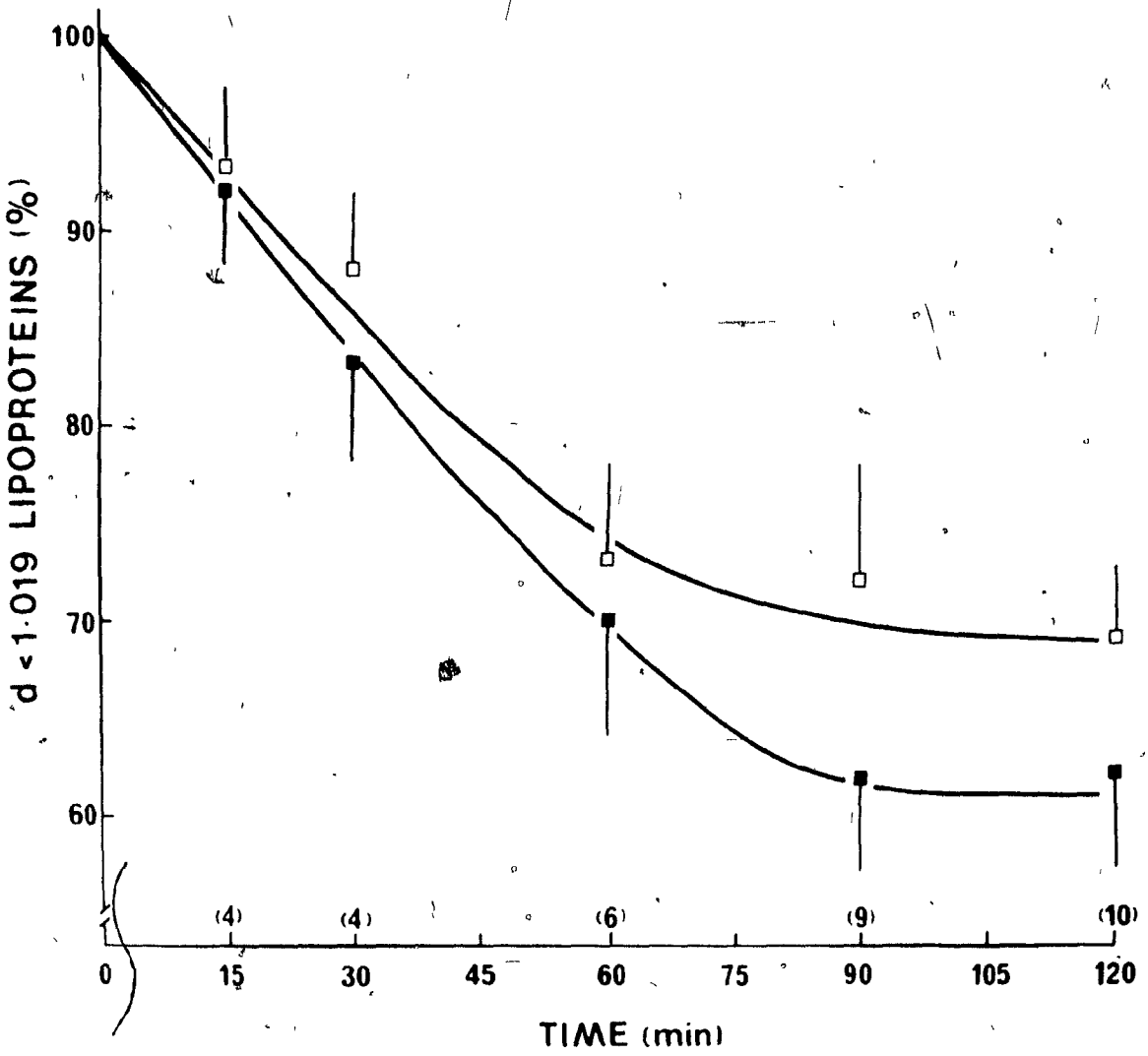
In early experiments the VLDL ($d < 1.006$ g/ml) and IDL ($d = 1.006 - 1.019$ g/ml) were isolated separately. However, no accumulation of TG or labelled lipoproteins was found in the IDL in the course of the heart perfusion. When IDL were present in the ^{125}I-labelled VLDL at the start of the perfusion (usually < 5%), these disappeared from the perfusate at the same rate as the VLDL. In the subsequent experiments therefore, VLDL remnants were isolated at $d < 1.019$ g/ml. In the control experiments, where concentrations of human and rat VLDL equal to those used in the heart perfusions were perfused through the apparatus for 120 minutes, it was shown that there was no loss of ^{125}I or TG from the perfusate, but there was a decrease of 6±2% in radioactivity of the VLDL ($n = 7$). The minimal loss of radioactivity from VLDL indicates that recirculating the substrate through the apparatus does not contribute to the changes observed in the heart perfusion experiments.

In the course of these studies, hearts were perfused with a range

FIGURE 6

LOSS OF TRIGLYCERIDE AND APOPROTEINS FROM HUMAN

VERY LOW DENSITY LIPOPROTEINS DURING HEART PERFUSION



Legend: □ - ^{125}I-labelled apoprotein
■ - TG

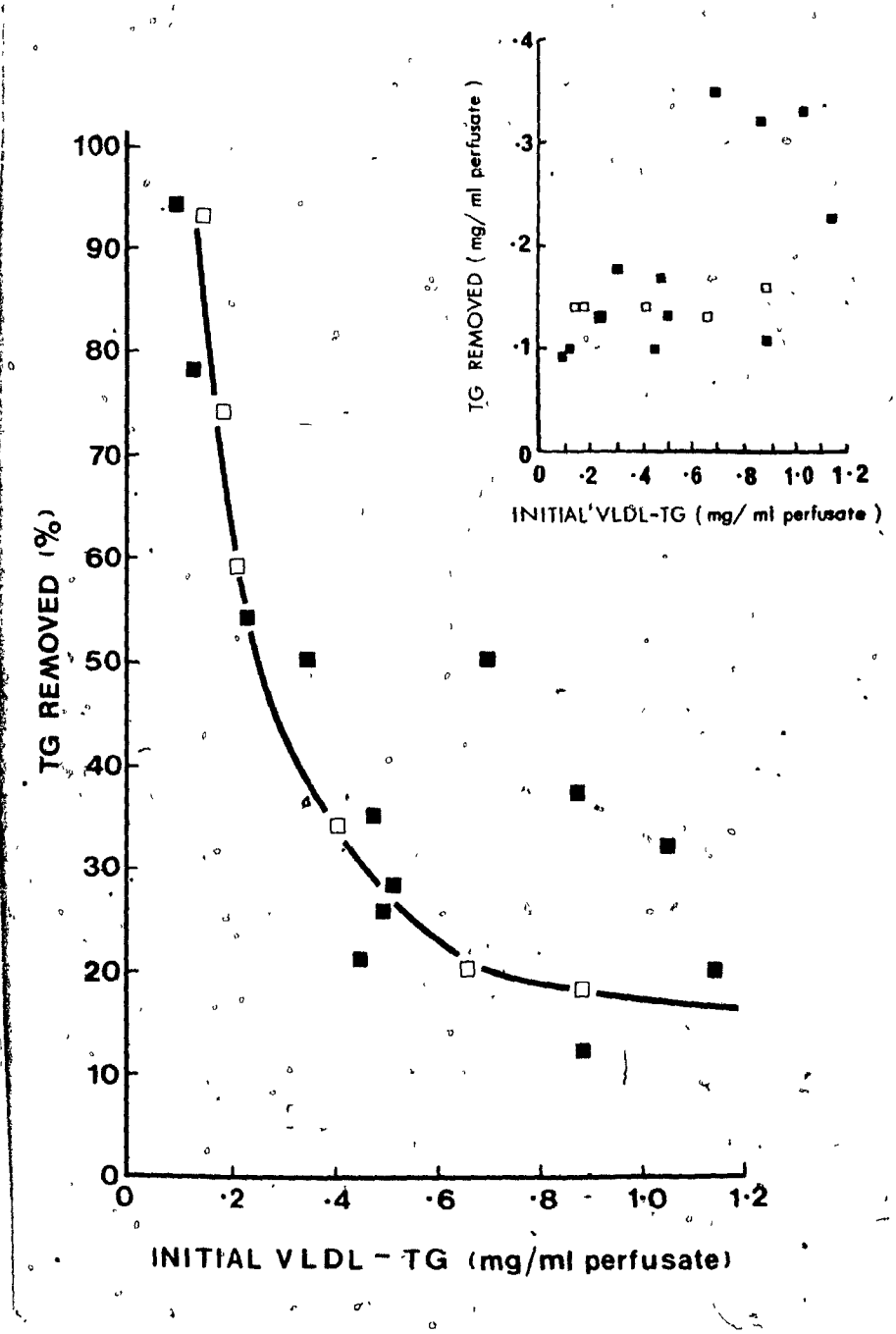
Hearts were replaced every 40 minutes. The loss of TG was determined chemically, and that of the apoproteins by the decrease in ^{125}I-labelled apoprotein. Values represent the average ±S.E. of the number of experiments, indicated in parentheses. The data were accumulated from experiments with initial TG concentrations ranging from 0.2 - 1.0 mg/ml perfusate. Because of this range, the significance of the differences between the losses of TG and protein were calculated by paired differences. These calculations gave probability values as follows: $p < 0.05$, 0.15, 0.0005, and 0.025 for the 30-, 60-, 90-, and 120-minute points respectively.

of concentrations of human and rat VLDL. The data on the removal of TG from the VLDL during the 120 minute perfusions are collected in Figure 7. In general, it will be noted that there is little difference in the rates of TG removal of rat and human VLDL. The results suggest a saturable system with a decreasing percentage of the TG being removed as the lipoprotein concentration increases, especially in the case of rat VLDL. In several experiments utilizing higher concentrations of human VLDL, the percent removed was higher than that expected by saturation kinetics. The inset in Figure 7 illustrates that over concentrations ranging from 0.1 to 1.0 mg human or rat VLDL TG / ml of perfusate, between 0.10 and 0.17 mg TG / ml of perfusate were removed during the experiment. However, a much greater removal was noted in four experiments using human VLDL concentrations of 0.7 to 1.2 mg / ml of perfusate. The reason for these differences is not apparent, but may be due to individual variations among the human blood donors, since the removal of TG from rat VLDL was almost identical at all concentrations.

Significant losses of lipid (TG and cholesterol) were expected to result in alterations in the shape as well as the size of the VLDL particle. The electron microscope appearance of human and rat VLDL and their remnants are compared in Figure 8. The micrographs show a decrease in the size of the remnants, with irregularities in the shape and some evidence of membrane-like borders around partially emptied interiors. The pattern is similar for both species, although somewhat more marked in rat VLDL remnants. The appearance of the human VLDL remnants is strikingly similar to the VLDL found in patients with familial LCAT deficiency (123). Since LCAT is not present in the heart perfusion system, these observations support the hypothesis that LCAT may be

FIGURE 7

LOSS OF VERY LOW DENSITY LIPOPROTEIN TRIGLYCERIDE IN RELATION TO THE INITIAL TRIGLYCERIDE PERFUSATE CONCENTRATION



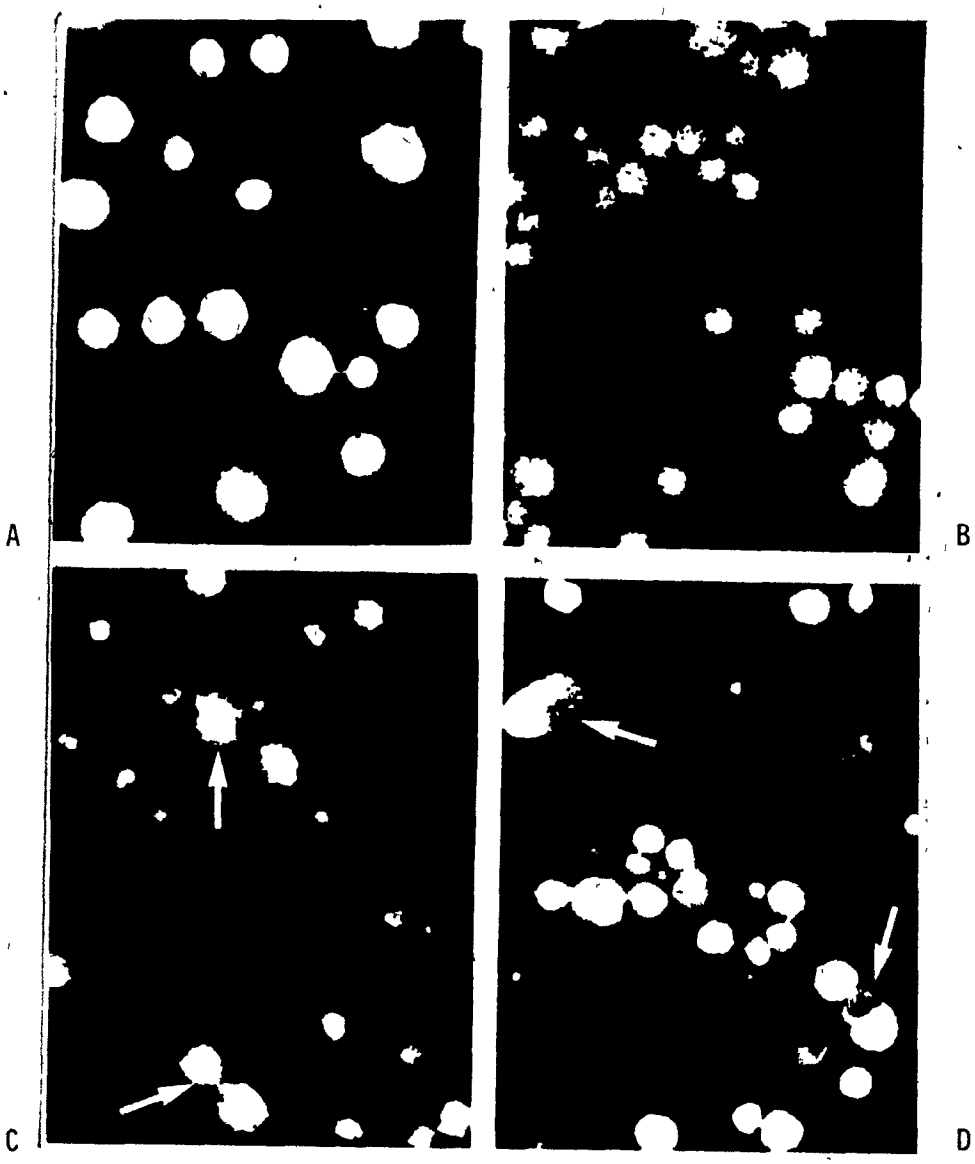
Legend: □ - Rat VLDL
■ - Human VLDL

All perfusions were carried out for 120 minutes. Points representing the percent removal of TG from the rat VLDL perfusate are joined. Inset shows the absolute amounts of TG removed. Each point represents a separate experiment using a different pool of VLDL.

FIGURE 8

ELECTRON MICROSCOPIC APPEARANCE OF HUMAN AND RAT

VERY LOW DENSITY LIPOPROTEINS AND THEIR REMNANTS



Negative staining with phosphotungstic acid, pH 7.2, was used to visulaize rat (A) and human (B) VLDL and their remnants (C and D represent rat and human VLDL remnants respectively), formed by perfusion through rat hearts. Magnification 150,000 X. Note the appearance in the remnants (D) of smaller particles and ones which show areas of decreased staining (arrows), probably representing partial delipidation. The rat VLDL and its remnants resemble those of the human, except that more small forms and particles surrounded by membrane-like borders are seen (arrows).

involved in reshaping the remnant surface (249).

A more detailed picture of rat VLDL remnants is shown in Figure 9. The partially collapsed, membrane-like structures around the TG core are particularly discernible. As pointed out (arrows), some remnants have completely collapsed into disc-shaped structures. Many of the remaining remnants may therefore be of discoidal shape also, but laying on their sides, they appear spherical.

Changes in the shape of the VLDL particles during the heart perfusions were also accompanied by changes in size. To determine the size distribution of the particles, 300 intact and remnant, human and rat VLDL were counted, each (see Figure 10). It was noted that following perfusion, there is a shift in the distribution of the particles toward the lower end of the same general size range. A similar observation was reported about VLDL remnants prepared in supradiaphragmatic rats (105). The results are even more interesting when the decrease in the average volumes of the particles is calculated (Table III), and compared with the total TG loss during the perfusion. An 11% decrease in the particle diameter in human VLDL during the heart perfusion resulted in a 30% decrease in the particle volume. As the initial VLDL TG concentration in the experiment producing these remnants was 0.5 mg/ml perfusate, 26% of the TG was removed, as indicated in Figure 7. The relationship between the TG loss and the decrease in the particle volume is even closer for rat VLDL. A 24% decrease in the particle diameter, equivalent to a 56% decrease in the volume, was accompanied by a 58% loss of TG (see Figure 7; initial TG concentration = 0.2 mg/ml). A close correlation was thus established between the amount of TG removed (a core component of VLDL), and the decrease in the particle volume.

FIGURE 9

ELECTRON MICROGRAPH OF RAT

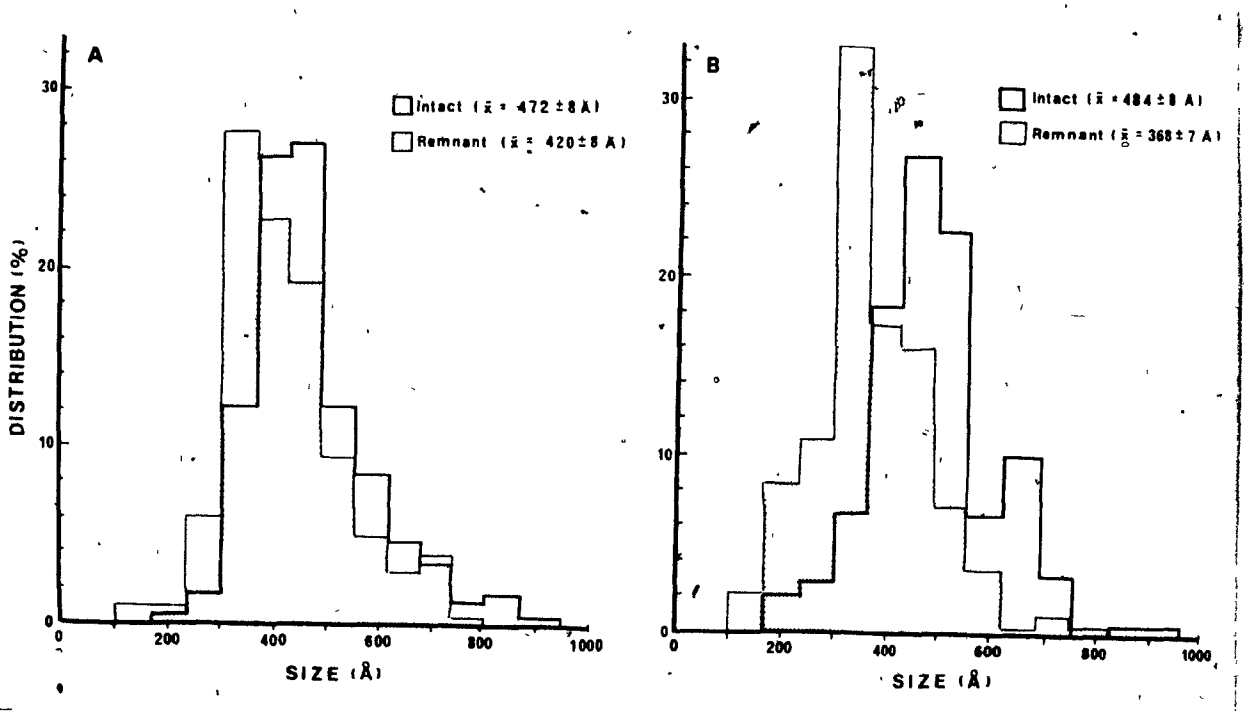
VERY LOW DENSITY LIPOPROTEIN REMNANTS

A higher magnification (320,000 ×) of the negatively stained rat VLDL remnants reveals the presence of completely collapsed, disc-shaped particles (arrows) as well as the presence of partially collapsed, membrane-like structures, about 30 - 35 Å thick.

FIGURE 10

SIZE DISTRIBUTION OF INTACT AND REMNANT

RAT AND HUMAN VERY LOW DENSITY LIPOPROTEINS



HUMAN VLDL

RAT VLDL

Heart perfusions were performed at 0.5 mg/ml and 0.2 mg/ml of initial human and rat VLDL concentrations respectively. Samples for electron microscopy were taken at the beginning and end of the heart perfusion, and 300 particles were sized for each distribution pattern.

TABLE III

LOSS OF TRIGLYCERIDE AND THE DECREASE IN THE AVERAGE VOLUME OF VERY LOW DENSITY LIPOPROTEINS DURING HEART PERFUSION

		mean radius (nm)*	calculated volume (nm^3)	% decrease in calculated vol.	% decrease in TG content†
HUMAN VLDL§ (d < 1.019 g/ml)	Pre-perfusion (intact)	23.6	55.0×10^3	----	----
	Post-perfusion (remnant)	21.0	38.8×10^3	30%	26%
RAT VLDL¶ (d < 1.019 g/ml)	Pre-perfusion (intact)	24.2	59.3×10^3	----	----
	Post-perfusion (remnant)	18.4	26.1×10^3	56%	58%

§ at 0.5 mg/ml initial perfusate VLDL TG concentration

¶ at 0.2 mg/ml initial perfusate VLDL TG concentration

* determined by sizing 300 particles of each category as shown in Figure 10

† determined chemically and shown in Figure 7.

Significant losses of protein from the VLDL particle during the heart perfusion suggests the formation of higher density products in the perfusate. The fate of the apoprotein removed from the VLDL is shown in Table IV. On the average, the removal of apoproteins from the d < 1.019 g/ml fraction of the perfusate amounted to 36±6% from human VLDL and 51±10% from rat VLDL. The data represent 13 and 7 experiments respectively, with initial VLDL concentrations ranging from 0.1 to 1.1 mg/ml perfusate. Approximately 20% of the labelled apoprotein lost from rat or human VLDL was recovered in the d = 1.019 - 1.063 g/ml range, 10% in the d = 1.063 - 1.21 g/ml fraction, small amounts (6%) in the d > 1.21 g/ml infranatant, and about 40 - 45% in the heart.

The observation that the perfused rat heart is capable of removing almost half of the apoproteins lost from VLDL (d < 1.019 g/ml) is surprising. In the human VLDL this represents, on the average, 16% of the the total VLDL apoprotein added to the perfusate. An even larger proportion of rat VLDL apoprotein added to the perfusate was taken up by the heart (21%).

In order to rule out the possibility that the uptake of the apoproteins by the heart is simply due to the ultrafiltration and trapping of VLDL, the following experiment, summarized in Table V, was performed. Hearts were perfused for three minutes with 200 µg of heparin dissolved in 20 ml of non-recycling perfusate, releasing the lipoprotein lipase (22). The hearts (three) were then perfused for 90 minutes with perfusate containing human ^{125}I-labelled VLDL. The resulting uptake of labelled apoprotein was negligible (< 2% of the total protein radioactivity). The same perfusate was then perfused through two normal rat hearts for an additional 90 minutes. Uptake of the labelled apoprotein was similar

TABLE IV

FATE OF THE APOPROTEINS REMOVED FROM

VERY LOW DENSITY LIPOPROTEINS DURING HEART PERFUSION

Lipoprotein Fraction	Distribution of ^{125}I-labelled Protein Lost From VLDL (%)	
	Human	Rat
d = 1.019 - 1.063 g/ml	21 ± 1 (13)	21 ± 7 (7)
d = 1.063 - 1.210 g/ml	11 ± 1 (12)	15 ± 4 (7)
d > 1.210 g/ml	5 ± 2 (11)	8 ± 4 (7)
Heart	44 ± 5 (7)	42 ± 12 (4)

Removal of apoproteins from the d < 1.019 g/ml fraction of the perfusate amounted to 36 ± 6% from human VLDL, and 51 ± 10% from rat VLDL in 13 and 7 experiments respectively. Each figure is the mean ±S.E., of the number of experiments indicated in parentheses. The initial TG concentrations ranged from 0.1 to 1.1 mg/ml of perfusate, and all perfusions were carried out for 120 minutes.

TABLE V

APOPROTEIN RECOVERY IN THE PERFUSATE AND HEPARIN-TREATED AND NORMAL HEARTS FOLLOWING PERFUSION WITH ^{125}I-LABELLED HUMAN VERY LOW DENSITY LIPOPROTEINS

Experiment	time*	Total perfusate ^{125}I-Apoprotein		^{125}I-Apoprotein recovered in heart tissue	
		cpm	% total	cpm	% total
A†	0	1.82×10^6	100	-----	-----
	90	1.75×10^6	96	0.03×10^6	1.6
B§	0	1.75×10^6	100	-----	-----
	90	1.51×10^6	86	0.20×10^6	11.0

* perfusion time in minutes

† perfusion with heparin-treated hearts (3 hearts)

§ perfusion with normal hearts (2 hearts), using perfusate from Experiment A.

Perfusate containing human VLDL at an initial TG concentration of 0.5 mg/ml was perfused first through hearts that were preperfused with a heparin-containing Krebs-Ringer solution to remove the LPL. Aliquots were taken at the beginning and the end of the two hour perfusion to determine the total perfusate protein-bound radioactivity. The protein-bound radioactivity was also assayed in the cardiac tissue as described in Chapter II. After the perfusion of the heparin-treated hearts, the perfusion continued for a further 90 minutes using normal hearts. At the end of this time, the total perfusate and the normal cardiac tissue protein-bound radioactivity was assayed again.

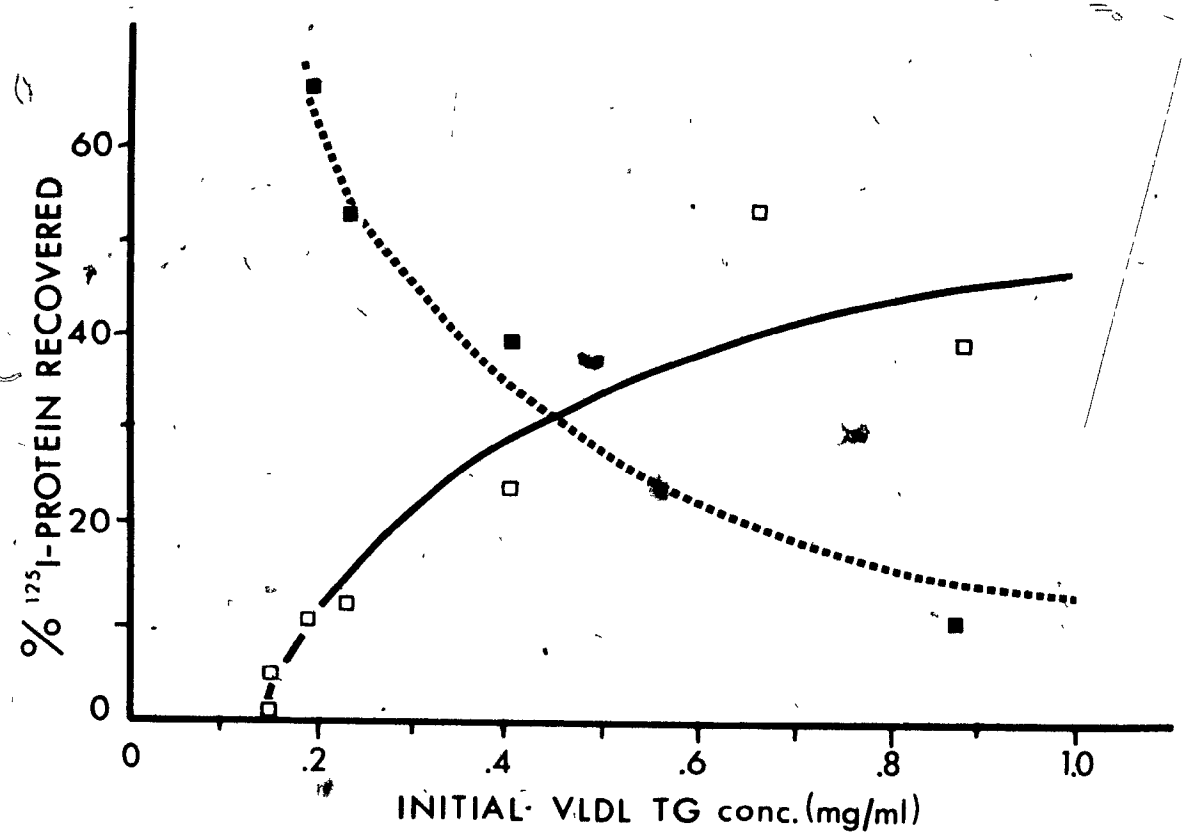
to that shown in Table IV, and about seven times greater than by the heparin-treated hearts. It therefore seems likely that lipolytic activity is a prerequisite for the uptake of the perfusate VLDL apoproteins by the heart. This finding, while incidental to this study, is particularly interesting, since the uptake of rat VLDL remnants by cultured rat aortic smooth muscle cells has been shown (255). The recovery of 80 - 85% of the ^{125}I-labelled protein removed from VLDL, of which approximately 50% is in the perfused hearts (see Table IV), suggests a relatively slow degradation of protein, just as observed in the cultured smooth muscle cells.

Data in Table IV reveal a considerable variation in the percentage of rat VLDL apoproteins recovered in the d = 1.019 - 1.063 g/ml fraction and that taken up by the heart, as indicated by the high standard errors (21±7% and 42±12%, respectively). The reason for the large variation is apparent when the percentages of rat VLDL apoproteins recovered in the d = 1.019 - 1.063 g/ml range and in the heart, in the individual experiments are plotted as a function of the initial VLDL TG concentrations, (Figure 11). The data clearly show an inverse relationship between the amounts of VLDL apoprotein recovered in the heart and that recovered in the LDL density range. This observation suggests that the heart tissue contains a high affinity receptor for the rat lipoproteins that is easily saturated. Thus at low, physiological concentrations of VLDL, only negligible amounts of d = 1.019 - 1.063 g/ml material are formed, most of it being removed by the heart. Increasing lipoprotein concentrations however, saturate the receptors and lipoproteins in the LDL range begin to accumulate.

When the production of d = 1.019 - 1.063 g/ml material from human

FIGURE 11

RECOVERY OF THE RAT VERY LOW DENSITY LIPOPROTEIN APOPROTEINS IN PERFUSED HEARTS AND IN THE d = 1.019 - 1.063 g/ml FRACTION OF THE PERFUSATE



Legend: ■ - recovery of ^{125}I-labelled apoprotein in the heart

□ - recovery of ^{125}I-labelled apoprotein in the d = 1.019 - 1.063 g/ml fraction of the perfusate

After the experiment the hearts were flushed with 30 ml of Krebs-Ringer solution under pressure and the ^{125}I-labelled apoprotein was determined in all hearts used in a single perfusion experiment, as described in Chapter II. Each point represents a separate experiment using a different pool of VLDL.

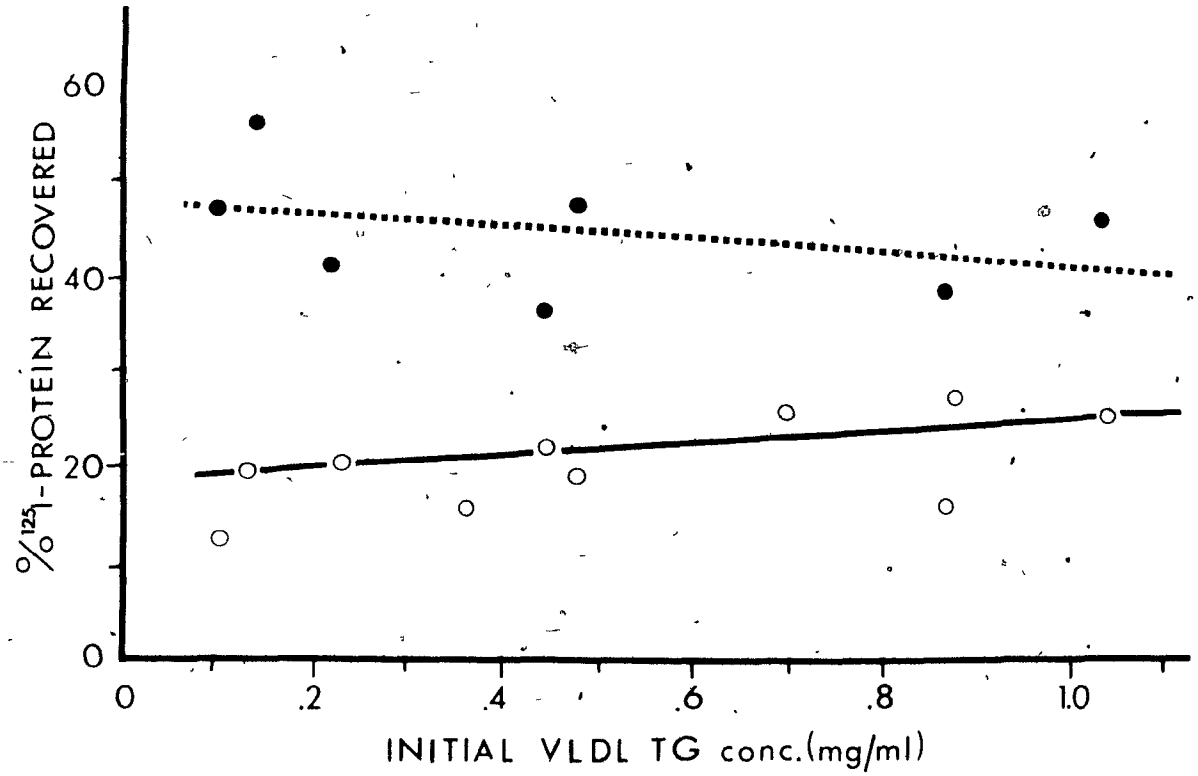
VLDL is compared to the uptake of human VLDL apoproteins by the perfused heart, as a function of the initial TG concentration, a qualitatively different picture is obtained (see Figure 12). A relatively constant proportion of the human VLDL apoproteins is recovered in the heart and in the LDL density range, regardless of the initial VLDL TG concentration, suggesting a low affinity mechanism of uptake of the human lipoproteins by the rat heart.

The inverse relationship between the percentage of rat VLDL converted to d = 1.019 - 1.063 g/ml material and that taken up by the heart, in contrast to the constant proportion of the human VLDL found in this density range and in the heart, independent of the initial TG concentration, highlights a fundamental difference in human and rat VLDL catabolism by the perfused rat heart. The precise nature of the uptake of lipoproteins of both species obviously demands a more rigorous study using a different experimental approach. On the basis of the presented data it is, for instance, impossible to decide which lipoprotein species is taken up: the VLDL remnant or the material from the d = 1.019 - 1.063 g/ml range.

The following experiment was carried out in an attempt to further characterize the nature of the specificity of lipoprotein uptake from the perfusate by the heart. Human and rat serum LDL (d = 1.019 - 1.050 g/ml) were iodinated and perfused through the hearts. The amount of the LDL apoprotein present in the perfusate was adjusted to be equivalent to that present when human or rat VLDL were perfused at low (0.15 mg/ml) initial TG concentrations. It was found that over 50% of both rat and human ^{125}I-labelled apoLDL was removed from the perfusate, of which over 90% was recovered in the heart tissue. The experiment further confirmed the ability of the perfused heart to take up apoproteins. The similar

FIGURE 12

RECOVERY OF THE HUMAN VERY LOW DENSITY LIPOPROTEIN APOPROTEINS IN PERFUSED HEARTS AND IN THE d = 1.019 - 1.063 g/ml FRACTION OF THE PERFUSATE



Legend: ● - recovery of ^{125}I-labelled apoprotein in the heart

○ - recovery of ^{125}I-labelled apoprotein in the d = 1.019 - 1.063 g/ml fraction of the perfusate

After the experiments the hearts were flushed with 30 ml of Krebs-Ringer solution under pressure and the ^{125}I-labelled apoprotein was determined in all hearts used in a single experiment, as described in Chapter II. Each point represents a separate experiment using a different pool of VLDL.

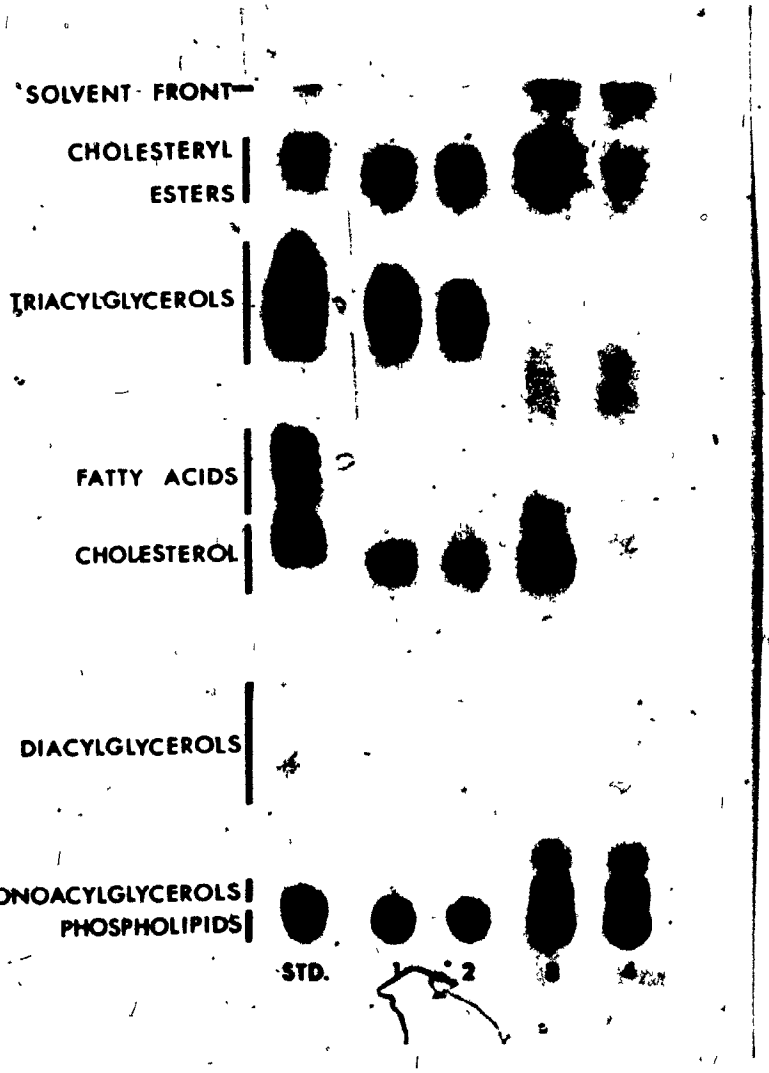
uptake of rat and human LDL indicates little species-specificity. It would thus appear that the specific, high affinity uptake of rat VLDL apoproteins is confined to the VLDL remnants and LDL-like material is not produced at low rat VLDL concentration. It also appears that the rat heart is capable of removing, non-specifically, other lipoproteins.

The lipid composition of human VLDL and its catabolic products is illustrated in Figure 13. Following heart perfusion, there is a noticeable decrease in all lipids with the possible exception of the cholesteryl esters. A trace of free fatty acids is also discernible. Qualitatively similar changes in the lipid composition of the rat VLDL remnants were also observed. The d = 1.019 - 1.063 g/ml fraction produced from human VLDL has virtually no TG, with cholesterol, cholesteryl esters and phospholipid as the dominant lipids. Small quantities of MG and free fatty acids are also seen. The d = 1.063 - 1.21 g/ml fraction contains largely phospholipids, with trace amounts of MG, TG and some cholesteryl esters. The lipid composition of these fractions corresponds to those normally associated with human serum LDL and HDL (124).

The distribution of the apoproteins of VLDL in the various density fractions following a heart perfusion was also investigated. An aliquot of each fraction was delipidated and the apoproteins, solubilized in a solvent containing 7 M urea and 1% SDS, were separated by PAGE in 7 M urea in the absence of SDS. The locations of the major apoproteins (apoB, apoE and apoC) were identified by comparison with the mobility of the purified apoproteins. Typical gels of the human and rat VLDL as well as the purified apoproteins are shown in Figure 14. The gels were divided into four zones: zone 1 contained apoB, the material found at the top of the running gel, as well as small amounts of aggregated material

FIGURE 13

LIPID COMPOSITION OF HUMAN VERY LOW DENSITY LIPOPROTEINS AND THEIR CATABOLIC PRODUCTS FOLLOWING HEART PERFUSION

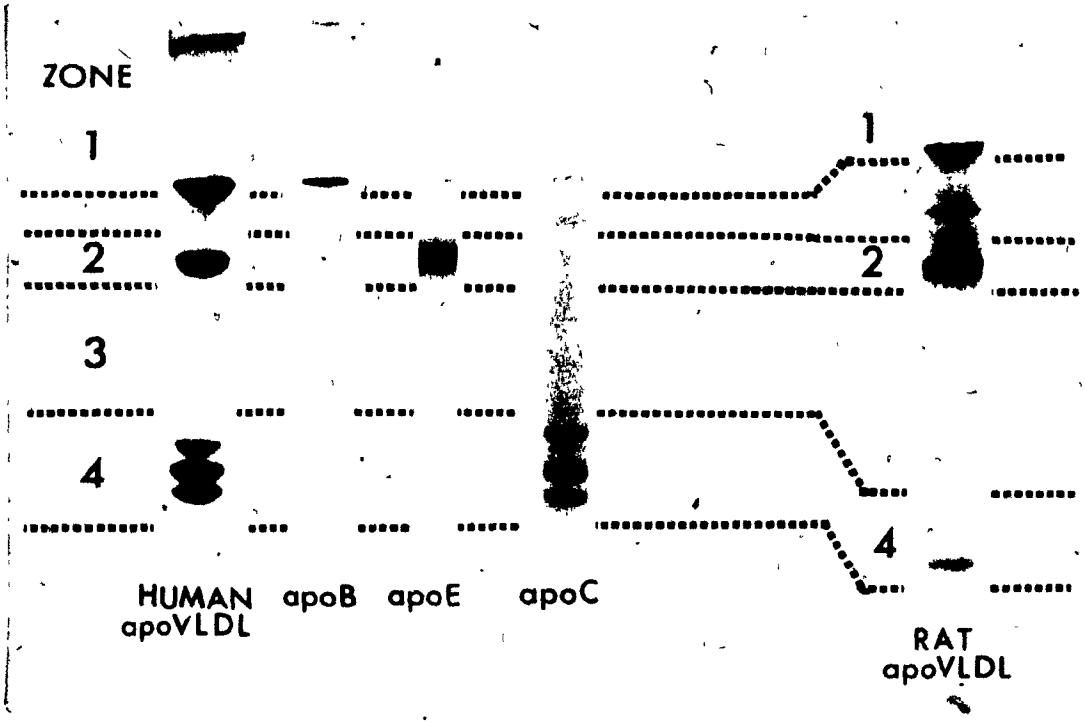


Legend: STD - mixture of lipid standards
1 - VLDL prior to perfusion
2 - d < 1.019 g/ml VLDL remnants
3 - d = 1.019 - 1.063 g/ml lipoproteins
4 - d = 1.063 - 1.21 g/ml lipoproteins

Lipid classes were separated by glass paper chromatography. The quantity of lipid spotted on the chromatogram is equal to the lipid extracted if the lipoproteins had been isolated from the following volumes of perfusate: 1 - 0.03 ml, 2 - 0.03 ml, 3 - 0.07 ml, and 4 - 0.06 ml.

FIGURE 14

TYPICAL POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERNS OF HUMAN AND RAT VERY LOW DENSITY LIPOPROTEINS AND SOME PURIFIED HUMAN APOPROTEINS



Human and rat VLDL were delipidated and their apoproteins separated as described in Chapter II. The gels were cut into zones, designated 1 - 4, for the assay of the radioactivity. The major apoproteins of zones 1 - 4, corresponded to apoB, apoE, apoD or apoA-III, and apoC-II and C-III. The area between zones 1 and 2 (apoC-I) as well as the clear portion of the gels beyond zone 4 were also assayed for radioactivity, but contained only negligible amounts. It will be noted that under identical electrophoretic conditions, the rat apoproteins migrated relatively faster than the equivalent human apoproteins.

that failed to penetrate the stacking gel. The area below zone 1, containing apoC-I, had only minimal amounts of radioactivity associated with it, undoubtedly due to the lack of tyrosine in this apoprotein (74) The major apoprotein of zone 2 was apoE, although minimal amounts of apoA-I may have been also present. Zone 3, a large segment of the gel, contained only one light protein band, probably apoD, and minimal amounts of radioactivity. The apoC-II and apoC-III were located in zone 4.

The gels were cut into the zones indicated and the radioactivity in each zone, as well as the interventing area between zone 1 and 2 was determined. The distribution of the apoproteins of the intact VLDL (circulated through the apparatus in the absence of hearts), VLDL remnants, and the higher catabolic products is shown in Table VI. It should be noted that the d < 1.019 g/ml remnants are richer in apoB and poorer in apoC. These changes are even more pronounced in rat VLDL remnants. Since small amounts of unidentified aggregated protein, containing no more than 10% of the total radioactivity applied to the gel were sometimes observed at the top of the stacking gel, in some experiments tetramethylurea (53) was used to delipidate and dissolve an aliquot of the VLDL and VLDL remnants, in addition to the conventional ethanol/ether delipidation of another aliquot of the same preparation. Tetramethylurea presumably solubilizes all of the apoproteins, with the exception of apoB. The precipitated apoB was separated from the tetramethylurea-soluble apoproteins, which were then separated by PAGE. The resulting gels contained no radioactivity or visible protein on the top of the stacking or running gel. The distribution of the radioactivity between the tetramethylurea-insoluble apoB and the various tetramethylurea-soluble, separated apoproteins was similar to that obtained when

TABLE VI

PERCENT DISTRIBUTION OF ^{125}I-LABELLED APOPROTEINS IN VERY LOW DENSITY LIPOPROTEINS AND THEIR PRODUCTS FOLLOWING A HEART PERFUSION

	HUMAN				RAT	
	VLDL		d=1.019-	d=1.063-	VLDL	
Zone	Intact	Remnant	1.063 g/ml	1.21 g/ml	Intact	Remnant
1	47 ± 5	51 ± 4	59 ± 3	38 ± 4	45 ± 6	66 ± 5
2	8 ± 2	10 ± 2	11 ± 2	10 ± 2	8 ± 2	8 ± 2
3	5 ± 1	6 ± 2	7 ± 2	10 ± 3	-----	-----
4	38 ± 3	29 ± 3	20 ± 3	33 ± 1	31 ± 1	8 ± 4
n†	7	7	4	4	4	4

* Gels were divided into zones as shown in Figure 14

† number of experiments

Zone 1 corresponds to apoB and zone 4 to apoC. Initial TG concentrations ranged from 0.1 to 1.0 mg/ml perfusate. Each value represents the mean ±S.E.

ethanol/ether delipidation and solubilization in urea was used.

Because of the variation in the extent of iodination of apoB and apoC between different VLDL preparations, changes in the proportion of these apoproteins can best be seen by analysis of the paired differences in each experiment (Table VII). As the result of the action of LPL, significant increases in the proportion of apoB and decreases in that of apoC in the VLDL remnant occur. A close correlation ($r = 0.75$, $p < 0.001$) between the percent loss of VLDL TG and the percent decrease in VLDL apoC during the formation of remnants can be seen when the data from individual experiments are plotted (see Figure 15).

The data in Table VI also indicate that the products of human VLDL catabolism in the d = 1.019 - 1.063 and 1.063 - 1.21 g/ml fractions do not have the apoprotein composition commonly associated with centrifugally isolated serum LDL and HDL, respectively. Thus in spite of the lower percent of apoC in the d = 1.019 - 1.063 g/ml fraction, the proportion of the non-B apoproteins exceeds that found in the circulating LDL (115) The d = 1.063 - 1.21 g/ml fraction contained a larger amount of labelled apoprotein at the top of the gel (zone 1) than is usually found when the apoproteins of circulating HDL are analyzed. Although apoA tends to polymerize after centrifugation at low concentrations (182), apoA-I is found in only minute amounts in the original VLDL. Thus the lipoproteins isolated at d = 1.019 - 1.063 g/ml and 1.063 - 1.21 g/ml may represent mixtures of products of VLDL catabolism. Indeed, about 20% of the material in the d = 1.019 - 1.063 g/ml range was not absorbed by Concanavaline A column and therefore represents lipoproteins deficient in apoB (304). This was confirmed by PAGE of the unretained material, containing 75% of the radioactivity in apoC and negligible amounts at the top of the running gel.

TABLE VII

CHANGE IN THE PROPORTIONS OF APOPROTEINS B AND C IN HUMAN AND RAT VERY LOW DENSITY LIPOPROTEINS DURING A TWO HOUR HEART PERFUSION

APOPROTEIN	HUMAN		RAT	
	Change	p	Change	p
ApoB (zone 1)*	+4.0 ± 2.0%(7)†	<0.05	+20.5 ± 6.5%(4)	<0.05
ApoC (zone 4)	-8.6 ± 1.2%(7)	<0.0005	-22.8 ± 4.8%(4)	<0.01

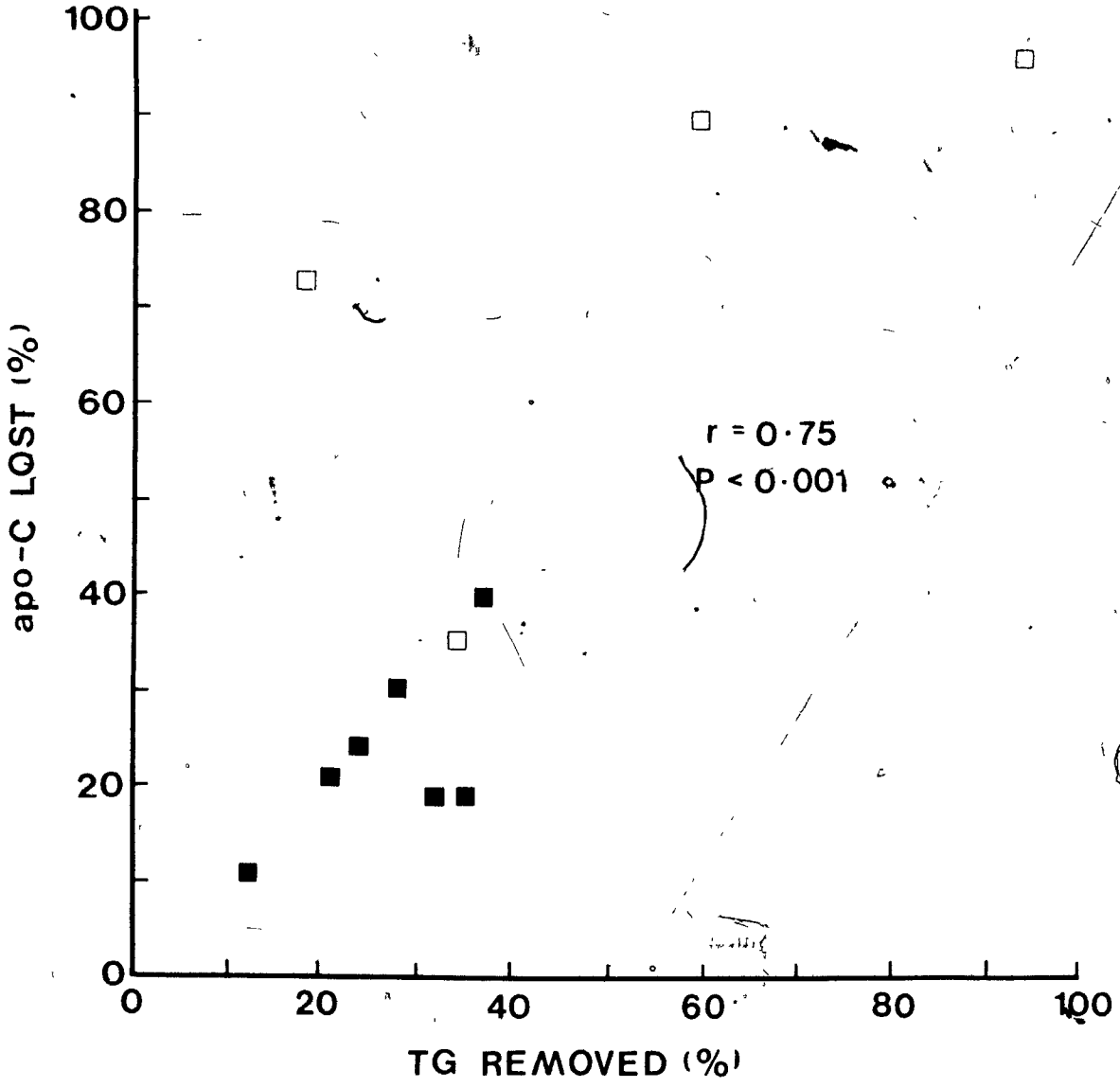
* as shown in Figure 14

† represents the number of experiments

Due to the variation in the extent of iodination of apoB and apoC between the different VLDL preparations, changes in the proportions of these apoproteins in human and rat VLDL during the heart perfusion were analyzed by paired differences in each individual experiment.

FIGURE 15

RELATIONSHIP BETWEEN THE LOSS OF TRIGLYCERIDE AND THE DECREASE IN THE PROPORTION OF APO C IN VERY LOW DENSITY LIPOPROTEIN REMNANTS



Legend: ■ - Human VLDL remnants
□ - Rat VLDL remnants

The loss of TG was determined chemically. The loss of apoC-II and apoC-III (zone 4 , see Figure 14), is expressed as percent decrease in the proportion of these apoproteins in the d < 1.019 g/ml remnants isolated after the perfusion. The correlation coefficient (r), was calculated using all points from both human and rat VLDL. The TG concentrations ranged from 0.1 to 1.0 mg/ml perfusate.

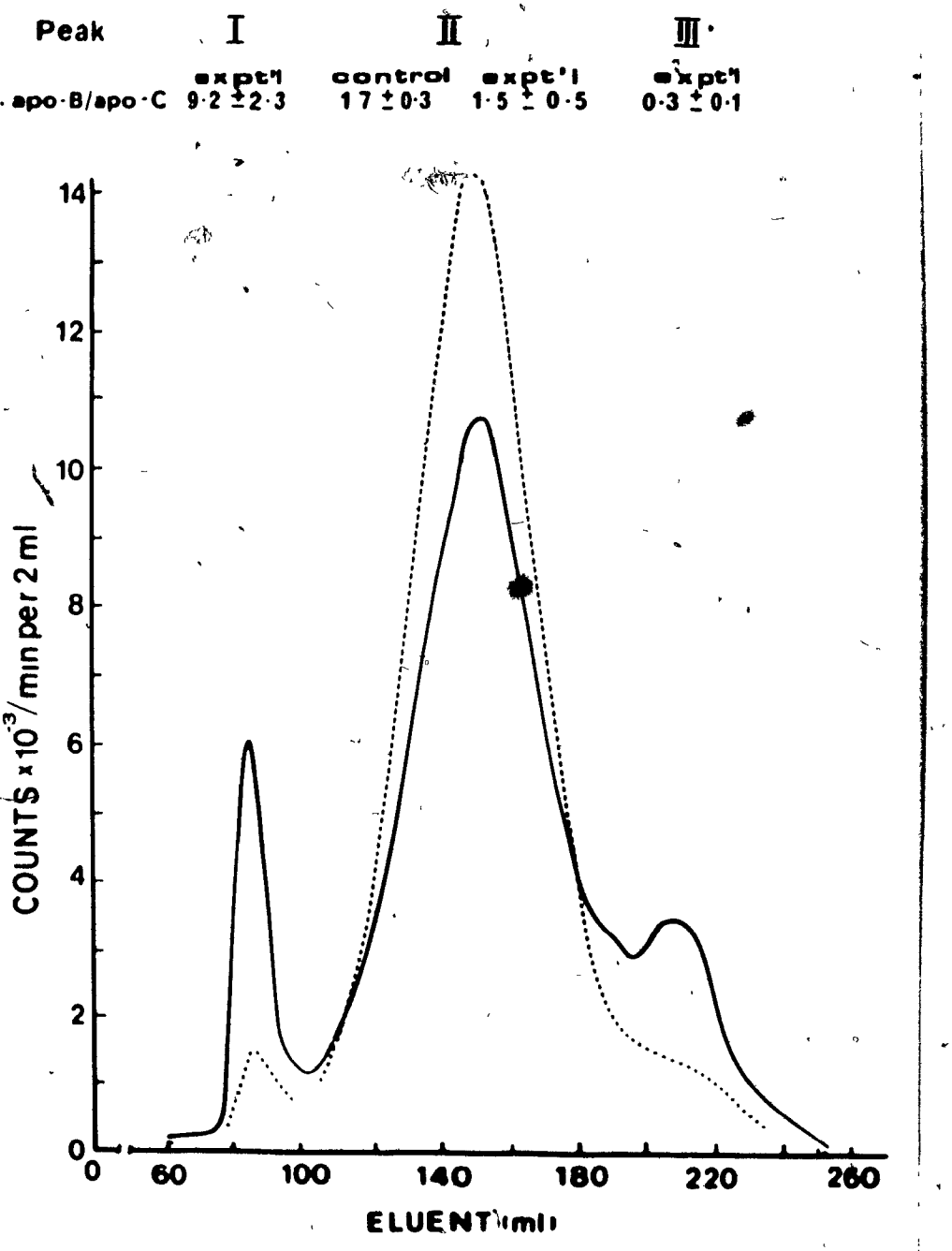
C. SEPARATION OF HUMAN PERFUSATE LIPOPROTEINS BY AGAROSE GEL FILTRATION

The observations that the higher density catabolic products of VLDL had lipid compositions similar to those of serum lipoproteins of the same density, but a different apoprotein composition (cf. Figure 13 and Table VI), suggests that the ultracentrifugally isolated lipoprotein fractions from the perfusate represented mixtures of different lipoprotein species of similar hydrated densities. This is a reasonable assumption, since the amount and the kind of lipid associated with the apoprotein(s) is the main determinant of the particle density. An attempt was therefore made to separate the lipoproteins found in the heart perfusate by agarose gel filtration without prior ultracentrifugation. The distribution of the lipoproteins in a typical control (circulated through the apparatus without hearts), and experimental perfusate containing human VLDL is shown in Figure 16. Except for a very small peak at the void volume, usually smaller than in the experiment shown, the control VLDL eluted as a single peak. Following the heart perfusion three lipoprotein peaks were discernible. The first was found in the void volume. The major peak corresponded to the control VLDL peak, although in some experiments a slight shift to the right was observed. A new lipoprotein peak appeared as the bed volume of the column was approached.

The apoprotein composition of each peak was analyzed by PAGE. The ratios of the radioactivity in zones 1 and 4, representing apoB and apoC respectively (cf. Figure 14), for each peak is shown in Figure 16. It will be noted that the perfusion did not produce a significant change in the ratios of the main peak (II). Peak I contained lipoproteins predominantly labelled in apoB, resembling circulating LDL. Lipoproteins

FIGURE 16

AGAROSE GEL FILTRATION (SMALL COLUMN) OF PERFUSATE LIPOPROTEINS FOLLOWING PERFUSION OF ^{125}I-LABELLED HUMAN VERY LOW DENSITY LIPOPROTEINS



Perfusate lipoproteins after perfusion in the absence (----) and presence (——) of hearts were separated on a 2 × 60 cm agarose column as described in Chapter II. A typical elution pattern is shown. The ratio of radioactivity in the apoB and apoC region of polyacrylamide gels (see Figure 14) of the delipidated lipoproteins in the three peaks represent a mean of three experiments ±S.E.

in peak III on the other hand contained very little, if any apoB relative to apoC, a pattern more consistent with serum HDL. Electron microscopy of the material eluted in peak I (Figure 17), shows large aggregates containing many particles resembling LDL in size, in addition to some VLDL sized particles.

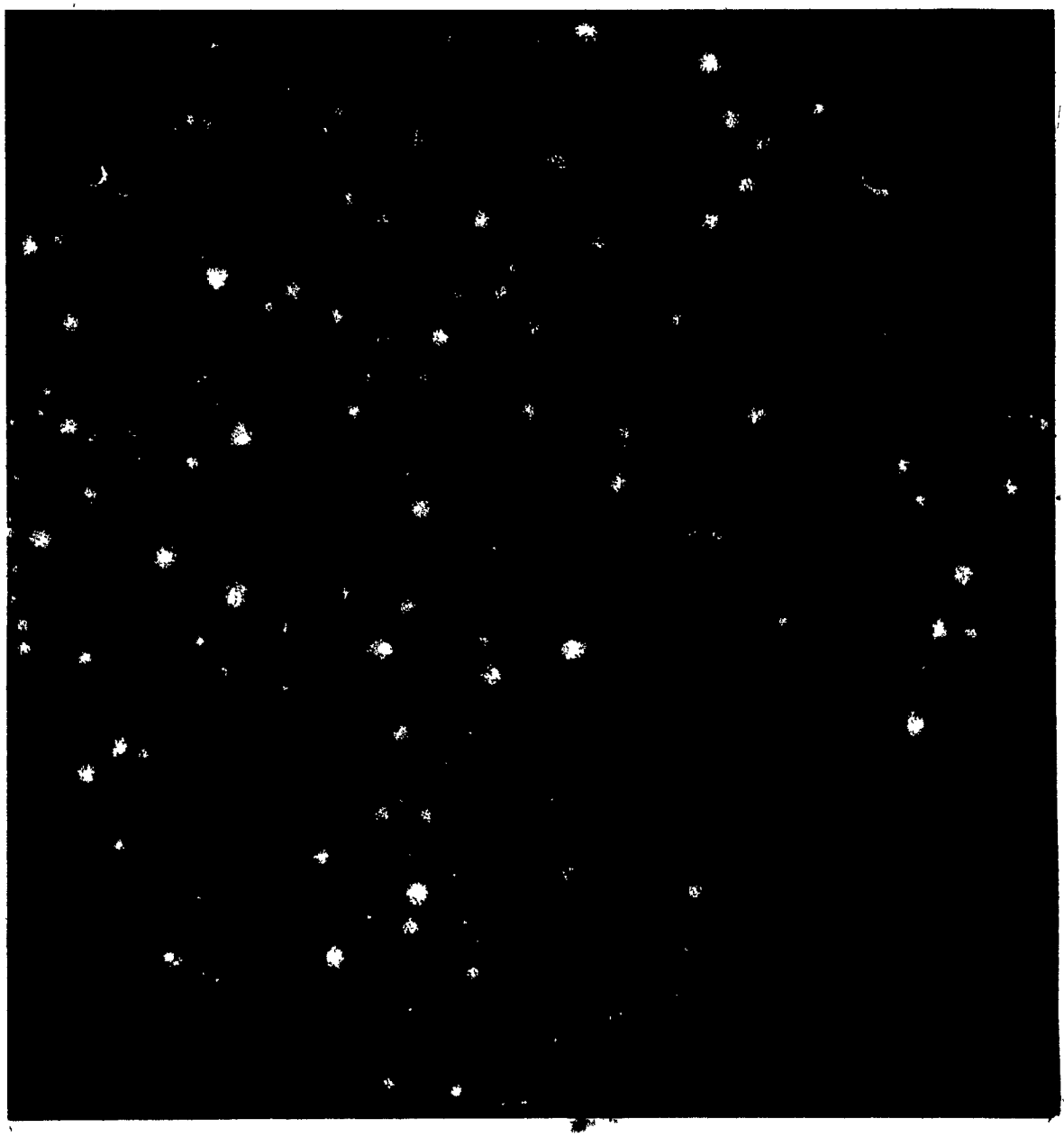
A similar pattern of lipoprotein distribution in the perfusate was obtained, with a somewhat better separation, when the perfusate (and control) lipoproteins were separated in larger and longer (4 × 100 cm) agarose column of similar composition. The elution pattern is shown in Figure 18. While the control VLDL eluted again as a single peak, the trailing portion of the material was collected as a separate fraction (IIIb). Following the heart perfusion four peaks were obtained: a peak at the void volume (I) and a second, smaller peak (II) preceded the main peak (IIIa and IIIb). The main peak however was shifted to the right, indicating a slight shift in the general size range of the total VLDL population. As in the smaller column, the main peak was followed by a new lipoprotein peak (IV).

Material from each fraction was pooled separately, concentrated by aquacide and dialyzed. The apoprotein composition of each fraction was studied by PAGE. The apoB and apoC content of each pooled fraction was determined by "rocket" immunoelectrophoresis. The apoB to apoC ratio of peaks I, IIIa, IIIb, and IV, determined by these two methods is indicated at the top of the elution profile (Figure 18). The total amounts of apoB and apoC in each of the peaks is shown in Table VIII. Apoprotein B is the predominant apoprotein in peak I as well as the major protein of the trailing portion of the main peak (IIIb). Apoprotein C on the other hand, is the predominant apoprotein of the peak IV lipoproteins.

FIGURE 17

ELECTRON MICROGRAPH OF LIPOPROTEINS

IN PEAK I OF AGAROSE GEL FILTRATION

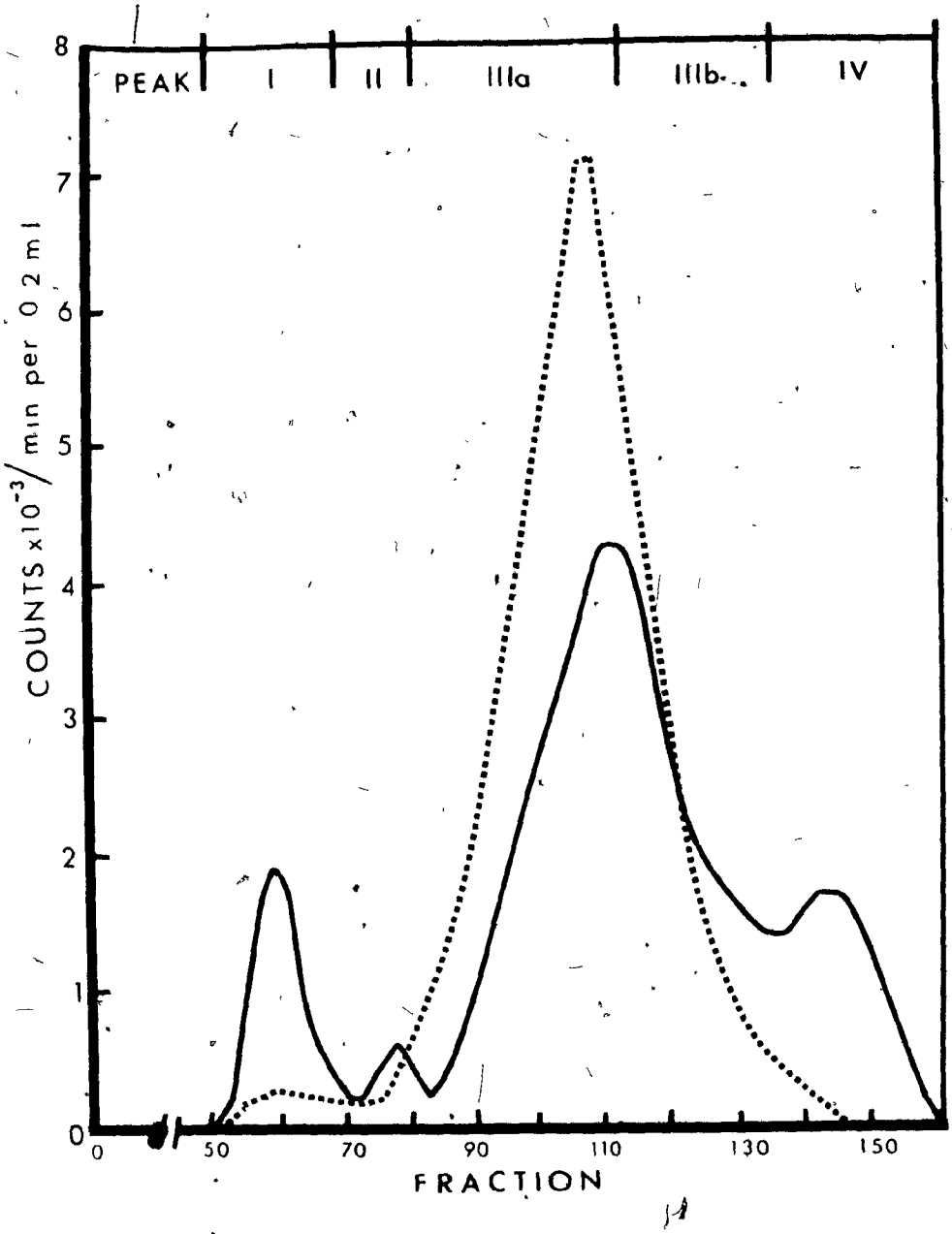


Visualization of the negatively stained lipoproteins of peak I obtained by agarose gel filtration of the human VLDL heart perfusate reveal the presence of numerous, apparently aggregated, LDL - sized particles as well as a smaller number of VLDL - sized lipoproteins (magnification 78,000 x).

FIGURE 18

AGAROSE GEL FILTRATION (LARGE COLUMN) OF PERFUSATE LIPOPROTEINS FOLLOWING PERFUSION OF ^{125}I-LABELLED HUMAN VERY LOW DENSITY LIPOPROTEINS

(μg) apoB/apoC*	5.1	—	1.0	2.1	0.3
(^{125}I) apoB/apoC	3.9	—	0.9	3.5	0.4



Legend: * - the content of apoB and apoC in each peak was determined by "rocket" immunoelectrophoresis and the ratios calculated.
After perfusion in the absence (----) and presence (——) of hearts, perfusate lipoproteins were separated on a 4 × 100 cm agarose column as described in Chapter II. The ratio of ^{125}I-apoB to ^{125}I-apoC in each peak was also determined, as described in Figure 16.

TABLE VIII

THE DISTRIBUTION OF APOPROTEINS B AND C IN THE VARIOUS LIPOPROTEIN FRACTIONS OF THE PERFUSATE SEPARATED BY AGAROSE GEL FILTRATION*

	HEART PERFUSATE¶				CONTROL§	
PEAK	I	IIIa	IIIb	IV	IIIa	IIIb
apoB (µg/peak)†	364	1156	935	172	1826	818
apoC (µg/peak)†	70	1326	446	510	2310	315
µg apoB/µg apoC	5.1	0.9	2.1	0.3	0.8	2.5
^{125}I-apoB/^{125}I-apoCΨ	3.9	0.9	3.5	0.4	0.9	1.2

* as shown in Figure 18

¶ Peak II did not contain enough material for meaningful analysis

§ Peaks I, II and IV were absent when control perfusate (absence of hearts) lipoproteins were separated

† as determined by "rocket" immunoelectrophoresis

Ψ as determined by separating the delipidated apoproteins of each peak by PAGE (see Figure 19), and counting zones 1 and 4, corresponding to apoB and apoC, respectively.

The apoB to apoC ratios determined by PAGE and immunoelectrophoresis are very similar. Furthermore, as determined immunologically, significant amounts of apoB were removed from the front portion of the main peak (IIIa) during the heart perfusion. Some of it redistributed to the trailing portion of the same peak, but most of it was recovered in peak I. On a weight basis, almost twice as much apoC as apoB was removed from fraction IIIa. While 13% was recovered in the trailing portion of the main fraction, most of it (52%) was found in peak IV.

The separation of the perfusate lipoproteins by agarose gel filtration indicates that catabolism of human VLDL by the perfused rat heart gives rise to LDL-like lipoproteins in apoprotein composition as well as size (see Figures 17 and 18). Furthermore, the production of a lipoprotein containing almost exclusively apoC was demonstrated. The small amount of apoB found in this peak appeared to be a contamination from the trailing portion of the main peak, as the separation of the two peaks was not complete. At the same time it should be remembered that when perfusate lipoproteins were isolated by ultracentrifugation, a method which separates lipoproteins on the basis of the amount and kind of lipid they contain, an LDL-like lipoprotein in lipid composition was found. Similarily, the HDL density range of the perfusate had a lipid composition typical of serum HDL (see Figure 13).

D. HEPATIC UPTAKE OF THE TRIGLYCERIDE-RICH LIPOPROTEIN REMNANTS

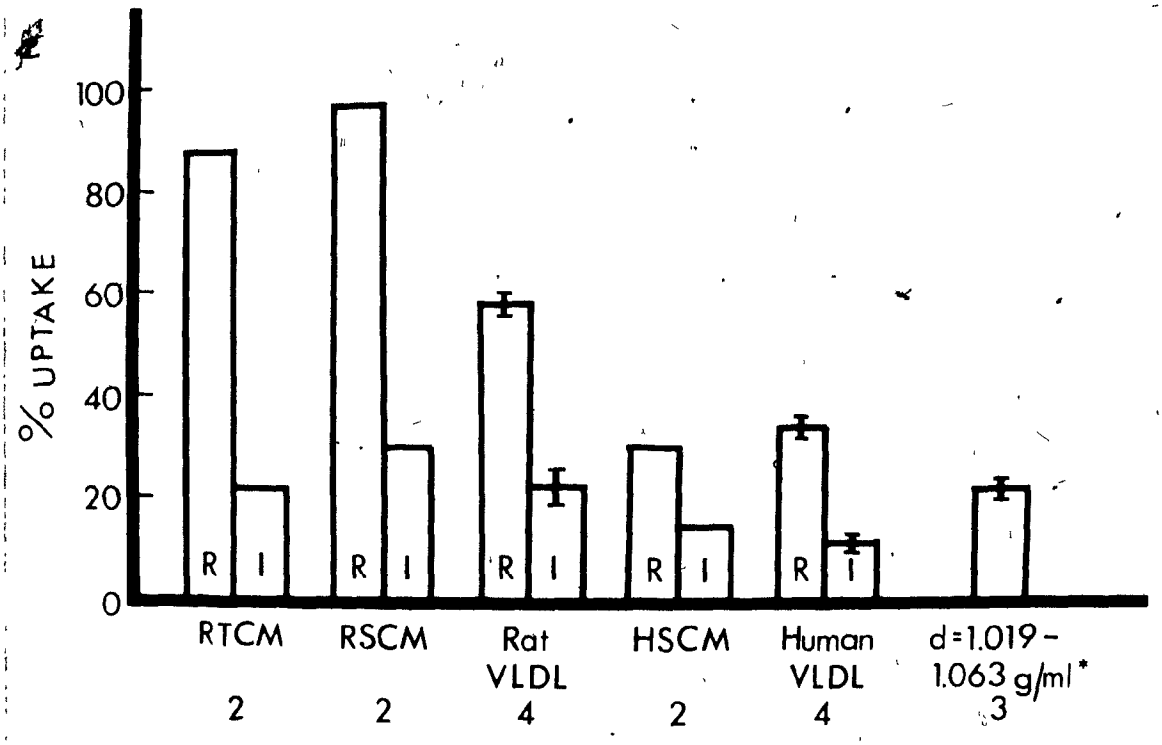
Preliminary data were obtained on the hepatic uptake of human and rat TG-rich lipoprotein remnants prepared by perfusing them through rat hearts. Remnants were prepared from rat thoracic duct chylomicrons and

rat and human serum chylomicrons and VLDL, as described in the previous section. Chylomicron remnants, isolated from the heart perfusate at $d < 1.006$ g/ml, were added directly into the liver perfusate. Very low density lipoprotein remnants, isolated at $d < 1.019$ g/ml were dialyzed to $d = 1.006$ g/ml prior to liver perfusion. In three experiments the $d = 1.019 - 1.063$ g/ml fraction, produced from human VLDL, was also dialyzed to $d = 1.006$ g/ml and their uptake by the perfused liver was studied. Control experiments consisted of perfusing the TG-rich lipoproteins of rat and human origin through the heart apparatus, in the absence of hearts, and subsequently by perfusing them through the liver. The results are presented in Figure 19. The rapid uptake of rat serum chylomicron remnants was similar to that observed with rat thoracic duct chylomicron remnants and reported previously (9). The action of LPL was necessary, as the intact particles are not taken up to a significant degree. Rat serum VLDL remnants were also rapidly removed by the perfused liver, although not to the same extent as the rat chylomicron remnants. Interestingly, a significant portion of intact rat VLDL was also removed (over 20%).

In direct contrast, human TG-rich lipoprotein remnants were removed from the perfusate in significantly smaller amounts. Unlike the rat, no difference in the rate of uptake between the human chylomicron and VLDL remnants was observed. In fact, the degree of uptake of human TG-rich lipoprotein remnants appeared to be similar to that of the intact rat TG-rich lipoproteins. These data suggest that some species-specificity in the hepatic uptake of lipoproteins may be operating in the rat.

FIGURE 19

UPTAKE OF THE HUMAN AND RAT INTACT AND REMNANT TRIGLYCERIDE-RICH LIPOPROTEINS BY THE PERFUSED RAT LIVER



Legend:
R - remnant lipoproteins (prepared by heart perfusion)
I - intact lipoproteins (circulated in the heart perfusion apparatus in the absence of hearts)
RTCM - rat thoracic duct chylomicrons ($S_f > 400$)
RSCM - rat serum chylomicrons ($S_f > 400$)
HSCM - human serum chylomicrons ($S_f > 400$)
* - lipoproteins of d = 1.019 - 1.063 g/ml produced during the catabolism of human VLDL by the perfused heart

The hepatic uptake of intact triglyceride-rich lipoproteins and their remnants, prepared by heart perfusion, was measured in terms of the disappearance of ^{125}I-labelled apoproteins from the liver perfusate. Liver perfusions were carried out for 60 minutes, as described in Chapter II. The numbers below each category of lipoproteins indicates the number of experiments performed for each, intact and remnant lipoproteins. The bars represent the mean of two experiments, or the mean ±S.E. when 3 or 4 experiments were pooled. Each experiment was carried out with a different pool of lipoproteins. The intact lipoproteins and their remnants for each experiment were from the same pool, however.

CHAPTER IV

DISCUSSION

The use of perfused hearts for the study of the first stage of the catabolism of the TG-rich lipoproteins has produced a number of interesting observations. The heart is a source of immobilized LPL which has similar kinetic characteristics to the heparin solubilized heart LPL (22). However, the beating heart is a more physiological preparation since some of the catabolic products are taken up by the tissue.

Preliminary experiments reported here demonstrate the capacity of the rat heart LPL to hydrolyze chylomicron TG from several sources. Little species-specificity is observed, as human chylomicrons are also a good substrate. Based on the relatively few experiments performed, it appears however, that the rat LPL has a lower affinity for the human chylomicrons. There may be a number of reasons for this, but since little is known about the precise nature of the LPL-substrate interaction, it is difficult to speculate on them. Differences in the accessibility of the substrate or subtle differences in the activator protein (apoC-II) may be responsible. The organization and composition of the surface components may not only affect the accessibility of the substrate (TG), but also determine the number of the enzyme molecules (LPL) the particle can get attached to at any one time (238).

Under identical perfusion conditions, at saturating levels the rat heart is consistently able to hydrolyze more TG in chylomicrons than in VLDL. Since in both cases little hydrolysis occurs after 90 minutes of

perfusion in spite of a fresh supply of the enzyme, the limitation must be a property of the remnant particle itself. The sensitivity of the LPL to the lipid composition of the substrate has been reported (305). A cholesterol to TG ratio of 1:10 in artificial substrates completely inhibited the LPL. A similar ratio of these lipids is reached relatively sooner during the hydrolysis of TG in VLDL than in chylomicrons, resulting in an earlier inhibition of VLDL hydrolysis.

In general, there appears to be little difference in the extent of removal of TG between human and rat VLDL. The relatively constant amount of TG removed from rat VLDL over a wide range of perfusate concentrations suggests that the enzyme system is close to saturation at the lowest concentrations used in these experiments. This would be predicted from the reported kinetic characteristics of the heart LPL (22). Although the greater variation in the extent of breakdown of human VLDL (see Figure 7) may be due to individual differences in the blood donors, it is also possible that the kinetics of the TG hydrolysis of human and rat VLDL differ.

The action of LPL on human and rat VLDL produced a number of physical and chemical changes in the particle. Following the heart perfusion there was a noticeable decrease in the proportions of all of the lipids of the VLDL remnant, with the possible exception of the cholesteryl esters. Substantial removal of TG, a core component of VLDL, resulted in a shift in the distribution of the particles towards the lower end of the same general size range. The average decrease in the volume of the particle, calculated from the average diameters, corresponded closely to that in the TG content, which was determined chemically (see Table III). Similar changes in the size and lipid composition of rat VLDL

remnants prepared in vitro by incubation of VLDL with post-heparin plasma or in supradiaphragmatic rats were reported (243,105).

The removal of TG from VLDL was not evenly distributed between each particle. Electron microscopy revealed the presence of VLDL particles at various stages of delipidation, ranging from apparently intact ones to completely collapsed, disc-shaped ghosts with excessive surface material surrounding the empty, relatively hydrophilic interior. Rat VLDL remnants in particular were consistently surrounded by a membrane-like material, a feature never observed with intact particles. The appearance of excessive surface coat was not reported in VLDL remnants formed in the presence of plasma. The possibility thus exists that the remnants formed in the present experiments are incomplete, requiring a second step, probably involving the action of LCAT, an enzyme absent in the heart perfusion system. Indeed, the appearance of remnants produced by heart perfusion is remarkably similar to the lipoproteins (VLDL and LDL) isolated from the sera of LCAT-deficient patients (123,135). Both contain a large amount of excessive surface material and tend to aggregate. The present results are thus consistent with the proposed involvement of LCAT in modifying the lipoprotein surface coat to accomodate the continuous removal of TG (249). While the surface of the remnants in the intact animal may be constantly remodeled in the circulation, it may play an important role while the particle is attached to the LPL on the endothelial lining of the arteries. A fusion of such lamellar structures with the endothelial cell membranes during the attachment of the TG-rich lipoproteins to the LPL has been suggested as a mechanism for the transport of the lipolytic products (MG and fatty acids) to the surrounding tissues (238).

Hydrolysis of the TG-rich lipoproteins is associated with a loss of apoC, and thus a relative enrichment of apoB (9,105,240,243,255). The present data show a significant correlation between the loss of TG and apoC from both human and rat VLDL during catabolism. The data furthermore demonstrate that the apoC leaving the VLDL particle during the hydrolysis contain considerable amounts of lipid (primarily phospholipid) to remain in the HDL density range without the presence of a postulated obligatory acceptor (serum HDL) (103,306). Negligible recovery of apoprotein in the d > 1.21 g/ml fraction of the perfusate, the large recovery of phospholipid containing apoC in the d = 1.063 - 1.21 g/ml fraction, and the isolation of a lipoprotein species containing almost exclusively apoC by column chromatography, all provide strong evidence of LpC formation during VLDL catabolism. Such observations are consistent with the postulated existence of discrete primary lipoprotein families (115), described in Chapter I, and represent the first demonstration of primary lipoprotein production during lipoprotein metabolism.

Although a recent report has also shown a correlation between the loss of apoC and the degree of VLDL TG hydrolysis (306), these investigators found that in the absence of HDL most of the apoC lost from VLDL was recovered in the d > 1.21 g/ml fraction of the incubation medium, apparently devoid of lipid. A closer examination of their experimental design explains this apparently contradictory finding. The VLDL used in their experiments was labelled in the apoC moiety by absorption of purified, delipidated exogenous apoC, which during the process of isolation (in 6 M urea) was considerably denatured. While delipidated apoC or even fragments thereof can activate LPL (307), it is not surprising that after the detachment from the VLDL it would be found in the d > 1.21

g/ml fraction.

The present data provide considerable information about the nature and composition of human VLDL remnants, properties that have not been defined to date. Thus while the transformation of large human VLDL into smaller ones has been suggested on the basis of in vivo kinetic studies (13,244,246), the compositional changes that take place during this process have not been defined. The composition of human VLDL remnants has been inferred from studies of the chemical composition of VLDL particles of decreasing size, or reduced electrophoretic mobility (slow pre-β mobility), isolated directly from the serum (243,245). In these studies however, small VLDL particles secreted directly by the liver could not be distinguished from the remnants. The present data provide evidence of the in vitro transformation of larger VLDL particles into smaller ones of defined chemical composition, similar to that of rat VLDL remnants produced in the present experiments or reported by others (105, 240).

The present studies have furthermore confirmed the in vivo kinetic studies, indicating that degradation of rat VLDL results in only minimal production of LDL (14,17,255), in contrast with human VLDL, a large part of which is transformed into LDL (10-13,156,253). At low levels of rat VLDL in the heart perfusate (corresponding to the normal rat serum concentrations), very little LDL (d = 1.019 - 1.063 g/ml) is formed, most of the lipoprotein being taken up by the heart or remaining in the VLDL range (d < 1.019 g/ml). This correlates well with the low concentrations of LDL found in the rat. In contrast, a constant proportion of the catabolized human VLDL in the heart perfusate is consistently found in this density range.

The finding of progressively more delipidated remnants throughout the lipoprotein density spectrum of the perfusate demonstrates the disadvantages of the ultracentrifugal isolation of lipoproteins, a technique that is convenient but may be dangerous when used to define distinct lipoprotein species. Thus despite the fact that the LDL and HDL density range lipoproteins isolated from the human VLDL perfusate had a very similar lipid composition to serum LDL and HDL respectively, their apoprotein composition was quite different. The LDL fraction of the perfusate contained larger than normal amounts of non-apoB apoproteins and the HDL fraction contained significant amounts of apoB-like material. These observations suggest the presence in each of these fractions of a mixture of lipoprotein species of similar hydrated densities. Indeed, the use of agarose gel filtration and affinity chromatography confirmed this possibility. When lipoproteins of d = 1.019 - 1.063 g/ml were applied to a Concanavaline A column, over 20% of the material was not absorbed, corresponding to a lipoprotein lacking apoB (304). Over 75% of this material was apoC. The aggregated material in peak I obtained by agarose gel filtration contained almost exclusively apoB. Numerous LDL-sized particles were observed with a few VLDL-like particles also present, probably accounting for the minimal non-apoB apoprotein found. Furthermore, the presence of partially delipidated smaller VLDL remnants was reflected by the slight shift of the main (VLDL) peak towards the bed volume of the column, and an increased apoB to apoC ratio in the trailing portion of this peak. Finally, an apoC-rich fraction (LpC) was also isolated, eluting well before the bed volume of the column, indicating a large molecular weight complex.

Although the major objective of the present work was to characterize the products of VLDL catabolism, the finding that the VLDL apoproteins are removed from the perfusate by the perfused heart is of interest.

While the present data do not completely rule out the possibility that some VLDL is trapped in the vascular system of the heart, this possibility seems unlikely, as lipolytic activity was found to be necessary for uptake to occur. The differences between the uptake of the human and rat VLDL apoproteins (cf. Figure 11 and Figure 12), provides further evidence that the uptake is not due to trapping. Trapping should be equally effective for human and rat lipoproteins.

As pointed out earlier, at low rat VLDL perfusate concentrations, only negligible amounts of LDL density material was produced, while a large portion of the catabolized material was recovered in the heart. Increasing concentrations of rat VLDL in the perfusate resulted in an increased proportion of the catabolized VLDL recovered in the LDL density range, while the proportion taken up by the heart rapidly decreased. Such a relationship (see Figure 11), would suggest the presence of an easily saturated high affinity receptor for the rat VLDL catabolic products. In view of the similar and incomplete uptake of both human and rat ^{125}I-labelled serum LDL at low concentrations from the perfusate, it is possible to speculate that the specificity of the uptake is confined to the rat VLDL remnants of $d < 1.019$ g/ml, without prior degradation to an LDL density range product. These products however, accumulate at higher rat VLDL concentrations when the receptors are presumably saturated. It is not known whether the iodinated VLDL protein removed from the perfusate is simply bound to the endothelium or is internalized into either the endothelial or myocardial cells. Cultured rat arterial smooth muscle cells have been shown to take up rat VLDL remnants in preference to the intact particles (255), but most studies using a variety of cultured cells from the rat could not detect a significant species-specificity, but rather suggested a limited capacity to take up any rat lipoproteins.

While it is generally believed that the rat VLDL, after removal of much of their TG content by the peripheral tissues are rapidly cleared from the circulation by the liver (308), the present data provide a first indication that the peripheral tissues in the rat may play an important role in the clearance of the VLDL remnant as well, much in the way the product of human VLDL catabolism, LDL, is thought to be cleared (261). The relative contribution of each of these tissues is impossible to establish from the present data, as the relative degree of VLDL delipidation necessary for the uptake by either of these tissues was not investigated. Nevertheless, the ability of the rat peripheral tissues to remove VLDL remnants may provide an additional mechanism to explain the low levels of LDL in this species. A recent report (309) may provide further support for this hypothesis. In addition to significant losses of lipids, VLDL in supradiaphragmatic rats lost over 50% of their protein content within 30 minutes. During the same time only a small increment in HDL proteins was noted, leaving a great proportion of the protein lost from VLDL unaccounted for, strongly suggesting uptake by the peripheral tissues.

The proportion of the human VLDL apoproteins taken up by the heart and that recovered in the LDL range of the perfusate appears to be independent of the VLDL concentration in the perfusate, suggesting a non-specific and, at least in the concentration range investigated, not saturable mechanism of uptake (see Figure 12). This observation and the fact that LDL-like material is formed from human VLDL even at the lowest VLDL concentrations investigated, highlight the difference in human and rat VLDL catabolism by the perfused rat heart.

Studies on the hepatic uptake of the TG-rich lipoprotein remnants

confirmed the rapid uptake of the rat thoracic duct and serum chylomicron remnants. Under similar conditions, significant amounts of rat VLDL remnants were also removed. The slower rate of rat VLDL remnant removal is probably a reflection of the in vivo situation, where the rate of the chylomicron clearance exceeds that of the VLDL removal from the circulation (16,17,96).

The ability of the perfused rat liver to take up human TG-rich lipoprotein remnants appears to be limited. Human chylomicron and VLDL remnants, as well as the fraction of d = 1.019 - 1.063 g/ml, produced during the catabolism of human VLDL, are taken up only to a limited extent. Interestingly, the degree of uptake was similar to that of intact TG-rich lipoproteins of the rat, suggesting that the limited uptake of the human lipoprotein remnants may involve a non-specific mechanism, proposed for the intact rat TG-rich lipoproteins (251). In view of the even more restricted uptake of the intact human TG-rich lipoproteins, it appears more feasible that the intact rat and the remnant human lipoproteins may exhibit some partial characteristics necessary for a specific uptake, thus augmenting the already operative non-specific uptake. The intact human TG-rich lipoproteins are probably taken up only by the non-specific uptake. These conclusions are of course speculative, and much more work is needed in this area.

The observed species preference for the hepatic uptake is surprising since studies for lipoprotein degradation by a variety of cultured cells have failed to detect any significant species-specificity. The data obtained in these experiments suggest the presence of receptors of some specificity for the rat TG-rich lipoprotein remnants however.

In summary, it was initially attempted to extend the two-stage,

heart and liver perfusion model of chylomicron catabolism (9) to the study of rat and human serum VLDL catabolism. As the presented data indicate, the catabolism of VLDL is more complex than that of chylomicrons. Rat VLDL at low concentrations are catabolized to remnants significantly depleted of lipids and apoC, and are rapidly removed by the liver or by the extrahepatic tissues, such as the heart. On the other hand, human VLDL catabolism results in a consistent production of LDL-like material. In the process of VLDL catabolism, apoC leaves the particle as a protein-lipid complex, LpC, which can exist in the density range usually associated with serum HDL, without the presence of an acceptor.

Some questions may be raised as to the validity of using the rat to study the fate of human remnants and their higher density catabolic products. Clearly, any species-specificity on the part of the rat tissues may preclude a meaningful study of the fate of the human VLDL apoproteins in the rat. Undoubtedly however, the model is extremely useful in delineating the delipidation steps of VLDL and the production of higher density products.

The present study, which largely restricted itself to the investigation of the action of LPL, contributed significantly to and complemented, the overall picture of VLDL catabolism obtained from in vivo studies in the human and rat. The perfusion system employed is particularly suitable for further investigation of some of the questions left unanswered. An obvious extension of the present study should be the investigation of the effects of additional serum components on VLDL catabolism. These could be added to the perfusate in a stepwise fashion, possibly enabling a distinction between the action of each. The addition of LCAT would be the next logical step. Because direct action of LCAT on VLDL remnants

appears to be unlikely, HDL should also be added, which would provide the activator protein (apoA-I) as well as cholesterol, either directly or through its interaction with the tissues. Also, a more detailed study into the nature of the uptake of lipoproteins by the perfused heart and liver may greatly increase our understanding of lipoprotein metabolism and diseases related to it.

SUMMARY

1. The rat heart LPL can hydrolyze TG in both rat and human serum chylomicrons but appears to have a greater affinity for the former.
2. In the range of the initial perfusate TG concentrations investigated, the rat heart was able to remove more TG from chylomicrons than VLDL of either species. Little hydrolysis of TG occurred after 90 minutes of perfusion in spite of a fresh source of enzyme, indicating an alteration of the lipoprotein particle inhibiting further enzyme action.
3. Triglyceride was removed from the VLDL to a greater extent than the protein moiety, leaving remnants containing relatively more apoB and less apoC. The extent of TG removal from rat or human VLDL was similar and appeared to saturate the heart LPL. The remnants were slightly smaller in size than the VLDL and included partially emptied particles. The proportions of all lipids, with the exception of cholesteryl esters, decreased in the VLDL remnants.
4. The loss of TG correlated with the change of the apoC content of the remnants. The apoC lost from the VLDL in the course of the hydrolysis was mostly recovered in the d = 1.063 - 1.21 g/ml fraction of the perfusate, suggesting the formation of LpC. This fraction resembled serum HDL in its lipid composition, phospholipid being the dominant lipid.
5. In addition to remnants of d < 1.019 g/ml, iodinated lipoproteins of d = 1.019 - 1.063 g/ml were also produced during VLDL degradation, containing largely cholesterol and cholesteryl ester. However, very little of this material was produced from low (physiological) concentrations of rat VLDL, most of the lipoproteins being removed by the heart. At high rat VLDL concentrations material of this density

accumulated and the proportion taken up by the heart rapidly decreased, suggesting a specific and saturable mechanism of uptake by the heart tissues.

6. Lipoproteins of d = 1.019 - 1.063 g/ml were formed from human VLDL at all concentrations. Human apoproteins were also taken up by the heart, but the proportion recovered in the heart and in the LDL density range was constant at all VLDL concentrations, suggesting a non-specific, and in the concentration range investigated, not saturable mechanism of uptake.
7. Agarose gel filtration of lipoproteins following heart perfusion with human VLDL revealed large aggregates containing particles which resemble LDL in size and apoprotein composition, since they contained largely apoB. A fraction containing predominantly apoC was also obtained by agarose gel filtration of the perfusate lipoproteins.
8. The hepatic uptake of the TG-rich lipoprotein remnants, produced by heart perfusion, was significantly greater than that of the intact lipoproteins. Furthermore, the uptake of the rat TG-rich lipoprotein remnants by the perfused rat liver was significantly greater than that of the human lipoprotein remnants. These data suggest some species specificity of the uptake of the rat lipoprotein remnants by the rat liver.
9. The presented data suggest that at normal concentrations, rat VLDL are completely catabolized by the heart and the liver without significant formation of LDL. Low density lipoproteins are produced from human VLDL at all concentrations. These observations may account for the large differences in the observed serum LDL levels between the two species.

CONTRIBUTION TO KNOWLEDGE

The author considers all of the findings outlined in the Summary as original contributions to the understanding of lipoprotein metabolism.

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