RAT APOLIPOPROTEIN A-IV METABOLISM

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PROEFSCHRIFT

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Voor mijn ouders

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ABBREVIATIONS

VLDL	very low density lipoprotein
LDL	low density lipoprotein
HDL	high density lipoprotein
Apo	apolipoprotein
EDTA	ethylene diamine tetraacetic acid
DOC	deoxycholate
TCA	trichloroacetic acid
TMU	tetramethylurea
IgG	γ-immunoglobulins
SDS	sodium dodecylsulfate
DTNB	dithionitrobenzoic acid

SAMENVATTING

Sinds de ontdekking dat de concentratie van plasma "high density" lipoproteïnen (HDL) cholesterol negatief correleert met het risico op hart- en vaatziekten in geïndustrialiseerde landen, werd de aandacht gericht op de rol van HDL in het cholesterol metabolisme. HDL worden operationeel gedefiniëerd als de klasse van lipoproteïnen die, met behulp van preparatieve ultracentrifugatie, geïsoleerd worden in het dichtheidsinterval 1.063-1.21 g/ml (humaan HDL), of 1.050-1.21 g/ml (ratte HDL). Zij zijn heterogeen, zowel in grootte als in chemische samenstelling. Alle componenten van HDL nemen deel aan uitwisselingsprocessen met andere plasma lipoproteïnen, ten gevolge van lipide uitwisselings eiwitten of enzym activiteiten aanwezig in het plasma van de meeste zoogdieren. Daarom is het moeilijk om het metabole lot van de verschillende HDL componenten te onderzoeken. Daar de rat geen plasma cholesteryl ester uitwisselingsactiviteit heeft, is dit dier een relatief eenvoudig model voor de studie van het HDL cholesterol metabolisme.

Dit proefschrift behandelt verschillende aspecten van het HDL apolipoproteïne metabolisme in de rat, in het bijzonder van het apolipoproteïne A-IV (apo A-IV). Daar apo A-IV gemakkelijk van het lipoproteïne complex wordt "gestript" gedurende isolatie in de ultracentrifuge, bleef haar aanwezigheid op HDL deeltjes tot voor kort onopgemerkt. De ontwikkeling van minder ruwe isolatie methodieken heeft het mogelijk gemaakt om de studie van haar metabole gedrag te verbeteren. In de studies, die in dit proefschrift beschreven zijn, wordt apo A-IV vergeleken met apo A-I, het meest voorkomende eiwit in HDL. Omdat de aminozuur volgorden van apo A-IV en apo A-I grote overeenkomst vertonen, is het interessant om te bepalen of hun metabolisme vergelijkbaar is.

Teneinde ratte HDL te karakteriseren werden de verschillende serum lipoproteïnen gescheiden door middel van gel filtratie op 6% agarose kolommen. Specifieke bepalingen werden ontwikkeld, met behulp van immunoeletroforese, voor de kwantificering van apo A-I, apo E en apo A-IV (Hoofdstuk 2). Zowel apo A-I als apo E (een ander HDL eiwit) blijven voor 100% gebonden aan lipoproteïne

deeltjes, indien deze gelfiltratie als isolatie methode gebruikt wordt. Ultracentrifugatie daarentegen, leidt tot "stripping" van significante hoeveelheden apo A-I en apo E. Deze eiwitten worden teruggevonden in de lipoproteïne-deficiente fractie met dichtheid > 1.21 g/ml. Hieruit kan de conclusie getrokken worden, dat ultracentrifugatie een nadelig effect heeft op de integriteit van serum HDL (Hoofdstuk 4). Analyse van serum op 6% agarose kolommen laat zien dat apo A-IV zowel voorkomt in een lipoproteïne-gebonden vorm als in een "vrije" vorm. Verdere karakterisering van de chromatografisch gescheiden dragers van serum apo A-IV werd gedaan door middel van specifieke immuunprecipitaties (Hoofdstukken 4 en 7). Uit deze studies blijkt dat apo A-IV in tenminste drie fracties aanwezig is: 1. deeltjes met de grootte en samenstelling van ratte HDL, die zowel apo A-I als apo A-IV (maar geen apo E) bevatten 2. deeltjes met de grootte van ratte HDL die apo A-IV bevatten, maar geen apo E of apo A-I 3. "vrij" apo A-IV met mogelijk kleine hoeveelheden gebonden cholesterol en fosfolipiden. De hoeveelheid "vrij" apo A-IV, aanwezig in ratte serum, is afhankelijk van de voedingstoestand van het dier. In gevaste dieren is de concentratie laag (slechts 9% van de totale serum apo A-IV concentratie), terwijl in gevoede dieren de hoeveelheid "vrij" apo A-IV ongeveer 7 keer hoger is en 35% van de totale serum apo A-IV concentratie bedraagt.

Studies, die de verdeling van apo A-I en apo A-IV tussen lipoproteïne fracties in mesentere lymph vergelijken, laten opvallende verschillen zien (Hoofdstuk 3). Apo A-I is in mesentere lymph alleen aanwezig op chylomicronen en op lymph HDL, terwijl apo A-IV daarnaast ook aanwezig is in de "vrije" vorm. Vet infuus in het duodenum induceert een 4-voudige toename in de uitscheiding van apo A-IV in mesentere lymph, wat tot uiting komt in de verhoging van apo A-IV uitscheiding in alle lipoproteïne fracties in de lymph. De uitscheiding van apo A-I is slechts 2 maal verhoogd tijdens vet infuus en wordt voornamelijk gereflecteerd in een verhoogde uitscheiding van apo A-I in triglyceride-rijke lipoproteïnen. Deze resultaten ondersteunen het denkbeeld dat apo A-I en apo A-IV synthese in de darm onafhankelijk worden gereguleerd. Mede wordt gesuggereerd dat "vrij" apo A-IV in vette lymph

bijdraagt tot verhoging van de "vrij" apo A-IV concentratie in serum, die wordt waargenomen na voeding.

Studies van het in vivo metabolisme van apo A-IV laten zien dat het HDL apo A-IV metabolisme anders is dan het HDL apo A-I metabolisme (Hoofdstukken 5 en 6). De halfwaarde tijd van HDL apo A-IV in rat serum is 8.5 uur, vergeleken met een waarde van 10.2 uur voor HDL apo A-I. Dubbel "label" experimenten werden in alle studies gebruikt, zodat het gedrag van HDL apo A-IV en apo A-I in hetzelfde dier kon worden bestudeerd, waardoor een directe vergelijking mogelijk was. De nieren leveren een kwantitatief belangrijke bijdrage aan de afbraak van HDL apo A-I, terwijl de lever een relatief kleine rol speelt. Het tegenovergestelde werd gevonden voor HDL apo A-IV. Zoals eerder vermeld, is een deel van de totale HDL apo A-IV aanwezig op deeltjes die noch apo A-I, noch apo E bevatten. Deze deeltjes hebben mogelijk een relatief snelle verdwijningssnelheid en worden specifiek afgebroken in de lever. Geen verschillen werden waargenomen tussen de in vivo degradatie van HDL, gemerkt met radioactief apo A-I afkomstig van òf lymphchylomicronen òf serum HDL, alsmede van HDL gemerkt met radioactief apo A-IV afkomstig van genoemde bronnen (Hoofdstuk 5), hetgeen laat zien dat posttranslatie processen of extracellulaire conversie geen groot effect hebben op het metabole gedrag van de eiwitten.

Tenslotte was het mogelijk om meerdere HDL subklassen te karakteriseren, wederom gebruik makend van specifieke immuunprecipitaties (Hoofdstuk 7). Drie relatief simpele HDL subklassen (apo A-I-HDL, apo E-HDL en apo A-IV-HDL) nemen meer dan 80% van het totale HDL cholesterol voor hun rekening. Het resterende HDL cholesterol is aanwezig op meer complexe HDL deeltjes die meer dan een van de drie belangrijke HDL apolipoproteïnen bevatten. Deze resultaten illustreren de complexiteit in HDL structuur en metabolisme.

SUMMARY

Attention has been focussed on the role of plasma high density lipoproteins (HDL) in cholesterol metabolism, following the discovery that the concentration of this fraction in plasma correlates negatively with the risk of coronary heart disease in industrialized countries. HDL are operationally defined as the class of lipoproteins isolated between densities 1.063-1.21 g/ml (human HDL), or 1.050-1.21 g/ml (rat HDL), using preparative ultracentrifugation. They are heterogenous in both size and chemical composition. All of its constituents participate in exchange processes with other plasma lipoproteins, as a result of lipid transfer proteins or enzyme activities present in the plasma compartment of most mammalian species. Therefore it is difficult to investigate the metabolic fate of the various HDL components. Because rat plasma lacks cholesteryl ester transfer activity, this animal is a relatively simple model for the study of HDL cholesterol metabolism.

The present thesis deals with several aspects of HDL apolipoprotein metabolism in the rat, in particular apolipoprotein A-IV (apo A-IV). Since apo A-IV is rapidly "stripped" from the lipoprotein particle during ultracentrifugal isolation, its presence on HDL particles remained unappreciated until recently. The development of milder isolation procedures has enabled us to improve the study of its metabolic behaviour. In the studies discussed in this thesis we have compared apo A-IV with apo A-I, which is the most abundant protein of HDL. Because apo A-IV and apo A-I show homology in their amino acid sequence, it is of interest to determine whether their metabolic behaviour is comparable.

In order to characterize rat HDL, the different serum lipoproteins were separated by molecular sieve chromatography on columns of 6% agarose. Specific electroimmunoassays were developed for apo A-I, apo E and apo A-IV in order to quantitate these apolipoproteins (Chapter 2). All apo A-I and apo E (another apolipoprotein constituent of rat HDL) remained associated with lipoprotein particles using gel filtration for lipoprotein isola-

tion. In contrast, ultracentrifugation caused significant amounts of apo E as well as apo A-I to be stripped off the lipoproteins. They are recovered in the lipoprotein-deficient bottom fraction of density > 1.21 g/ml, suggesting that ultracentrifugation affects the integrity of serum HDL (Chapter 4). Analysis of serum on 6% agarose columns reveals that apo A-IV occurs both in a lipoprotein-associated form and in a "free" form. Further characterization of the chromatographically separated carriers of apo A-IV was obtained from specific immunoprecipitation studies (Chapters 4 and 7). From these studies it is evident that apo A-IV is present in at least three fractions: 1. particles with the size and composition of typical rat HDL, containing both apo A-I and apo A-IV (but no apo E) 2. HDL-sized particles containing apo A-IV, but neither apo E nor apo A-I 3. "free" apo A-IV, probably containing small amounts of bound cholesterol and phospholipids. The amount of "free" apo A-IV present in rat serum is dependent upon the nutritional status of the animal. In fasting animals it is low (only 9% of total serum apo A-IV), while in chow-fed animals the "free" apo A-IV content is about 7-fold higher and comprises as much as 35% of the serum apo A-IV concentration.

Studies comparing the distribution of apo A-I and apo A-IV between lipoprotein fractions in mesenteric lymph revealed marked differences (Chapter 3). Apo A-I in mesenteric lymph is only present on chylomicron particles and lymph HDL, whereas apo A-IV, in addition, is also present in the "free" form. Lipid infusion into the duodenum induces a 4-fold increase in the output of apo A-IV in mesenteric lymph, which is reflected in elevation of apo A-IV output in all lymph lipoprotein fractions. The output of apo A-I was only 2-fold elevated by lipid infusion and mainly reflected increased output of apo A-I in triglyceride-rich lipoproteins. These results favour the view that apo A-I and apo A-IV synthesis in the intestine are independently regulated. They also suggest that the "free" apo A-IV in fatty lymph contributes to the elevation of"free" apo A-IV levels in serum, observed after feeding.

Studies on the in vivo turnover of apo A-IV show that HDL apo A-IV metabolism differs from HDL apo A-I metabolism (Chapters

5 and 6). The half-life of HDL apo A-IV in rat serum is 8.5 h, compared to a value of 10.2 h for HDL apo A-I. Double label experiments were used in all studies, so that the behaviour of HDL apo A-IV and apo A-I could be studied simultaneously in the same animal, enabling us to make a direct comparison. It was found that the kidneys are quantitatively most important for the degradation of HDL apo A-I, whereas the liver plays a relatively minor role. The opposite was found for HDL apo A-IV. As mentioned previously, part of the total HDL apo A-IV is present on particles containing neither apo A-I nor apo E. It is proposed that these particles (apo A-IV-HDL) have a relatively rapid turnover and are specifically degraded in the liver. No differences in the fractional turnover rate or the tissue uptake of HDL labeled with apo A-I derived from either lymph chylomicrons or serum HDL, or with apo A-IV, derived from these sources, was observed (Chapter 5), indicating that posttranslational processes or extracellular conversion have no big effects on the metabolic behaviour of the protein.

Finally, again using specific immunoprecipitations, it was possible to characterize additional rat HDL subclasses (Chapter 7). Three relatively simple HDL subclasses (apo A-I HDL, apo E HDL and apo A-IV HDL) account for more than 80% of the total HDL cholesterol. The remaining HDL cholesterol is present on more complex HDL particles containing more than one of the three major HDL apolipoproteins. These finding illustrate the complexity in HDL structure and metabolism.

CHAPTER 1

INTRODUCTION

TABLE 1.1.

Major human plasma lipoproteins

	density of flotation g/ml	major lipid constituent	major apo-lipoprotein	electro-phoretic mobility
Chylomicrons	⟨0.95	triglycerides	B_{48}[1],A-I,A-II, A-IV,C,E	origin
Very low density lipoproteins	⟨1.006	triglycerides	B_{100},E,C	pre-β
Low density lipoproteins	1.019-1.063	cholesterol (esters)	B_{100}	β
High density lipoproteins	1.063-1.21	phospholipids	A-I,A-II,C	α

Major rat plasma lipoproteins

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Low density lipoproteins	1.019-1.050	cholesterol (esters)	B_{100},E	β
High density lipoproteins	1.050-1.21	cholesterol (esters)/phos-pholipids	A-I,A-IV,E,C	α

[1] apolipoprotein B exist in two forms which, though distinct, share common antigenic determinants. B_{48} is synthesized predominantly in the intestine and B_{100} in the liver. The subscripts relate to their relative molecular weight.

Lipids are organic compounds that are poorly soluble in water. In the human body, they function as structural components, as storage forms of energy, as metabolic fuel and as substrates for the synthesis of hormones (prostaglandines and steroid hormones) or intracellular messengers (phosphatidylinositolphosphates). Lipids are transported in plasma in large complexes of physically combined lipid and protein. These complexes are called: plasma lipoproteins. They have a micellar structure in which triglycerides and cholesteryl esters form a hydrophobic core surrounded by a surface coat of phospholipid, free cholesterol and protein molecules. The classification and nomenclature of the plasma lipoproteins have been based on the ultracentrifugal flotation in salt solutions (1-3) and on their electrophoretic mobility in agarose gels (4). Four major different lipoprotein classes can be distinguished:

- chylomicrons
- very low density lipoproteins (VLDL)
- low density lipoproteins (LDL)
- high density lipoproteins (HDL)

These lipoprotein classes have a specific size and chemical composition (see Table 1.1.) and play different roles in lipid metabolism.

CHYLOMICRONS

The small intestine has long been recognized as an important organ for lipoprotein and apolipoprotein synthesis (5-8). After absorption into the enterocyte, dietary lipids are incorporated into chylomicrons and subsequently secreted in the mesenteric lymph. These particles consist mainly of triglycerides (about 90% of the total lipid mass) and consequently have a very low density ($d < 0.95$ g/ml). The triglycerides are located in the core of the particles together with cholesteryl esters. The surface is mainly composed of phospholipids and a number of apolipoproteins, which are also synthesized in the enterocytes (apo B_{48}, apo A-I, apo A-II and apo A-IV, (see ref. 9-13)). The composition of the chylomicron particles is strongly influenced by the nutritional status

of the animal. After fat feeding large chylomicrons, carrying exogenous triglycerides, will predominate in the lymph, while during fasting the size of the particles decreases and the triglycerides are derived from endogenous sources. Chylomicrons function as vehicles to transport lipids through the intestinal lymph into the plasma compartment. There they acquire apo C's and apo E by transfer from other lipoproteins, mainly HDL (9, 12, 14). Apo C-II in particular serves to activate the enzyme lipoprotein lipase, located on the endothelial cells of capillaries of extrahepatic tissues. This enzyme catalyses the hydrolysis of chylomicron triglyceride (15), resulting in the production of smaller remnants which possess a surplus of surface coat components, like phospholipids and apolipoproteins (mainly apo A-I, apo A-IV and apo C's). The latter relocate by transfer to HDL (12, 14-20) thus completing the cycle of events. The chylomicron remnant particles, which have retained apo E, can now be rapidly removed from the circulation by a specific hepatic receptor that recognizes apo E (21, 22).

VERY LOW DENSITY LIPOPROTEINS

VLDL (density < 1.006 g/ml) are synthesized in the liver. They have a lower triglyceride content (60-70% of the total lipid mass) and a smaller size than chylomicrons. It should be stated that in the context of the present work we define chylomicrons as triglyceride-rich particles derived from the intestine, while VLDL particles are of hepatic origin. The apolipoprotein composition of VLDL is significantly different from that of chylomicrons (Table 1.1). Lymph chylomicrons contain mainly apo B_{48}, apo A-I and apo A-IV, while serum VLDL consist of apo B_{100}, apo E and apo C. VLDL are responsible for the transport of triglycerides and cholesterol from the liver for redistribution to various tissues. Within the plasma compartment VLDL are attacked by lipoprotein lipase which hydrolyses their triglyceride core and produces a series of smaller cholesterol enriched particles (i.e. intermediate density lipoproteins, (IDL) and low density lipoproteins, (LDL)) (23). Recent studies have shown that a significant frac-

tion of IDL-apo B is removed directly from the circulation and never reaches LDL (23-28). Therefore two different pathways for VLDL degradation have been proposed. Firstly, large VLDL particles are rapidly attacked by lipoprotein lipase, as described above, resulting in remnant particles which are subsequently catabolized by the liver and do not contribute to the formation of LDL (28). The uptake of the remnants in the liver is probably receptor-mediated (29), greatly facilitated by apo E but inhibited by the C apolipoproteins (30). Secondly, small VLDL particles, either directly secreted from the liver or derived from lipolysis of larger triglyceride-rich particles, are somehow predisposed to go all the way through the VLDL delipidation cascade and may lead to the formation of LDL (25, 28).

LOW DENSITY LIPOPROTEINS

LDL isolated from the density range between 1.019-1.063 g/ml, is the major cholesterol carrying lipoprotein in the human circulation and has therefore been the subject of studies dealing with cholesterol metabolism. Its core is composed of cholesterol esters and small quantities of triglycerides, while the surface coat comprises protein, unesterified cholesterol and phospholipids. LDL contains one specific apolipoprotein molecule: apo B_{100}. Sometimes small amounts of apo E are also present both in human as well as rat LDL. The concentration of LDL in rat plasma is low, compared to human plasma.

As noted above, LDL constitute the end stage of lipase induced VLDL hydrolysis. In some subjects (eg. individuals with familial hypercholesterolemia), however, turnover of LDL apo B appears to exceed the VLDL apo B production rate, suggesting that LDL apo B may be derived from other sources than VLDL. From studies in cholesterol-fed animals (31-34) and in hypercholesterolemic patients (35, 36) evidence for direct synthesis of LDL in the liver was obtained. In normolipidemic human subjects and animals, however, no evidence for LDL synthesis in the liver was obtained (38, 39). A possible explanation for these observations is that under certain conditions, apo B may enter the circulation

as IDL (density range 1.006-1.019) and subsequently are converted into LDL (26, 40).

LDL appear to be largely responsible for the transport of cholesterol to various tissues which require it for structural (membrane synthesis) and metabolic purposes (e.g. steroid hormone production). It also plays a keyrole in delivering cholesterol to the liver for ultimate excretion from the body. Its tissue uptake is governed by specific high affinity LDL receptors on the cell surface which recognizes both apo B_{100} and E (41-45), although other, less well defined processes, also contribute to its catabolism (46-48).

HIGH DENSITY LIPOPROTEINS

HDL consist of a heterogenous group of relatively small particles, isolated by ultracentrifugation in the density interval 1.063-1.21 g/ml (human HDL) or 1.05-1.21 g/ml (rat HDL). Phospholipids are the major lipid component.

Human HDL, can be divided into two major subclasses if isolated by ultracentrifugation: HDL_2 and HDL_3. They differ in size, density, overall chemical composition and apolipoprotein content (Table 1.2., (49-56)). HDL_c represents another particle type which floats at a lower density than the mean mass of HDL and accumulates in the plasma of various species during cholesterol feeding (57). It is characterized by a high apo E content.

In the rat, in contrast to man, HDL is the major cholesterol carrying lipoprotein fraction. The main HDL fraction in the rat, referred to as HDL_2 (Table 1.3.) has an overall lipid composition similar to that of human HDL_2. However, its apolipoprotein content differs inasmuch as it contains more apo E and apo A-IV but less apo A-II than human HDL. In addition to HDL_2 a particle called HDL_1 can be isolated in the density interval 1.03-1.08 g/ml in normal rat plasma. HDL_1 has apo E as its major protein component together with variable quantities of apo A-I (57, 59-61). Cholesterol feeding to rats, as stated above, results in the appearance of a cholesterol-rich particle called HDL_c, which contains predominantly apo E.

TABLE 1.2.

Properties of human serum high density lipoprotein subclasses[1]

Physical properties	HDL_c	HDL_2	HDL_3
Density range (g/ml)	1.04-1.06	1.063-1.125	1.125-1.21
Chemical composition, (weight %)			
protein	20.3%	41.0%	55.0%
phospholipids	29.3%	29.5%	22.5%
cholesterol	50.0%	21.6%	14.6%
triglycerides	0.5%	4.5%	4.1%
Apolipoproteins (weight %)			
A-I	+[2]	75%	65%
A-II		15%	25%
A-IV			+[3]
C		10%	5%
E	+[2]	+[3]	

[1] obtained from ref. 58.

[2] main apolipoprotein components occuring in varying proportions in HDL_c.

[3] minor apolipoprotein components occuring in varying proportions.

ORIGIN OF HIGH DENSITY LIPOPROTEINS

The circulating HDL particles which are described above, are not secreted as such into the plasma, but are produced in a nascent form by their tissues of origin, the liver and the intestine (62-67). The particles, synthesized in the liver, are poor in cholesteryl esters and contain mainly apo E (68-71), in contrast to plasma HDL that contains mainly cholesteryl esters in its core and has apo A-I as its major surface protein. The nascent HDL is disc-shaped on electron microscopy, but, under the influence of the plasma enzyme lecithin-cholesterol acyltransferase (LCAT), which esterifies free cholesterol, it adopts the

spherical configuration characteristic of the mature particles present in plasma. The surface coat components, required for this conformational change, come by transfer from triglyceride-rich particles. Lymph HDL has a lipid composition similar to that of nascent HDL from the liver. It is enriched in phospholipids, deficient in cholesteryl esters and also has a discoidal appearance. Its major apolipoprotein, however, is apo A-I. Apo E is almost completely absent from lymph HDL (64, 65).

TABLE 1.3.

Properties of rat serum high density lipoprotein subclasses

Physical properties	HDL_c[1]	HDL_1[1]	HDL[2]
Density range (g/ml)	1.020-1.080	1.020-1.080	1.050-1.210
Chemical composition (weight %)			
protein	15.1%	30.1%	42%
phospholipid	41.4%	26.0%	31%
cholesterol	42.8%	39.9%	26%
triglycerides	1.0%	4.0%	0-1%
Apolipoproteins (weight %)			
A-I	+[3]	+[3]	50%
A-IV			15%
E	+[3]	+[3]	20%
C			10%

[1] values obtained from ref. 57.

[2] values obtained from ref. 192 (chemical composition) and from ref. 37 (apolipoproteins)

[3] main apolipoprotein components occuring in varying proportions.

METABOLISM OF HIGH DENSITY LIPOPROTEINS

Since the discovery that HDL cholesterol concentrations in plasma correlate negatively with the risk of coronary heart disease in industrialized countries (72-74, 158, 159), more attention has been focussed on this lipoprotein class and its role in cholesterol metabolism. Circulating HDL is not a stable particle, but lives in a constant flux as a result of enzyme reactions and lipid transfer processes, which makes it difficult to study its metabolism. However, the extent of the interchange is limited in the rat since this animal lacks a plasma protein (cholesteryl ester transfer protein), which is responsible for cholesteryl ester transfer between lipoprotein particles in other species (75, 76). Consequently this animal is a relatively simple model for studies designed to investigate HDL metabolism, since cholesterol esters do not exchange from the particle and therefore are a good marker of its turnover.

HDL seems to play a major role in the process referred to as reverse cholesterol transport (77-79). This links HDL particle metabolism to the transport of cholesterol from peripheral tissues to the liver where cholesterol is excreted as such or degraded to bile acids. Furthermore, HDL is also involved in the delivery of cholesterol to organs that participate in the synthesis of steroid hormones (80-83).

As already mentioned above, HDL is the major cholesterol carrying lipoprotein fraction in rat plasma. Studies by Van 't Hooft et al. (81), later confirmed by others (82, 83), have shown that partial hepatectomy does not change apo A-I turnover, whereas it does decrease HDL cholesteryl ester flux. Therefore, it is possible to dissociate the metabolism of these two HDL components. The liver turns out to be the major site of catabolism for HDL cholesteryl esters and phospholipids (81-84), while other organs, notably the kidneys, play a role in the removal of apo A-I from the circulation (82, 85).

APOLIPOPROTEINS IN RAT HIGH DENSITY LIPOPROTEINS

Knowledge of the structure and function of apolipoproteins has increased rapidly during the last few years. Apolipoproteins play a specific role in the maintenance of the structure of the lipoproteins, but they also have specific functions in the regulation of lipoprotein metabolism. Apolipoproteins from rat HDL can be visualized on SDS gel electrophoresis as shown in Fig. 1.1. Three major apoproteins can be detected: apo A-I, apo E and apo A-IV, while small amounts of C apoproteins are also present.

APOLIPOPROTEIN A-I

Apo A-I was first described as a component of rat HDL (89-92). It is a single polypeptide of 235 amino acids (calculated mol. wt. 27,369 (93, 94)) with a known amino acid sequence. Rat apo A-I is 8 amino acids shorter than human apo A-I, which contains 243 amino acids and has a calculated molecular weight of 28,100 (95).

The liver and the intestine are the two organs of apo A-I synthesis (11, 13, 96-103). Apo A-I is synthesized as a preproprotein with a 24 amino acid NH_2-terminal extension. Cotranslational cleavage of this polypeptide results in the proteolysis of the 18 amino acid pre segment, leaving an unusual pro peptide with a 6 amino acid NH_2-terminal extension, which is called pro apo A-I (104, 105). On isoelectric focusing gel electrophoresis it is clear that 80% of the mesenteric lymh synthesized apo A-I is present in the proform, with a pI of 5.5 (the more acidic form, see Fig. 1.2.). The 6 amino acid pro segment is then cleaved probably by an enzymatic process in the plasma compartment (101, 106, 107), yielding mature apo A-I (pI 5.6). However, up to 36% of the pro form escaped this cleavage and remains unchanged in the circulation. In other words, the conversion of pro apo A-I into the mature form in the rat seems to be a slow and possibly incomplete process (101).

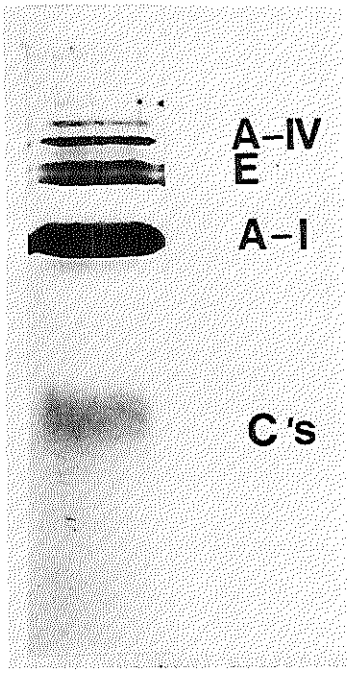

Fig. 1.1. Analysis of apolipoprotein from rat HDL, isolated in the density range 1.050-1.21 g/ml, on SDS polyacrylamide gel electrophoresis.

Fig. 1.2. Analytical isoelectric focusing in polyacrylamide gels of apo A-I in a pH gradient from 4-6.

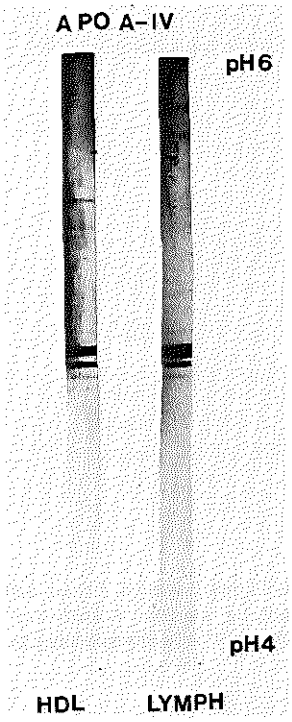

Fig. 1.3. Analytical isoelectric focusing in polyacrylamide gels of apo A-IV in a pH gradient from 4-6.

APOLIPOPROTEINS A-IV

Apo A-IV was first described by Swaney et al. (108). It is a single polypeptide of 371 amino acids (calculated mol. wt. 46,000 (109)); its amino acid composition is remarkably similar in rat and man (110-113). Interestingly, the human protein shows immunochemical crossreactivity with anti-rat apo A-IV (110).

Wu et al. (11, 114) showed, by means of ^{3}H-leucine incorporation into rat apolipoproteins that in rats 59% of the apo A-IV was synthesized by the small intestine. The other organ involved in apo A-IV synthesis is the liver. Measurements of specific mRNA levels in the small intestine and in the liver, translated in a

mRNA-dependent protein synthesizing system and immunoprecipitated with specific antibody, confirmed that the intestine is more active in apo A-IV synthesis than the liver (115, 116). The apo A-IV product (pre apo A-IV) from rat intestine has a molecular weight of 48,500 daltons (117), 2,500 daltons larger than mature apo A-IV. Both the rat and human proteins are secreted as a pre protein with a largely homologous 20 amino acid NH_2-terminal extension (117, 118). The extension is cleaved cotranslationally and results in the mature apo A-IV protein. Thus, unlike pre pro apo A-I, pre apo A-IV does not contain a propeptide.

Further examination of apo A-IV, isolated from lymph chylomicrons or serum HDL, showed that apo A-IV contains two major protein bands with pI's close to 5.0 (Fig. 1.3.). The more prominent component is the basic protein that amounts to 80% of the apo A-IV isolated from lymph chylomicrons and 60% of the apo A-IV obtained from serum HDL. Analysis of the isoforms after blotting on nitrocellulose paper, with a specific antibody raised against apo A-IV demonstrated that both react with the antibody, indicating that they are isoproteins of apo A-IV. Whether the difference is caused by differences in carbohydrate moiety remains to be determined.

APOLIPOPROTEIN E

Apo E, or arginine-rich apolipoprotein, was first recognized as a constituent of VLDL particles in both human and rat (89, 90, 119). Rat apo E is a single peptide containing 293 amino acids (calculated mol. wt. 34,000) with a known amino acid composition and a complete amino acid sequence (90, 94, 120, 121). The original designation arginine-rich apolipoprotein is derived from the discovery that apo E contains characteristically 10-12 mol% of the amino acid arginine. Rat apo E is 8 amino acids shorter than human apo E (120).

The liver is the major organ for apo E synthesis (11, 62, 69, 70, 120-124). The intestine, an important organ for apo A-I and apo A-IV synthesis, does not make a significant contribution to apo E synthesis (11, 120, 123). However, the protein can be

synthesized in many other tissues like adrenals and the brain (123, 124). The primary translation product is a preprotein with a 18 amino acid NH_2-terminal extension which is cleaved cotranslationally (94, 120, 121). The protein is then glycosylated, secreted and subsequently desialylated in serum.

AIM OF THE STUDY

Earlier studies by Van 't Hooft et al. (85, 160), in our laboratory, mainly focussed upon the catabolism of HDL apolipoproteins A-I and E. They found that the kidneys were an important organ involved in the catabolism of these proteins together with the liver. The present thesis mainly deals with HDL apo A-IV. Until very recently, apo A-IV metabolism was relatively unknown. This was due to the fact that, during isolation of HDL particles by density ultracentrifugation, apo A-IV was readily "stripped" and recovered in the "bottom" fraction, together with the bulk of the serum protein. Better isolation procedures enabled us to investigate the behaviour of apo A-IV in the rat, leading to expansion of our knowledge of lipoprotein metabolism in general.

The first part (Chapter 2) outlines a sensitive electroimmunoassay for the determination of apo A-I, apo E, and apo A-IV in diluted samples. This method has been successfully used to analyse the distribution of these proteins in rat mesenteric lymph (Chapter 3) and rat serum (Chapter 4), following their fractionation by molecular sieve chromatography on agarose gels. The latter procedure was employed to obviate the well known problem of particle protein stripping which occurs during ultracentrifugation (60, 86-88). Chapter 4 describes the results of a study designed to compare the effects of gel filtration and ultracentrifugation on HDL particle composition. Chapters 5 and 6 describe our studies on the catabolic sites of apolipoprotein A-IV containing lipoproteins. The final paper (Chapter 7) describes the analysis of HDL subclasses using specific immunoprecipitation.

CHAPTER 2

ELECTROIMMUNOASSAY OF RAT APOLIPOPROTEINS A-I, A-IV AND E. A PROCEDURE FOR SAMPLE TREATMENT TO INCREASE THE SENSITIVITY IN DILUTED FRACTIONS

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SUMMARY

Methods for the quantitative determination of rat apolipoproteins A-I, A-IV and E by electroimmunoassay are described. Apolipoproteins present in diluted samples of biological fluids (approx. 2 ml) were concentrated by precipitation with deoxycholate and trichloroacetic acid. The protein pellets were solubilized in 0.1 ml of 0.5 M NaOH and these samples were delipidated with tetramethylurea and assayed. This protocol enables the measurement of apolipoprotein concentrations that are at least 10 times lower than normally detectable; 0.2 µg of apolipoprotein A-IV, 0.2 µg of apolipoprotein A-I and 0.8 µg of apolipoprotein E can be easily detected in samples of 2 ml.

INTRODUCTION

Quantitative measurements of specific apolipoproteins in biological fluids are possible with a variety of immunochemical procedures, e.g., radioimmunoassay, enzyme-linked immunosorbent assay, radial immunodiffusion, immunoelectrophoresis and nephelometry. If the number of samples to be analyzed is limited, radial immunodiffusion and immunoelectrophoresis are the methods of choice. Their use is, however, limited to samples with substantial concentrations of apolipoproteins due to the relatively low sensitivity of these methods. To make them applicable to diluted samples, we developed a procedure of sample treatment that enables measurement of apolipoprotein concentrations by electroimmunoassay, which are at least 10 times lower than normally detectable. Apolipoproteins present in diluted samples were concentrated by precipitation with deoxycholate-trichloroacetic acid (DOC-TCA), the precipitates were then delipidated with TMU and assayed. This method has been successfully used to analyze the distribution of apolipoproteins A-I, A-IV and E in rat serum and mesenteric lymph, fractionated by agarose gel column chromatography.

MATERIALS AND METHODS

Isolation of apolipoproteins

HDL was isolated from serum of chow-fed male Wistar rats (body weight 350-400 g) by sequential density ultracentrifugation at 4°C in the density interval of 1.063-1.21 g/ml (2), washed once by reflotation at a density of 1.21 g/ml and dialyzed extensively against 0.15 M NaCl, containing 2mM Na-phosphate buffer (pH 7.4).

The HDL was delipidated by extraction with 20 vol. of ethanol-diethyl ether 3:1 (v/v) for 20 h at 4°C (125) and the apolipoproteins were dissolved in 0.05 M NaCl containing 5 M urea and 2 mM Na-phosphate buffer (pH 7.4).

Apo E was isolated from the apo HDL fraction by affinity chromatography on Sepharose CL-4B, containing covalently bound heparin, as described by Shelburne et al. (126) and further

purified by chromatography at 4°C on Sephadex G 200 (column size 0.9 x 70 cm, flow 7 ml/h), equilibrated with 150 mM Tris-HCl (pH 8.2) containing 1 mM EDTA, 0.02% NaN_3 and 6 M urea. The fraction of HDL not bound to the heparin-Sepharose column was used for the isolation of apo A-I and apo A-IV. Apo A-I and apo A-IV were separated by gel filtration at 4°C on Sephadex G-200 (column size 2.5 x 90 cm, flow 15 ml/h), equilibrated with the same buffer as described above. The isolated apolipoprotein fractions were dialyzed against 0.15 M NaCl containing 1 mM EDTA (pH 7.4) and stored at -70°C. The purity was checked by urea polyacrylamide gel electrophoresis (apo A-I and apo A-IV) (127) and by Na-dodecylsulphate polyacrylamide gel electrophoresis (apo A-I, apo A-IV, apo E) (128). Each apolipoprotein showed a single band after staining with Coomassie Brilliant Blue R-250.

Preparation of antisera

Apolipoprotein solutions (50 µg in 1.5 ml of 0.15 M NaCl) were mixed with an equal volume of Freund's complete adjuvant and injected intradermally at multiple spots in New Zealand white rabbits weighing about 2 kg. After 14 days this procedure was repeated once. Then booster injections without the adjuvant were given every 14 days, untill 5 µl of a 1:30 dilution of the rabbit serum caused the formation of a visible precipitation arc against 5 µl of undiluted rat serum in an Ouchterlony double immunodiffusion test. Animals were bled and serum was prepared. The IgG fraction of these antisera was isolated by affinity chromatography on Sepharose-4B, containing covalently bound protein A (Pharmacia, Uppsala, Sweden) according to standard procedures. The titers (µg of IgG protein needed to precipitate 1 µg of apolipoprotein) of the resulting IgG preparations were 78 (apo A-I), 140 (apo A-IV) and 598 (apo E). The specificity of the antibody preparations was checked by double immunodiffusion against pure rat apo A-I, apo E and apo A-IV and against rat serum albumin. All antibody preparations reacted only with their specific antigen.

Electroimmunoassays

Immunoelectrophoresis according to Laurell (129) was performed for each of the rat apolipoproteins. The antibody supporting gel in the apo A-I assay contained 1% agarose dissolved in 80 mM Tris-HCl, 24 mM Tricine-HCL, 0.5 mM Ca-lactate (pH 8.6), 5% (^{v}/v) polyethylene glycol (mol. wt. 300) and anti-rat apo A-I rabbit IgG. 60 µl of anti-apo A-I, containing 1.2 mg of IgG, was mixed with 30 ml of agarose solution. For the assay of apo A-IV, 2 mM Ca-lactate was used, while for the apo E assay neither Ca-lactate nor polyethylene glycol was added. One hundred eighty µl of anti-apo A-IV (5.1 mg of IgG) or 420 µl of anti-apo E (15.9 mg of IgG) was mixed with 30 ml of agarose solution. Samples of 13 µl were applied to the wells in the agarose gels. The electrophoresis of apo A-I and apo A-IV was carried out at 15°C and 7 V/cm for 17 h. The apo E assay was carried out at 2 V/cm for 12 h. The gels were washed in saline, dried on overhead projector sheets (3M, Minneapolis-St. Paul, USA), stained with Coomassie Brilliant Blue R-250 (0.034%) in ethanol-acetic acid-water (1:2:4 ^{v}/v) and destained in ethanol-acetic acid-water (3:1:5, ^{v}/v). The within-day coefficients of variation for rat serum were 3%, 6% and 6% for apo A-I, apo A-IV and apo E, respectively; the between-day coefficients of variation were 4%, 7% and 7% for apo A-I, apo A-IV and apo E, respectively.

Sample treatment

Proteins, including (apo) lipoproteins, in the samples to be analyzed were routinely precipitated with TCA in the presence of DOC as a carrier (130). This precipitation step increased the sensitivity of the assay by a factor of 10, because the precipitate could be solubilized in a small volume. Samples were diluted, if necessary, to a final volume of 1.975 ml and 0.025 ml of 2% DOC solution was added. After standing for 30 min at room temperature, 1 ml of 18% TCA solution was added and after 15 min. the resulting precipitate was collected by centrifugation at 3000 g for 30 min. The supernatant was discarded and the precipitate was dissolved in 0.1 ml of 0.5 M NaOH. Freshly distilled TMU (0.1 ml) was added and the samples were incubated for 30 min. at

30°C (127). Next, 0.1 ml of 20 mM Tris-HCl (pH 8.3), containing 8 M urea, was added and the samples were heated at 100°C for 2 min. The resulting volume was measured and accounted for in the final calculations. A sample of pooled rat serum and pure apolipoprotein solutions were treated identically and used as standards in each plate. Protein was determined according to Lowry et al. (131), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

In order to make the immunoassays suitable for assaying apolipoproteins in a variety of biological fluids, we developed a procedure for sample treatment that makes it possible to measure apolipoprotein concentrations in very diluted samples. Apolipoproteins in diluted samples are quantitatively precipitated with TCA in the presence of DOC. The small pellet, collected by centrifugation, is routinely solubilized in 0.1 ml of 0.5 M NaOH.

Proteins are quantitatively recovered in the pellet. The use of a rather concentrated NaOH solution is necessary to neutralize the TCA-containing pellet and to dissolve the protein pellet in a small volume. If the NaOH concentration is lowered, the rocket immunoprecipitate becomes less clear and because larger volumes (0.2 ml of 0.2 M NaOH and 0.2 ml of TMU) are needed to dissolve the protein pellet, the sample concentration is decreased. The solubilized pellet is subsequently delipidated with TMU and apolipoproteins are fully unfolded by the addition of 8 M urea followed by boiling for 2 min. The described sample treatment, using 0.1 ml of 0.5 M NaOH, results in a 10-fold concentration of apolipoproteins. This factor can be increased much further if larger samples are used, assuming that a reproducible method for the collection of the precipitate is available.

We used the buffer Tris-Tricine instead of the frequently used Veronal buffer because the use of the former buffer results in sharper rockets and essentially prevents current induced changes in the pH of the medium during electrophoresis.

The described procedure for sample treatment could affect the immunoreactivity of the different apolipoproteins. Therefore

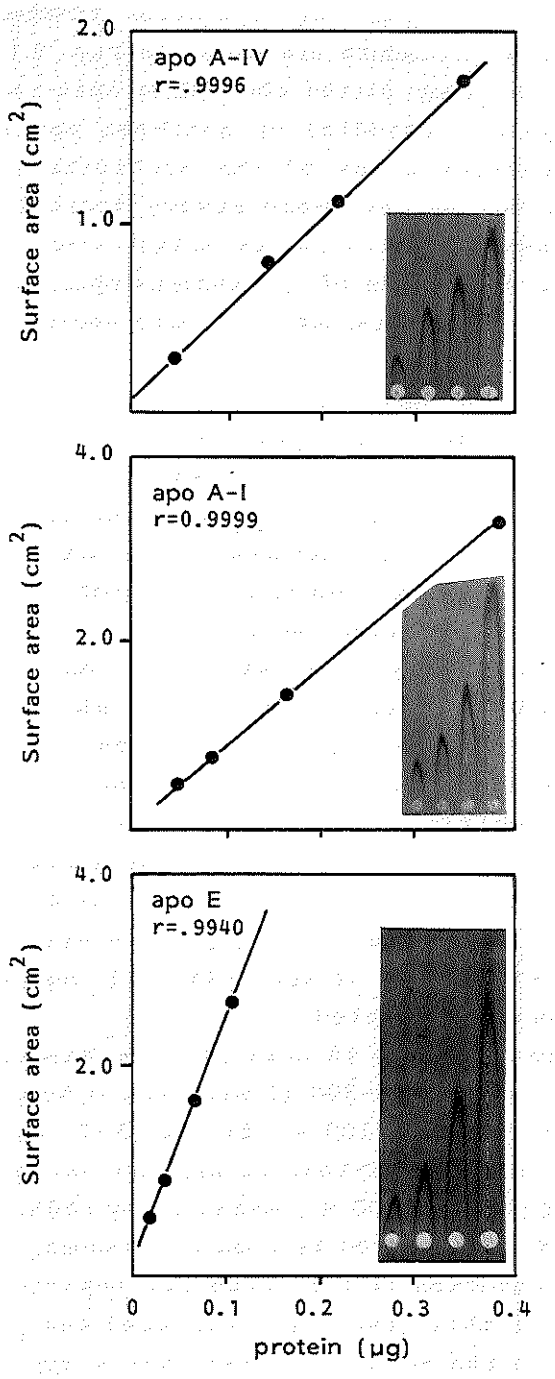

Fig. 2.1. Relationship of rocket surface area (height x width at half height (cm^2)) and protein concentration (µg) of pure apo A-IV (top), apo A-I (middle) and apo E (bottom) standards, applied to the wells in a sample volume of 13 µl.
Experimental conditions were as described in "Materials and Methods".

standards treated identically were always included in the assay. Rockets obtained with pure rat apolipoprotein standards treated according to the above mentioned procedure are shown in Fig. 2.1. Immunoprecipitates are shown in assay plates containing anti-rat apo A-IV (top), anti-rat apo A-I (middle) or anti-rat apo E (bottom) rabbit IgG. The surface areas of the precipitates (height x width at half-maximal height) were always strictly linearly related to the antigen concentration as illustrated in the figures. The morphological appearance of the rockets obtained with rat serum was identical to that obtained with pure apolipoprotein standards (not shown).

For further evaluation of the method we studied the effect of sample concentration on the recovery of immunoreactive apolipoproteins, using prediluted pure apolipoprotein solutions and prediluted rat serum. The recovery of pure apolipoproteins after predilution followed by precipitation and subsequent resolubilization, appears to be slightly less than 100%, if compared with untreated apolipoprotein solutions. However, we observed a comparable loss (4-14%) in the serum samples (see Table 2.1.). Apolipoprotein concentrations measured in biological fluids with or without the precipitation with DOC-TCA are therefore identical. Two NaOH concentrations were used to resolubilize the precipitates. Using the highest concentration (0.5 M NaOH) the volume could be limited to 0.1 ml and the resulting rockets had sharper edges and were easier to quantitate. Using the protocol as described above (using a 2 ml sample volume), apolipoprotein masses in biological fluids as low as 0.2 µg of apo A-IV, 0.2 µg of apo A-I and 0.8 µg of apo E can be measured.

The apolipoprotein concentrations in sera of male Wistar rats fasted overnight (body weight 250-300 g) were found to be 31.8 $\pm$ 3.8, 9.0 $\pm$ 2.2 and 20.4 $\pm$ 3.5 mg/100 ml for apo A-I, apo A-IV and apo E, respectively (n=12). Previous literature values for rat serum apo A-I range from 34 mg/100 ml, measured by radioimmunoassay (87), to 59 ml/100 ml measured by electroimmunoassay (96, 132). The apo A-IV concentration in rat serum was reported to be 24.4 mg/100 ml (133), but this level was increased due to the addition of 40% sucrose to the drinking water. Serum apo E

levels in male rats of about 17 mg/100 ml were measured using either electroimmunoassay (122) or radioimmunoassay (86). It appears that the serum apolipoprotein levels measured by our assay are in reasonable agreement with the reported literature values, keeping in mind that fasting causes a decrease in apo A-I as well as apo A-IV concentration (unpublished observations).

TABLE 2.1.

Effects of DOC-TCA precipitation and subsequent resolubilization in NaOH on the recovery of immunoreactive apolipoproteins

	0.2 M NaOH	0.5 M NaOH
Pure apo A-I standards	93± 4[a]	86±2
Apo A-I in rat serum lipoproteins	93± 3	88±3
Pure apo A-IV standards	90± 7	88±8
Apo A-IV in rat serum lipoproteins	93± 4	92±4
Pure apo E standards	96±12	86±9
Apo E in rat serum lipoproteins	96± 4	86±5

[a] Values are % ± SD (three experiments) of concentrations of standards or rat serum assayed without precipitation and resolubilization. All samples were delipidated. Two concentrations of NaOH were tested. Precipitates were dissolved in 0.2 ml of 0.2 M NaOH or 0.1 ml of 0.5 M NaOH.

In conclusion, a precipitation step including addition of DOC-TCA, preceeding TMU delipidation, makes it possible to measure apolipoprotein concentrations in very dilute samples. This method was specially designed to measure apolipoprotein concentrations in samples obtained after fractionation of serum and mesenteric lymph on 6% agarose columns. The results of these experiments will be published elsewhere.

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CHAPTER 3

DISTRIBUTION OF APOLIPOPROTEINS A-I AND A-IV AMONG LIPOPROTEIN CLASSES IN RAT MESENTERIC LYMPH, FRACTIONATED BY MOLECULAR SIEVE CHROMATOGRAPHY

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SUMMARY

The distribution of apo A-I and apo A-IV among lymph lipoprotein fractions was studied after separation by molecular sieve chromatography, avoiding any ultracentrifugation. Lymph was obtained from rats infused either with a glucose solution or with a triacylglycerol emulsion. Relative to glucose infusion, triacylglycerol infusion caused a 20-fold increase in the output of triacylglycerol, coupled with a 4-fold increase in output of apo A-IV. The output of apo A-I was only elevated 2-fold.

Chromatography on 6% agarose showed that lymph apo A-I and apo A-IV are present on triacylglycerol-rich particles and on particles with the size of HDL. Apo A-IV in addition is also present as "free" apo A-IV. The increase in apo A-I output is caused by a higher output of apo A-I associated with large chylomicrons only, while the increase in apo A-IV output is reflected by an increase in apo A-IV output in all lymph lipoprotein fractions, including lymph HDL and "free" apo A-IV. The increased level of "free" apo A-IV, seen in fatty lymph, may contribute to, and at least partly explain, the high concentrations of "free" apo A-IV present in serum obtained from fed animals.

INTRODUCTION

The small intestine has since long been recognized as an important organ for lipoprotein and apolipoprotein (apo) synthesis (8, 134, 135). After absorption into the enterocyte the dietary lipids are incorporated into chylomicrons which serve as vehicles for transport through the intestinal lymph to the plasma compartment. The size and composition of lymph chylomicrons have been described to depend upon the nutritional status of the animals. After fat feeding the lymph will contain predominantly large chylomicrons (Sf > 400) transporting mainly exogenous triacylglycerol. The apoprotein moiety of these particles mainly consist of apo A-I, apo B and apo A-IV (13, 114). Under fasting conditions predominantly smaller chylomicrons are found in rat mesenteric lymph (20 < Sf < 400), containing triacylglycerol, cholesterol and phospholipids derived from endogenous sources. The major apolipoproteins on these small chylomicrons are again apo A-I, apo B and apo A-IV. Small amounts of apo E and apo C's could also be detected (13, 17, 136).

In addition to chylomicrons, high density lipoproteins (HDL) have been found in rat mesenteric lymph (64). Lymph HDL differs from plasma HDL because, in addition to spherical HDL, the main structure found in plasma, discoidal particles that are enriched in phospholipids and deficient in cholesteryl esters are present. The apoprotein moiety of disc shaped HDL mainly consists of apo A-I, while almost no apo E or apo C could be detected. It has been proposed that discoidal mesenteric lymph HDL is synthesized in the enterocyte, while the spherical HDL particles may in part be derived from the plasma entering the lymph by ultrafiltration (64). Whether HDL of intestinal origin contributes significantly to the size of the pool of HDL in plasma is not well known.

In the present study we determined the distribution of apo A-I and apo A-IV among lipoproteins in intestinal lymph. Lymph was obtained from rats during intraduodenal infusion of triacylglycerol or glucose and directly fractionated on agarose columns, without any ultracentrifugation. This procedure avoids the stripping and redistribution of apolipoproteins and surface lipids due

to ultracentrifugation in salt solutions (12). Two different agarose columns were used: 6% agarose permitting the separation of large triacylglycerol-rich particles, lymph HDL and possible "free" apolipoproteins, and 2% agarose for further separation of the triacylglycerol-rich subfractions.

MATERIALS AND METHODS

Taurocholate was obtained from Calbiochem (La Jolla, U.S.A.). 6% agarose (BioGel A-5M, 200-400 mesh) and 2% agarose (Bio Gel A-50M, 100-200 mesh) were obtained from BioRad, Richmond, Ca, U.S.A.).

Male Wistar rats (weighing about 250 g) deprived of food for 20 h were anesthetized with diethylether. The main mesenteric lymph duct was cannulated between 9 and 10 A.M. using PP-50 polyethylene tubing (137). A second PP-50 cannula was inserted into the duodenum. The rats were placed into a restraining cage and allowed to recover from the anesthesia. All animals had free access to drinking water containing 5% glucose and 0.6% NaCl. Animals received intraduodenally a 0.7% NaCl solution containing either 10% (^{w}/v) glucose, or an emulsion of 69 mM glyceroltrioleate and 6.25 mM taurocholate in 0.7% NaCl both at a rate of 1.46 ml/h. Lymph was collected in ice chilled tubes between 5 P.M. and the next morning. After removal of the fibrin clots EDTA was added till a final concentration of 1 mM.

Lymph lipoproteins were separated by gel filtration chromatography as described for serum previously (138). Fifteen ml batches of lymph obtained from one animal in each individual experiment were applied to both a 6% and a 2% agarose column (2.5 x 120 cm), equilibrated and eluted with 0.15 M NaCl, 1 mM EDTA, 0.01% NaN_3, 2 mM Na phosphate (pH 7.4) at 4°C. Flow rate was approximately 15 ml/h and fractions of 5 ml were collected.

In the eluate, cholesterol was determined according to Zak et al. (139) using the extraction procedure of Abell (140). Triacylglycerol was determined according to Laurell (142). Total protein was determined by the method of Lowry et al. (131) using bovine serum albumin as standard. The turbidity after the color

development, due to excess of lipids, was removed by extraction with diethylether. Total phospholipids were determined according to Bartlett et al. (141). Prior to this procedure, samples were precipitated with TCA (final concentration 5% w/v) in the presence of albumin as a carrier. The precipitate was collected by centrifugation and phospholipid phosphorus was determined in the pellet without further extraction. Apolipoproteins were determined with specific electroimmunoassays exactly as described before (143). Prior to electrophoresis the samples were concentrated by means of DOC-TCA precipitation. The precipitates were dissolved in 0,1 ml of 0,5 M NaOH and delipidated with TMU as described earlier (143). A sample of pooled rat serum and pure apolipoprotein standards were treated identically and used as standards in each plate. The within-day coefficients of variation for rat serum were 3%, 6% and 6% for apo A-I, apo A-IV and apo E respectively; the between-day coefficients of variation were 4%, 7% and 7% for apo A-I, apo A-IV and apo E respectively. Recoveries of lipids and apolipoproteins after agarose chromatography were: total cholesterol 87 $\pm$ 16%, phospholipids 88 $\pm$ 14%, triacylglycerol 93 $\pm$ 21%, apo A-I 89 $\pm$ 15%, apo A-IV 77 $\pm$ 13% (means $\pm$ SD of 6 experiments).

Statistical analysis

The results were statistically evaluated using Student's two-tailed unpaired t-test.Differences were considered statistically significant at $p < 0.05$.

RESULTS

Table 3.1. shows data on the chemical composition of mesenteric lymph obtained from rats infused intraduodenally with either a glucose solution or a triacylglycerol emulsion. During glucose infusion a lymph flow of 2 $\pm$ 0.5 ml/h was obtained, somewhat lower than that found during triacylglycerol infusion (3 $\pm$ 0.5 ml/h). The lymphatic output of triacylglycerol during lipid infusion amounted 75.7 $\pm$ 10.7 mg/h, which is slightly less than the infusion rate into the duodenum (90 mg/h), suggesting that

TABLE 3.1.

Transport rates of lipids and apolipoproteins in mesenteric lymph of rats during glucose or triacylglycerol absorption

Mesenteric lymph was collected from rats fed intraduodenally either with a glucose solution or a triacylglycerol emulsion. Data are expressed as transport rate (concentration x lymph flow). Lymph flows were 2.0±0.5 and 3.0±0.5 ml/h during glucose and triacylglycerol absorption, respectively.

	Transport rate mg/h	
	Glucose infusion	Triacylglycerol infusion
Triacylglycerol	3.8 ± 1.6[1]	75.7 ± 10.7[2]
Phospholipids	1.3 ± 0.2	5.5 ± 2.7[2]
Total cholesterol	0.4 ± 0.1	0.7 ± 0.3[2]
Total protein	14.9 ± 2.7	22.3 ± 5.5[2]
Apo A-I	0.094 ± 0.015	0.197 ± 0.038[2]
Apo A-IV	0.072 ± 0.015	0.261 ± 0.083[2]
Apo E	n.d.[3]	n.d.

[1]All values are given as mean ± S.D. of 7 experiments.

[2]All parameters are significantly increased due to triacylglycerol absorption ($p < 0.01$).

[3]n.d. means not detectable. Apo E levels were less than 1 µg/ml.

most of mesenteric lymph produced during our observation period is recovered extracorporally. During glucose infusion the output of triacylglycerol was decreased 20-times (Table 3.1.). Output of lymphatic phospholipids were 4-times higher on triacylglycerol infusion than on glucose infusion, while smaller differences were observed for cholesterol and total protein (1.5 times). The lymphatic output of apolipoproteins A-I and A-IV was also increased during lipid infusion (2-and 4-times, respectively). Lymphatic concentrations of apolipoprotein E were too low to be detected using the immunoassay available in our laboratory (less than 1 µg/ml).

The distribution of apolipoproteins among lymph lipoproteins was studied after fractionation of lymph by agarose gel chromatography. Artificial losses of (apo) lipoprotein constituents during lipoprotein separation are expected to be low using this procedure This is important as chylomicrons are known to be very susceptible for such processes (12).

In the present study, two types of agarose gels were used, 6% agarose, capable of separating chylomicrons, HDL and possible free apolipoproteins and 2% agarose, capable to subfractionating chylomicrons according to size. Apo A-I in fatty lymph, fractionated on a 6% agarose column, is distributed bimodally (Fig. 3.1.A.). Three quarters of the apo A-I is present in the chylomicron fraction eluting in the void volume of this column, while the rest of the apo A-I is eluted in particles of the size of serum HDL (as determined in separate experiments in which rat serum was fractionated on the same column; compare ref. (138)). The other major apoprotein present in fatty lymph, apo A-IV, is distributed differently. Sixty % of apo A-IV recovered from the column is carried by chylomicrons found in the void volume, while only 8% is associated with particles of the size of HDL. The remaining 32% of lymph apo A-IV is present in fractions in which so called "free" apolipoprotein A-IV is found when rat serum is fractionated on the same column (Fig. 3.1.A., (138)). In lymph, during glucose infusion (Fig. 3.2.A.), roughly equal quantities of apo A-I are associated with the chylomicron and HDL fractions. The data for apolipoprotein A-IV were found to be 39%, 17% and

44% for the chylomicron, HDL and "free" apolipoprotein fractions, respectively.

More information about the distribution of apo A-I and apo A-IV between chylomicron particles of different size was obtained by fractionating lymph on 2% agarose columns. Elution profiles of both apolipoproteins in lymph during triacylglycerol infusion (Fig. 3.1.B.) showed a marked peak in the void volume, followed by a slow descending limb. These peaks in the void volume of the column were not observed in glucose lymph (Fig. 3.2.B.). Arbitrary two areas were distinguished in the elution profile: large chylomicrons, eluting between 205-276 ml and small chylomicrons, eluting between 277-470 ml. The chemical composition of large chylomicrons and small chylomicrons isolated from glucose lymph or triacylglycerol infused lymph are presented in Table 3.2. As expected, a decrease in triacylglycerol and an increase in phospholipids were observed with decreasing size of the chylomicrons. In the large chylomicron fraction in fatty lymph, a weight ratio for apo A-I over apo A-IV of about 1.0 was observed, which declined to about 0.8 in the smaller chylomicron particles. In glucose lymph, marked shifts of apo A-I and apo A-IV to smaller particle sizes were found. Only insignificant quantities were found in the large chylomicron fraction. In addition to these changes in apolipoprotein mass distribution, the ratio of apo A-I over apo A-IV, which is 4.0 in small chylomicrons present in glucose lymph, is significantly higher than the ratio found in fatty lymph (0.8).

Compositional data of the lymph HDL and the "free" apolipoprotein fractions are shown in Table 3.3.. Lymph HDL, isolated from glucose as well as fatty lymph, is rich in phospholipids and contains only small quantities of cholesterol. The lymphatic output of apo A-I, the major apoprotein of lymph HDL, during triacylglycerol infusion, was not very different from the output during glucose infusion. Apo A-IV in lymph HDL was higher during fat absorption but (part of) this increase may be due to the increase in "free" apo A-IV (see below) not completely separated from lymph HDL. Both in glucose and fatty lymph, apo A-IV was found in a peak eluting from the column after HDL. The position

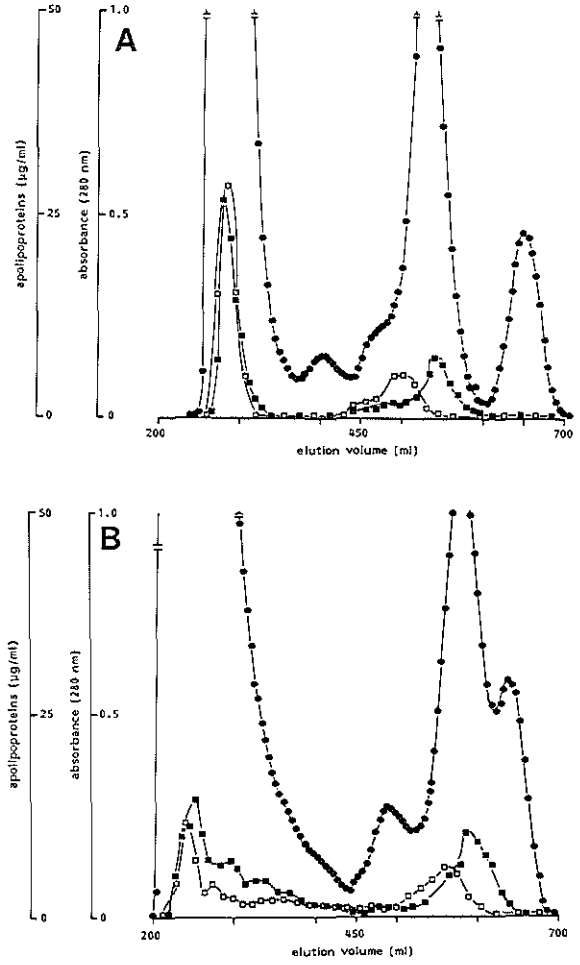

Fig. 3.1. Separation by molecular sieve chromatography on 6% agarose (3.1.A.) or 2% agarose (3.1.B.) of mesenteric lymph obtained during lipid infusion. Fifteen ml of lymph were applied to a column (2.5 x 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate pH 7.4, 1 mM EDTA and 0.01% NaN_3, at 4°C. Absorbance at 280 nm (●), apolipoprotein A-I (□), apolipoprotein A-IV (■).

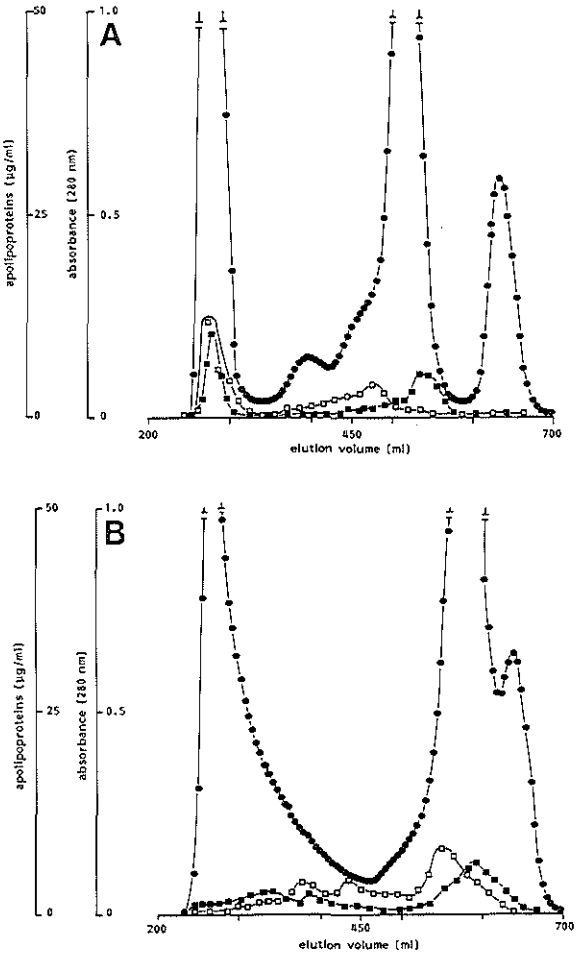

Fig. 3.2. Separation by molecular sieve chromatography on 6% agarose (3.2.A.) or 2% agarose (3.2.B.) of rat mesenteric lymph obtained during glucose infusion. Fifteen ml of lymph were applied to a column (2.5 x 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate pH 7.4, 1 mM EDTA and 0.01% NaN_3, at 4°C. Absorbance at 280 nm (●), apolipoprotein A-I (□), apolipoprotein A-IV (■).

TABLE 3-2.

Effects of glucose or triacylglycerol absorption on the transport rates in mesenteric lymph and chemical composition of chylomicrons fractions isolated by 2% agarose gel chromatography.

The data shown in this table are expressed as transport rates in mg/h (concentration in lymph multiplied by the lymph flow). Large chylomicrons were eluted between 205-276 ml, small chylomicrons were eluted between 277-470 ml. For further details see Fig. 3.1.B. and 3.2.B. and the text.

Mass transported in lymph associated with	Constituent	Infusion in duodenum of: Glucose	Triacylglycerol
Large chylomicrons	apo A-I	0.004±0.004	0.086± 0.039[1,2]
	apo A-IV	0.003±0.005	0.089± 0.048[2]
	total lipid mass	1.1 ±1.7	56.6 ±18.9[2]
	triacylglycerol (weight %)	(78± 9)	(92±3)
	phospholipids (weight %)	(20±10)	(7±2)
	cholesterol (weight %)	(2± 1)	(1±1)
Small chylomicrons	apo A-I	0.057±0.013	0.060±0.032
	apo A-IV	0.016±0.019	0.072±0.022[2]
	total lipid mass	2.81 ±0.4	10.1 ±3.8[2]
	triacylglycerol (weight %)	(63±9)	(75±5)
	phospholipid (weight %)	(30±7)	(22±5)
	cholesterol (weight %)	(3±1)	(3±1)

[1]All values are given as mean ± S.D. of 3 experiments.

[2]Statistically significant at $p < 0.05$.

TABLE 3.3.

Effects of glucose or triacylglycerol absorption on the transport rates in mesenteric lymph and chemical composition of "HDL-like" fractions and "free" apolipoproteins isolated by 6% agarose gel chromatography

The data shown in this table are expressed as transport rates in mg/h (concentration in lymph multiplied by the flow). Lymph HDL fraction eluted between 400-480 ml, "free" apo A-IV fraction eluted between 480-535 ml. For further details see Fig. 3.1.A. and 3.2.A. and the text.

Mass transported in lymph associated with	Constituent	Infusion in duodenum of	
		Glucose	Triacylglycerol
Lymph HDL	apo A-I	0.038±0.008	0.049±0.019
	apo A-IV	0.010±0.004	0.028±0.005[2]
	total lipid mass	0.32 ±0.19	0.47 ±0.19
	triacylglycerol (weight %)	(n.d.)	(n.d.)[3]
	phospholipids (weight %)	(90±6)	(96±1)
	cholesterol (weight %)	(10±6)	(4±1)
"Free" apo A-IV	apo A-I	0.004±0.001	0.007±0.001
	apo A-IV	0.017±0.008	0.082±0.039[2]
	total lipid mass	0.027±0.017	0.119±0.085
	triacylglycerol (weight %)	(n.d.)	(n.d.)
	phospholipid (weight %)	(100)[4]	(100)[4]
	cholesterol (weight %)	(n.d.)	(n.d.)

[1]All values are given as means ± S.D. of 3 experiments.

[2]Statistically significant at $p < 0.05$.

[3]n.d. means not detectable.

[4]parts will be associated with albumin, which coelutes with this fraction.

of this peak coincides with that of albumin, as determined in calibration runs using ^{125}I-labeled albumin and was shown to be identical with the position of the "free" apo A-IV fraction present in rat serum (results not shown, compare ref. 138). An interesting new observation in this study is that the lymphatic output of "free" apo A-IV is increased during triacylglycerol absorption.

DISCUSSION

In the present study, the effect of lipid absorption on the output and lipoprotein distribution of apolipoproteins A-I and A-IV in mesenteric lymph was analyzed.

It was found that the output of apo A-IV in the mesenteric lymph was increased fourfold during triacylglycerol infusion. In contrast, the lymphatic output of apo A-I was moderately increased (twofold), in agreement with earlier data of Imaizumi et al. (13). It has been shown by others that the rate of synthesis of apo A-IV is increased during triacylglycerol absorption (98), while the rate of synthesis of apo A-I is only moderately (13) or not increased (98, 100, 103). In line with these data are studies on the intestinal mRNA levels, showing that the apo A-IV mRNA levels are increased during fat absorption, while the apo A-I mRNA levels remain unaltered (115). Taken together, these studies indicate that relative differences in the rate of synthesis of apolipoproteins by the enterocytes are reflected in the output of the apolipoproteins in mesenteric lymph. It must be noted, however, that other pathways may affect the output of apolipoproteins in mesenteric lymph. It was shown that some mesenteric lymph apolipoproteins are derived from plasma by ultrafiltration, but the contribution of this pathway to the total lymph apolipoprotein mass is small (13). Furthermore, it was shown that the enterocyte is able to secrete apolipoproteins directly into the circulation (100), but again this pathway is considered of minor importance as compared to the output of apolipoproteins in mesenteric lymph. It can be concluded that changes in the output of apolipoproteins in mesenteric lymph are to a large extent, a

reflection of changes in the rate of secretion of the apolipoproteins by the enterocytes.

In subsequent studies, the distribution of apo A-I and apo A-IV among lipoproteins in mesenteric lymph was analyzed. The lymph lipoproteins were separated by molecular sieve chromatography to avoid the use of ultracentrifugation, a procedure which has been shown to dissociate apolipoproteins from lipoproteins in serum (87, 88) and in lymph (12). As illustrated in Figs. 3.1.B. and 3.2.B., lipid absorption is associated with a shift in apo A-I mass distribution towards larger chylomicron particle size. When recalculated in lymphatic output rates (Table 3.2.) this is reflected in a twentyfold increase in apo A-I in the large chylomicron fraction, while the mass of apo A-I associated with small chylomicrons is not very much different. The bulk of the increase in triacylglycerol output during lipid infusion is also confined to the large chylomicrons fraction.

Several studies have provided evidence that mesenteric lymph HDL is predominantly of intestinal origin (64-67), suggesting that lymphatic HDL may be an important precursor of serum HDL. Indeed, Green et al. (64) have shown that approximately 85% of apo A-I present in mesenteric lymph from glucose-infused rats can be recovered in the d > 1.006 g/ml fraction, suggesting that most of the apo A-I synthesized by the intestine is found in association with (precursor) HDL particles. However, in the present study, in agreement with Imaizumi et al. (13), it was found that only about 40% of the apo A-I in mesenteric lymph of glucose-infused rats was recovered in the HDL fraction. The output of apo A-I in mesenteric lymph HDL was not significantly affected by lipid infusion, as illustrated in Table 3.3., while under the latter conditions the apo A-I mass in chylomicrons is increased (see above); only 25% of the total mesenteric lymph apo A-I is recovered in the HDL fraction. In contrast, in a recent study (67) it was demonstrated that approximately 80% of the apo A-I in mesenteric lymph of lipid-infused rats was associated with HDL. The large quantitative differences in the literature regarding lymph HDL as a carrier for apo A-I cannot be explained easily, but may be due to differences in lipoprotein separation procedu-

res, associated with a more or less pronounced stripping of surface material from chylomicrons, followed by recovery of this material in the HDL fraction.

Lipid infusion has a much more pronounced effect on the output of apo A-IV in mesenteric lymph than on apo A-I output. In contrast to apo A-I, the increased apo A-IV output was seen in all mesenteric lymph lipoproteins, although the increase in HDL may partly be due to "free" apo A-IV. These data suggest that the synthesis of apo A-IV by the intestine is more closely connected with chylomicron production than the synthesis of apo A-I.

It was proposed by Fidge (133) that the "free" apo A-IV in rat serum is derived from lymph chylomicrons. This hypothesis was based on the observation that the apo A-IV moiety of intravenously injected chylomicrons is rapidly transferred to the "free" apo A-IV fraction. Although it was shown in the present study that most of the apo A-IV in lymph of glucose-infused rats was associated with small chylomicrons, a considerable quantity of total lymph apo A-IV was recovered in a fraction that eluted at the same position as "free" apo A-IV of rat serum. As illustrated in Figs. 3.1.A. and 3.2.A., the "free" apo A-IV of mesenteric lymph was rather well separated from the apo A-I- and cholesterol-containing HDL fraction. Similar results were found previously with rat serum (138). This indicates that "free" apo A-IV of mesenteric lymph shares some properties with "free" apo A-IV from rat serum, suggesting that mesenteric lymph "free" apo A-IV may also be a precursor of serum "free" apo A-IV. Recently, we have shown that the "free" apo A-IV fraction of rat serum is approximately eightfold increased in response to normal chow feeding, when compared with 20 h food-deprived rats (138). As the intestine is the major tissue for apo A-IV synthesis (11, 116), it is likely that the increased serum "free" apo A-IV concentration is related to an increase in the output in mesenteric lymph of either "free" apo A-IV or chylomicron apo A-IV or both. "Free" apo A-IV in mesenteric lymph is either secreted by the enterocyte as such, or liberated from chylomicrons in the lymph compartment by chylomicron surface film alterations following chylomicron secretion. Whatever its mechanism of formation, our data do

suggest that the increase in "free" apo A-IV in serum after (fat) feeding is partially explained by an increased influx of "free" apo A-IV from mesenteric lymph into the serum compartment. As "free" apo A-IV may be involved in the transport of cholesterol from peripheral cells to the liver (144) more data on the origin and metabolism of this fraction are needed.

The authors wish to thank Miss A.C. Hanson and Miss M.I. Wieriks for typing the manuscript.

CHAPTER 4

THE DISTRIBUTION OF APOLIPOPROTEIN A-IV BETWEEN LIPOPROTEIN SUBCLASSES IN RAT SERUM

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ABSTRACT

The distribution of apolipoproteins (apo) A-I, A-IV and E in sera of fed and fasted rats was studied using various methods for the isolation of lipoproteins. Serum concentrations of apo A-I and apo A-IV decreased significantly during fasting (16% and 31%, respectively), while apo E concentrations remained essentially the same. Chromatography of sera on 6% agarose columns showed that apo A-IV is present on HDL and as socalled "free" apo A-IV. The concentration of "free" apo A-IV decreased six-to seven-fold during fasting, explaining the decrease in total serum apo A-IV. Serum apo A-I and apo E are almost exclusively associated with HDL-sized particles. When sera are centrifuged at a density of 1.21 g/ml, marked quantities of apo A-I (8-9%) and apo E (11-22%) are recovered in the "lipoprotein deficient" infranatant, suggesting that ultracentrifugation affects the integrity of serum HDL.

The nature of the chromatographically separated carriers of serum apo A-IV was investigated by quantitative immunoprecipitation. From these studies it is concluded that apo A-IV in rat serum is present in at least three fractions:

1. particles with the size and composition of HDL, containing both apo A-I and apo A-IV and possibly minor quantities of apo E.
2. HDL-sized particles containing apo A-IV, but no apo A-I or apo E.
3. "Free" apo A-IV, probably containing small amounts of bound cholesterol and phospholipid.

INTRODUCTION

Apolipoprotein A-IV (apo A-IV) was first described by Swaney et al. (91) as a polypeptide with a molecular weight of 46.000 daltons present in rat HDL. The distribution of apo A-IV in human and rat serum is distinct from that of apo A-I and apo E, as apo A-IV is present in HDL as well as in a so called "free" form (111, 145-147). The possible metabolic relationship between apo A-IV present in HDL and non-lipoprotein-bound apo A-IV has been investigated using ultracentrifugation to separate both pools of apo A-IV (133). Because it is known that HDL apo A-I and apo E will dissociate from HDL during ultracentrifugation (60, 86-88), we compared the effect of the isolation procedure (gel filtration versus ultracentrifugation) on the distribution of apo A-IV, apo A-I and apo E. We also isolated the various fractions carrying apo A-IV in serum by agarose gel chromatography, combined with immunoprecipitation. This report describes the existence of three fractions in serum containing apo A-IV, two HDL species and a "free" apo A-IV probably containing small amounts of bound cholesterol and phospholipid.

MATERIALS AND METHODS

Serum samples

Male Wistar rats, weighing 250-300 g, maintained on standard laboratory chow and tapwater were used. Blood was collected at 9 AM from fed or 20 h food-deprived animals by aortic puncture under light ether anaesthesia. The blood was kept on ice for 2 h. The serum was obtained by low speed centrifugation at 4°C and EDTA solution (pH 7.4) was added to a final concentration of 1 mM.

Gel filtration of serum

Ten ml of pooled serum from fasted or fed rats, containing 10% sucrose (w/v) was applied on a 6% agarose column (2.5 cm x 120 cm, Bio-Rad, Richmond, Ca, USA), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate buffer (pH 7.4), 0.01% NaN_3 and 1 mM EDTA (148), operated at 4°C. The flow rate was 15-20 ml/h and fractions of 5 ml were collected. The column was calibrated with iodinated rat serum albumin, rat serum VLDL and HDL and with human LDL. Recoveries of cholesterol, apo A-I, apo A-IV and apo E were 96 ±4%, 87 ± 12%, 91 ± 7% and 80 ± 2%, respectively (means ± S.D.; n=4).

Ultracentrifugal isolation of serum lipoproteins

Serum of fasted or fed rats was adjusted to density 1.21 g/ml with solid KBr. Aliquots (3 ml) of these sera were carefully overlayered with KBr solution of density 1.21 g/ml containing 1 mM EDTA. The lipoprotein-containing ($d < 1.21$ g/ml) and the lipoprotein-deficient ($d > 1.21$ g/ml) fractions were isolated in the Beckman 40.3 rotor operating at 12°C and 38.000 rpm for 48 h. The recoveries of cholesterol, apo A-I, apo A-IV and apo E were 98 ± 6%, 97 ± 13%, 102 ± 9% and 91 ± 13%, respectively (means ± S.D.; n=8).

Immunoassays

Apo A-I, apo A,IV and apo E concentrations were determined by the electroimmunoassay technique of Laurell (129), as modified by Dallinga-Thie et al. (143).

Quantitative immunoprecipitation

Rabbit anti-apo A-I and anti-apo A-IV IgG, prepared as described (143), were used for the quantitative immunoprecipitaton of apo A-I- and apo A-IV-containing lipoproteins. Immunoprecipitations were performed by adding predetermined quantities of the IgG's to the apo A-IV-containing fractions, obtained by 6% agarose column chromatography of sera. Mixtures were incubated overnight at 4°C and the immunoprecipitates were isolated by low speed centrifugation at 4°C. Apolipoprotein concentrations were determined in both the precipitate and supernatant. Both supernatant and precipitate were extracted according to Bligh and Dyer (149) in order to determine the lipid composition. The chloroform phase was evaporated under a stream of nitrogen and used for lipid determinations. Cholesteryl esters were saponified with alcoholic KOH. After extraction with petroleum ether, total cholesterol was determined as described. Appropriate cholesterol standards were run in parallel throughout the whole procedure. The amount of cholesterol precipitated by a control rabbit IgG preparation isolated from a non-immunized rabbit was negligible.

Chemical analysis and statistics

Total cholesterol was determined with an enzymatic method (CHOD-PAP kit, Boehringer, Mannheim, F.R.G., cat. no. 310328). Total phospholipid phosphorus was determined according to Bartlett (141). Prior to this procedure, samples were precipitated, in the presence of albumin as a carrier, with TCA (final concentration 5%, $^{w}/v$). The precipitate was collected by centrifugation.

The data collected in this study were statistically evaluated using Student's unpaired, two tailed t-test.

RESULTS

Effects of nutritional status on serum lipid and apolipoprotein concentrations

In Table 4.1. lipid and apolipoprotein concentrations in sera from fed and fasted rats are presented. The serum cholesterol levels were not affected by the nutritional status, but total serum apo A-IV concentration decreased significantly during a 20 h fasting period. After more prolonged fasting (48 h) the serum apo A-IV concentration was further reduced to 5 mg% (unpublished observation). Fasting also resulted in a minor, but statistically significant, decrease in serum apo A-I concentration but the apo E level remained essentially the same.

Gel filtration of sera from fed and fasted rats

Figs. 4.1. to 4.4.show the elution patterns of apolipoproteins and cholesterol in rat serum fractionated on 6% agarose gel columns. A minor cholesterol peak is eluted in the void volume together with the triglycerides (not shown), consisting of chylo

TABLE 4.1.

Cholesterol, apo A-I, apo A-IV and apo E concentrations in sera of fed and fasted rats

	Fed[1]	Fasted[1]
	mg/100 ml ± SD	
Cholesterol	55.0±6.0	53.0±6.0
Apo A-I	39.8±5.7	33.3±3.1[2]
Apo A-IV	15.7±2.0	10.8±1.7[3]
Apo E	22.0±5.0	24.5±4.6

[1]Six experiments

[2]Statistically significant at $p < 0.05$.

[3]Statistically significant at $p < 0.001$.

Fig. 4.1.

Fig. 4.2.

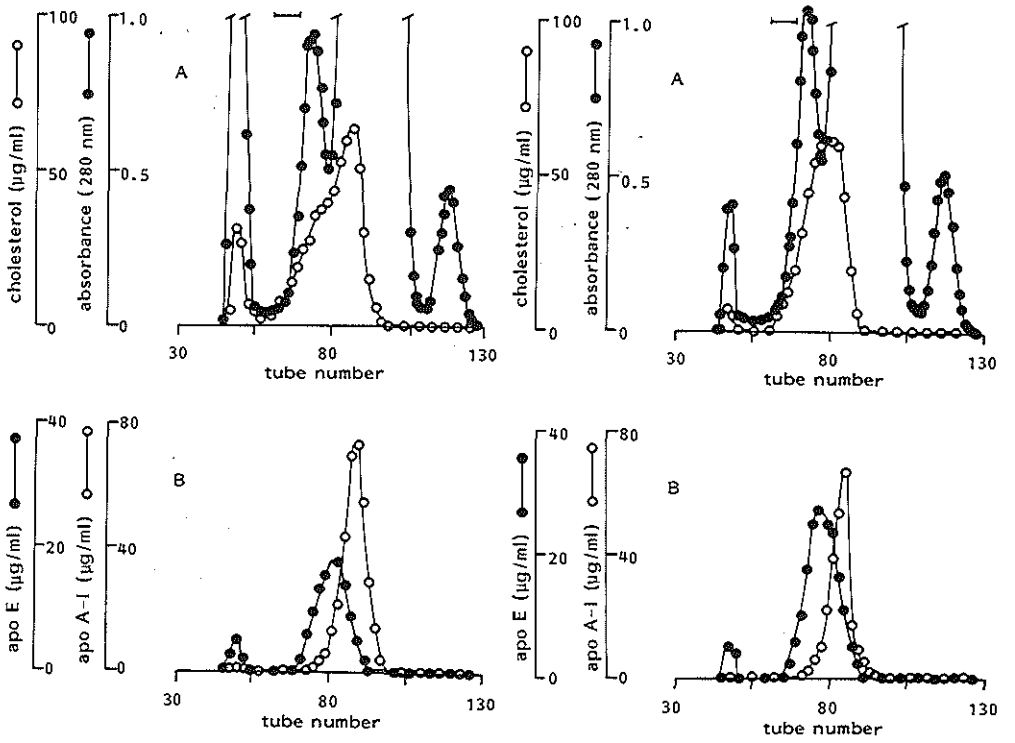

Fig. 4.1. Separation on a column of 6% agarose gel of serum from chow-fed rats. Ten ml of serum was applied to a column of 6% agarose (2.5 x 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate pH 7.4, 1 mM EDTA and 0.01% NaN_3, at 4°C. The horizontal bar indicates the elution volume of human LDL.

Fig. 4.2. Separation on a column of 6% agarose gel of serum from rats deprived of food for 20 h. Ten ml of serum were applied to a column of 6% agarose (2.5 x 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate pH 7.4, 1 mM EDTA and 0.01% NaN_3, at 4°. The horizontal bar indicates the elution volume of human LDL.

microns and/or VLDL. The major peak in cholesterol is largely due to HDL because the LDL level in rat serum is low. There is no difference in total serum cholesterol concentration between sera from fed and fasted rats but a skewed major peak in cholesterol is present in the fed state, indicating more variety in particle size (compare Figs. 4.1.A. and 4.2.A.). This phenomenon is not due to an increase in LDL in the fed state as LDL is eluted at a smaller elution volume (indicated by the horizontal bars, representing 90% of human LDL applied to the column in separate experiments, in Fig. 4.1.A. and 4.2.A.) as determined in separate experiments. The peak in absorbance at 280 nm coinciding with the shoulder of the cholesterol peak (fractions 70-80) is due to immunoglobulin M (mol. wt. 900,000 daltons).

The distributions of apo A-I and apo E are shown in Figs. 4.1.B. and 4.2.B. and those of apo A-IV in Figs. 4.3. and 4.4. In the fed state a minor fraction of apo A-I and apo E is located in the VLDL/chylomicron peak, while the majority (> 90%) is found in the HDL peak. The elution profiles of apo E and apo A-I in the HDL region were not identical as apo E clearly preceeded apo A-I. No big differences in elution patterns of apo E or apo A-I were observed between the fed and fasted states. Although comparison of Figs. 4.1.B. and 4.2.B. suggests an increase in HDL apo E after fasting, this observation appeared to be statistically insignificant. In sera from fasted animals, apo E was present in VLDL while no apo A-I could be detected in this fraction (Fig. 4.2.B.). In sera from fed animals, both apo A-I and apo E were detectable in the chylomicron/VLDL peak, although the amount of apo A-I in the void volume was extremely low (Fig. 4.1.B.).

With a 6% agarose column it is possible to obtain a clear separation between two distinct fractions containing apo A-IV (Figs. 4.3. and 4.4.). The first peak of apo A-IV elutes together with apo A-I and these fractions also contain the bulk of the serum cholesterol. The second apo A-IV peak elutes together with the bulk of the serum proteins (and iodinated albumin; not shown) in fractions containing little cholesterol, no apo A-I and no apo E. In the fed state an average of 36 $\pm$ 4% of the total apo A-IV mass was found in this second peak compared to 9% in the fasted

Fig. 4.3.

Fig. 4.4.

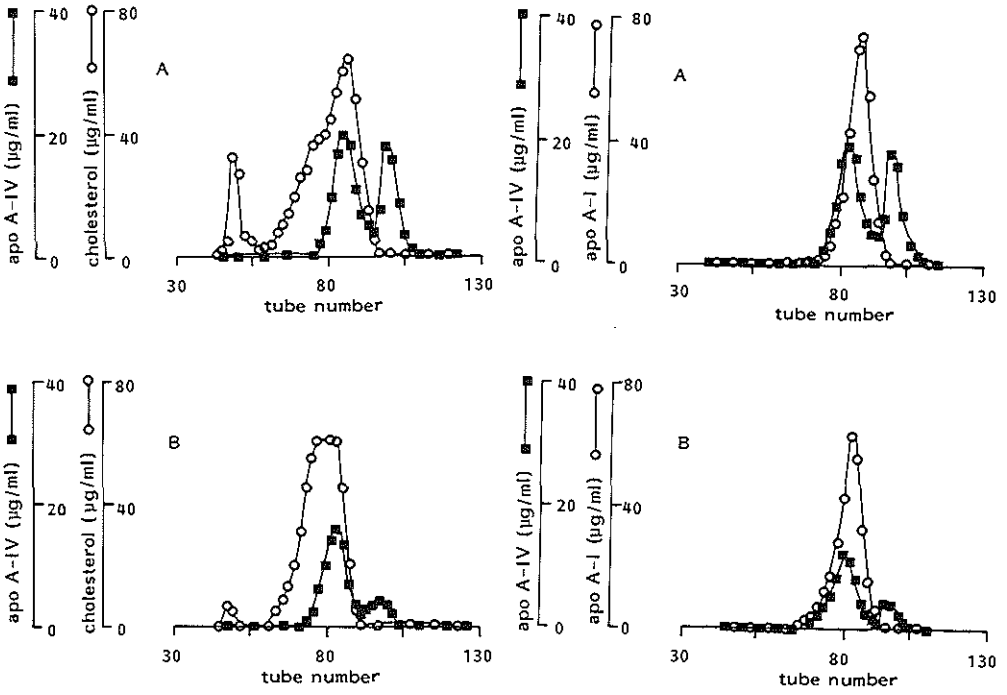

Fig. 4.3. Distribution of apo A-IV (■) and cholesterol (○) between serum lipoproteins of fed rats (Fig. 4.3.A.) and fasted rats (Fig. 4.3.B.) separated on a column of 6% agarose gel.

Fig. 4.4. Distribution of apo A-IV (■) and apo A-I (○) between serum lipoproteins of fed rats (Fig. 4.4.A.) and fasted rats (Fig. 4.4.B.) separated on a column of 6% agarose gel.

state (Table 4.2.). The amount of apo A-IV recovered in the size-range of typical HDL is not influenced, indicating that the decrease in serum apo A-IV level after a 20 h fasting period (Table 4.1.) is due to a decrease in the second apo A-IV peak.

TABLE 4.2.

The distribution of apo A-IV in serum between HDL and "free" apo A-IV, separated by 6% agarose chromatography

	HDL (first apo A-IV peak)	"Free" apo A-IV (second apo A-IV peak)
	mg/100 ml	
Fed[1]	8.7±1.6	4.9±0.4 (36± 4%)[2]
Fasted[3]	8.5,7.0	0.8,0.7 (8-10%)[4]

[1]Mean values of 4 experiments ± S.D.

[2]Numbers in parentheses are % recoveries of total serum apo A-IV in the second peak.

[3]Values of 2 separate experiments.

[4]Significantly lower than the value in serum obtained from fed animals $p < 0.005$.

Ultracentrifugation

Data on the cholesterol and apolipoprotein distribution between the d < 1.21 g/ml and d > 1.21 g/ml fractions are presented in Table 4.3. Essentially all cholesterol was recovered in the d < 1.21 g/ml fractions but significant amounts of serum apo A-IV, apo A-I and apo E, varying between 8% and 46% for the different apolipoproteins, are found in the "lipoprotein-deficient" (d > 1.21 g/ml) fraction. The amount of apo A-IV in this lipoprotein-deficient fraction is dependent upon the nutritional status; it decreased from 7,0 mg/100 ml in fed animals to 3,9 mg/100 ml after 20 h of fasting. These values represent 46% and 35% of total serum apo A-IV in the fed and the fasted states, respectively. In both cases the amount of apo A-IV found in this

lipoprotein-deficient fraction is significantly higher ($p < 0.005$) compared to the amount found in the "free" apo A-IV fraction, using the gel filtration method (see Table 4.2.).

TABLE 4.3.

Distribution of cholesterol, apo A-I, apo A-IV and apo E after centrifugation of serum at a density of 1.21 g/ml

	Total serum	d < 1.21 g/ml	d > 1.21 g/ml
		mg/100 ml ± SD (n=4)[1]	
Fed			
Cholesterol	51.5±3.2	50.5±3.3	n.d.[2]
Apo A-I	42.2±5.4	35.0±4.0 (83± 9%)	3.4±0.4 (8±1%)
Apo A-IV	15.3±1.9	9.1±0.5 (59± 3%)	7.0±0.6 (46±4%)
Apo E	20.0±4.4	13.4±4.0 (66±12%)	4.4±0.8 (22±4%)
Fasted			
Cholesterol	51.3±6.5	50.1±6.3	n.d.[2]
Apo A-I	34.4±3.1[3]	31.7±3.0 (91± 9%)	3.3±0.9 (9±3%)
Apo A-IV	11.2±0.8[4]	7.0±0.6 (63± 5%)	3.9±0.8[4] (35±7%)
Apo E	22.7±2.3	19.2±2.2 (85±13%)	2.3±1.0 (11±5%)

[1]Numbers in parentheses are the percentage recoveries in the separate fractions.

[2]Not detectable, without prior concentration.

[3]Significantly lower than the values in serum obtained from fed animals, $P < 0.05$.

[4]Significantly lower than values in serum obtained from fed animals, $P < 0.005$.

Immunoprecipitation studies

In order to obtain information about the composition of the carriers of apo A-IV in serum, we fractionated serum by agarose gel chromatography and precipitated the apo A-IV-containing particles in the HDL size range, as well as in the "free" apo A-IV peak, using anti-apo A-IV IgG. Similar studies were performed using anti-apo A-I IgG; immunoprecipitates as well as supernatants were analyzed chemically and immunochemically. The results of these experiments are shown in Tables 4.4. and 4.5. It can be concluded from the data in Table 4.4. that apo A-IV in serum is associated with at least two species with the size of HDL: 1. particles containing apo A-I, apo A-IV and possibly minor quantities of apo E and 2. particles containing apo A-IV, but neither apo A-I nor apo E.

TABLE 4.4.

Analysis of HDL isolated by chromatography of rat serum on 6% agarose columns

	HDL[1]	HDL precipitated with anti-apo A-I	HDL precipitated with anti-apo A-IV
Cholesterol	79.8±1.2[2]	58.9±0.2	12.0±2.1
Phospholipids	142.8±5.7	113.3±2.5	15.4±1.6
Apo A-I	79.2±1.5	79.2±1.5 (77.5±6.3)[3]	4.3±2.7
Apo A-IV	22.3±5.2	14.5±0.9	22.3±5.2 (25.3±5.7)[3]
Apo E	14.6±1.5	4.7±0.8	0.9±1.0

[1]Fractions containing the bulk of apo A-I (and apo A-IV) were pooled.

[2]Results are expressed as µg/ml column fraction. Values are means ± S.D. (n=3).

[3]The values in parentheses were assayed in the precipitates and agreed very well with the amounts present in the original HDL preparation. HDL apo A-I and HDL apo A-IV were quantitatively precipitated using anti-apo A-I or anti-apo A-IV, respectively.

These conclusions are based on the partial precipitation of apo A-IV (60%) using anti-apo A-I, indicating that about 40% of apo A-IV in HDL is not associated with apo A-I. Furthermore insignificant quantities of apo E were precipitated using anti-apo A-IV, in line with the existence of an apo A-IV containing HDL, free of apo A-I and apo E. The chemical composition of HDL fractions precipitated with anti-apo A-I is similar to that of serum HDL, e.g., a protein content of 35-40% and a phospholipid: cholesterol ratio of 2:1. HDL-sized fractions precipitated with anti-apo A-IV seem to have a higher protein content and a phospholipid: cholesterol ratio closer to 1 (Table 4.4.). Only about 6% of HDL apo A-I can be precipitated with anti apo A-IV indicating that more than 90% of HDL apo A-I is present on an HDL subfraction deficient in apo A-IV, assuming a particle weight for HDL of about 275,000. It can also be calculated that the bulk of this HDL is deficient in apo E because the amount of apo E precipitated by anti-apo A-I is very low.

Similar analyses of the "free" apo A-IV fraction (see Table 4.5.) revealed that small amounts of cholesterol and phospholipid are associated with apo A-IV (about 11% by weight). Neither apo E and apo A-I (checked by immunoassay) nor apo C and apo A-II (checked by SDS-polyacrylamide gelelectrophoresis) could be detected in the immunoprecipitate.

DISCUSSION

Rat apo A-IV is mainly synthesized in the intestine (11, 98) and its concentration in serum is affected by the dietary history of the animal. Studies of Delamatre and Roheim (145) have shown that single meal feeding of rat chow, a high fat meal or a high fat meal plus cholesterol results in 40-80% increase in serum apo A-IV, with no observed effect on serum apo A-I. Long-term feeding of a cholesterol/high fat diet also results in an increase in serum apo A-IV compared to a chow-fed control group. The data presented in this report show that the levels of apo A-IV in sera of fasted rats are decreased by 31% compared to postprandial levels in chow-fed rats.

Chylomicrons in intestinal lymph carry both apo A-I and apo A-IV. After entering the plasma compartment, part of the apo A-I rapidly dissociates from the chylomicrons and is transferred to HDL (12, 13, 19, 150); the same mechanism has been proposed for apo A-IV (12, 19). It appears from our data that the dissociation of apo A-IV from the chylomicrons is even more complete than for apo A-I, as apo A-IV could not be detected at all in the chylomicron/VLDL fraction after analysis of serum from fed rats on 6% agarose columns (Fig. 4.3.). More recently it has been suggested that the precursor-product relationship between chylomicron- and HDL-apo A-IV is not direct, but linked by a passage through the pool of "lipoprotein-free" apo A-IV (133).

TABLE 4.5.

Chemical composition of the "free" apo A-IV fraction isolated by chromatography of rat serum on 6% agarose columns, followed by immunoprecipitation

Cholesterol	0.9±0.7[1]
Phospholipid	0.6±0.4
Apo A-I	0
Apo A-IV	10.2±2.8 (12.2±3.4)[2]
Apo E	0

[1]The fractions containing the bulk of the "free" apo A-IV, obtained from serum of fed rats by agarose gelfiltration, were first treated with anti-apo A-I, in order to remove any contaminating apo A-I containing lipoproteins. The resulting "free" apo A-IV fraction was subsequently precipitated with anti-apo A-IV. Lipids, apo A-I and apo E were measured in the precipitate. Results are expressed as µg/ml column fraction. Values are means ± S.D. (n=3).

[2]Apo A-IV was measured both in the "free" apo A-IV fractions directly obtained from the agarose columns as well as in the final precipitate (values in parentheses).

We recovered 9% of serum apo A-I, the main protein of HDL, in the d > 1.21 g/ml fraction after one ultracentrifugal run of 24 h, which is in agreement with reported values (151). No apo A-I could be detected in column fractions following the HDL peak during fractionation of serum by gel filtration (Table 4.2., Figs. 4.1., 4.2. and 4.4.). It was reported that about 50% of the apo E was lost to the "lipoprotein-free" infranatant during a standard isolation of HDL by sequential ultracentrifugation (60, 151, 152). In the present study only one ultracentrifugal step was performed leading to "stripping" of about 11-22% of serum apo E. All fractions containing apo E or apo A-I on gel filtration of serum also had substantial amounts of cholesterol. Although the existence of lipid free aggregates of apolipoproteins with the size of HDL and a density > 1.21 g/ml cannot be excluded, this suggests an artificial origin of apo E and apo A-I in the ultracentrifugal "lipoprotein-deficient" fraction. The underlying cause of this proposed ultracentrifugal loss of lipoprotein apolipoproteins is complex and probably attributable to combined effects of high ionic strength and high sheering forces (88).

The situation for apo A-IV is clearly different. Fractionation of rat serum by gel filtration results in the recovery of part of the apo A-IV in a fraction that elutes after the main cholesterol peak. The concentration of this "free" apo A-IV is very dependent upon the nutritional status of the animal. It can be calculated from data in Table 4.2. that there is a six- to seven-fold decrease in "free" apo A-IV during fasting. Others have shown that cholesterol feeding (145) and aging (146) will result in an increasing amount of apo A-IV in this fraction. In human serum using either ultracentrifugation or crossed immunoelectrophoresis, it was observed that even more (> 95%) of the serum apo A-IV was present in a form not bound to VLDL, LDL or HDL (110, 111), Using gel filtration chromatography, about 77% of human apo A-IV was found in fractions recovered from the column after VLDL, LDL and HDL (147). In our experiments 46% and 35% (in serum from fed and fasted rats, respectively) of the total serum apo A-IV were recovered in the d > 1.21 g/ml fraction after one ultracentrifugal run at d 1.21 g/ml. Using agarose column chroma-

tography 36% and 9% (fed versus fasted; see Table 4.2.) of serum apo A-IV were found in the "free" apo A-IV fraction, indicating that not only apo A-I or apo E, but also apo A-IV may be "stripped" from rat HDL during ultracentrifugation.

Results of our immunoprecipitation experiments showed that the "free" apo A-IV fraction contained small amounts of bound cholesterol and phospholipid, which were detectable after precipitation with anti-apo A-IV. In these experiments the "free" apo A-IV fraction was first treated with anti-apo A-I, in order to exclude any contamination of HDL-sized particles containing apo A-I. However, some contamination of "free" apo A-IV with apo A-IV-containing lipoproteins with the size of HDL cannot be excluded (see below).

Further characterization of apo A-IV containing particles with the size of HDL was performed by precipitation with specific antisera against apo A-I and apo A-IV. From our experiments it can be concluded that, based on apolipoprotein composition, HDL is heterogeneous. It is concluded that two apo A-IV containing HDL particles are present in rat serum:

1. particles that have apo A-IV, but no apo A-I and apo E.
2. particles that contain both apo A-I and apo A-IV and possibly some apo E. In addition, the data suggest that the major part of HDL apo A-I is present on a particle that contains neither apo A-IV nor apo E.

More research is needed for further characterization of the different HDL subfractions and for definition of their origin and specific metabolic functions.

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CHAPTER 5

COMPARISON OF THE METABOLIC BEHAVIOR OF RAT APOLIPOPROTEINS A-I AND A-IV, ISOLATED FROM BOTH LYMPH CHYLOMICRONS AND SERUM HIGH DENSITY LIPOPROTEINS

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ABSTRACT

1. Rat apolipoprotein (apo) A-I and A-IV, isolated from both lymph chylomicrons and serum high density lipoproteins (HDL) were analyzed by isoelectric focusing. Lymph chylomicron apo A-I consisted for 81±2% of the pro form and for 19±2% of the mature form, while apo A-I isolated from serum HDL was present for 36±4% in the pro form and for 64±4% in the mature form. Apo A-IV also showed two major protein bands after analysis by isolectric focusing. The most prominent component is the more basic protein that amounts to 80±2% in apo A-IV isolated from lymph chylomicrons and to 60±3% in apo A-IV isolated from serum HDL.

2. Apo A-I (or apo A-IV), isolated from both sources (lymph chylomicrons or serum HDL), was iodinated and the radioactive apolipoproteins were incorporated into rat serum lipoproteins. The resulting labeled HDL were isolated from serum by molecular sieve chromatography on 6% agarose columns and injected intravenously into rats. No difference in the fractional turnover rate or the tissue uptake of the two labeled HDL preparations was observed, neither for apo A-I nor for apo A-IV.

3. It is concluded that the physiological significance of the extracellular pro apo A-I conversion or the posttranslational modification of apo A-IV is not related to the fractional turnover rate in serum or to their rate of catabolism in liver and kidneys.

INTRODUCTION

Rat apolipoproteins (apo) A-I and A-IV are synthesized in the intestine (11, 116) and secreted in the mesenteric lymph mainly in association with chylomicrons (13). After exposure to plasma, apo A-I and apo A-IV are transferred from the chylomicrons to HDL (12, 18). Both apolipoproteins are synthesized in the enterocytes as preproteins (105, 117), that are cleaved cotranslationally. Apo A-I, in contrast to apo A-IV is secreted as a pro protein (105). After secretion the six amino acid prosegment is cleaved, probably by an enzymatic process in the plasma compartment (101, 107), yielding mature apo A-I. Although apo A-IV is synthesized without a pro segment, rat plasma apo A-IV exhibits several isoproteins (12, 112, 153) suggesting that apo A-IV is modified posttranslationally. The molecular basis of this apo A-IV polymorphism is unknown.

The metabolic functions of the pro apo A-I to mature apo A-I conversion and the posttranslational modification of apo A-IV are not clear. While these processes apparently proceed after the newly synthesized apolipoproteins have reached the plasma compartment, we have compared the isoprotein distribution, the in vivo serum decays and the in vivo tissue sites of degradation of apo A-I and apo A-IV, isolated from both lymph chylomicrons and serum HDL.

MATERIALS AND METHODS

Treatment of rats

Male Wistar rats, weighing 350-400 g, maintained on standard laboratory chow and tap water were used. Blood was collected from the animals by aortic puncture under light ether anesthesia. The blood was kept on ice for 2 h and serum was obtained by low speed centrifugation at 4°C.

Isolation of apolipoproteins

Serum HDL apo A-I and apo A-IV were isolated from rat serum as described previously (143). Lymph apo A-I and apo A-IV were

isolated from rat mesenteric lymph. The cannulation was performed exactly as described by Lambert (137). Lymph collection started 5 h after the operation and the lymph was collected on ice. Clots were removed and EDTA (to a final concentration of 1 mM) was added. Chylomicrons were isolated by ultracentrifugation in the 50.2 rotor at a density of 1.019 g/ml for 2 h at 48,000 rpm and 4°C. The toplayer, containing the large chylomicrons, was further fractionated on a 6% agarose column (2.5 x 120 cm; Bio Gel A 5M, 200-400 mesh, Bio Rad, Richmond, CA., U.S.A.), equilibrated and eluted with 0.15 M NaCl containing 2 mM Na-phosphate (pH 7.4), 0.01% NaN_3, 1 mM EDTA at 4°C with a flow rate of 15 ml/h. The large chylomicrons, containing the bulk of apo A-I and apo A-IV, were recovered in the void volume. Lymph apo A-I and apo A-IV were purified from these large chylomicrons using the same procedure as described for the purification of apo A-I and apo A-IV from serum HDL. The purity of all apolipoprotein preparations was checked by SDS-PAGE electrophoresis (154), isoelectric focusing (155) and immunoblotting (156) using specific antisera against apo A-I and apo A-IV (143). The immunoblots were visualized with swine anti-rabbit immunoglobulin conjugated to horse radish peroxidase (DAKO, Glostrup, Denmark) and diaminobenzidine (Sigma, St. Louis, MO., U.S.A.).

Radioiodination of apolipoproteins was carried out using the ICl method, modified as described by Van 't Hooft et al. (85). The efficiency was 10-20% and the iodine/protein ratio was less than 1 gatom per gmol of protein.

Labeling and separation of lipoproteins by column chromatography

The ^{125}I- and ^{131}I-labeled apolipoproteins were mixed with 2 ml of rat serum and incubated for 1 h at 4°C. After addition of sucrose to a final concentration of 10% (w/v), the sample was applied to a 6% agarose column (1.5 x 50 cm) and eluted as described above. The flow rate was 3-4 ml/h and fractions of 1 ml were collected. Absorbance at 280 nm, ^{125}I- and ^{131}I-radioactivity were measured in each fraction. Approximately 80% of both apo A-IV preparations and 40-70% of both apo A-I preparations were incorporated into particles with the size of HDL. Rechromatogra-

phy of the labeled HDL preparations did not result in any loss of radioactivity.

Metabolic studies

HDL fractions containing either ^{125}I-lymph chylomicron apo A-I and ^{131}I-serum HDL apo A-I or ^{125}I-lymph chylomicron apo A-IV and ^{131}I-serum HDL apo A-IV were pooled and dialyzed for 24 h against 0.15 M NaCl. All preparations were used for metabolic studies exactly as described by Van 't Hooft et al. (85). The distribution of radioactive apo A-I or apo A-IV among serum lipoproteins was determined 4 h after the intravenous injection of labeled HDL. Serum obtained from leupeptin-treated or saline-treated animals was analyzed by molecular sieve chromatography on 6% agarose (column size 2 x 120 cm, flow rate 10 ml/h) as described above. All ^{125}I- and ^{131}I-radioactivity was found to be associated with HDL.

The data collected in this study were statistically evaluated using Student's unpaired t-test. Where indicated, values given are means $\pm$ S.D. for 3 experiments.

RESULTS AND DISCUSSION

Apo A-I and apo A-IV were purified from rat serum HDL and mesenteric lymph chylomicrons using conventional methods. On SDS-PAGE electrophoresis all purified apolipoproteins migrated as a single band with estimated molecular weight of 27,000 for both apo A-I preparations and 46,000 for both apo A-IV preparations (results not shown). The purified apolipoproteins were subsequently analyzed by isoelectric focusing (Fig. 5.1.). Two prominent protein bands were detectable in all apo A-I preparations: a more basic protein with pI 5.6 (mature apo A-I) and a more acidic protein with pI 5.5 (pro apo A-I containing a 6-amino acid prosegment) (101, 105, 107). Quantification of the isoproteins by densitometric scanning of the stained gels at 650 nm revealed that serum HDL apo A-I was composed for 64$\pm$4% of the mature protein and for 36$\pm$4% of the proprotein, while lymph chylomicron apo A-I was composed for 19$\pm$2% of the mature protein and for

81±2% of the proprotein. These data are in good agreement with the values published by Sliwkowski et al. (101).

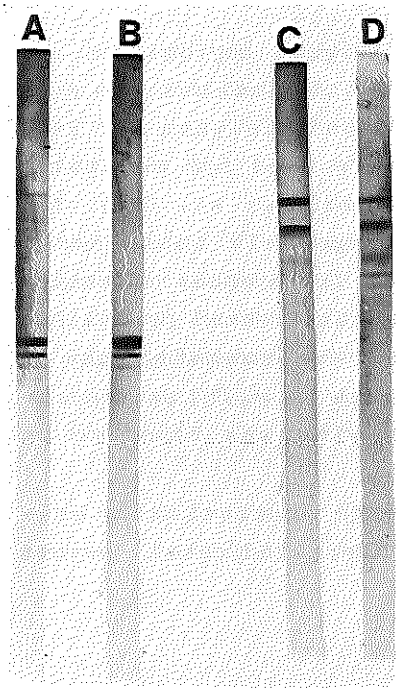

Fig. 5.1. Analytical isoelectric focusing in polyacrylamide gels of apolipoproteins in a pH gradient from 4-6.
A. serum HDL apo A-IV
B. lymph chylomicron apo A-IV
C. serum HDL apo A-I
D. lymph chylomicron apo A-I

In agreement with earlier studies ((12, 112, 153) it was found that rat serum HDL apo A-IV and lymph chylomicron apo A-IV also consist of two major isoforms (Fig. 5.1.), with pI's close to 5.0. Lymph chylomicron apo A-IV was composed for 80±2% of the more basic form and for 20±2% of the more acidic form. The distribution of the two major isoforms is different in apo A-IV isolated from serum HDL: 60±3% was present in the basic form, 38±3% in the acidic form and about 2% is present as an additional minor component. The question was raised whether these isoforms really represented different isoproteins of apo A-IV. Therefore the isoforms were analyzed, after blotting on a nitrocellulose paper, with a specific antibody raised against apo A-IV. It was found that all isoforms reacted with anti-apo A-IV (results not shown), indicating that the isoforms represent isoproteins of apo

A-IV. From these studies it could be concluded that the apo A-I and apo A-IV isoprotein composition in lymph chylomicrons and in serum HDL was not identical. The physiological relevance of the different isoprotein compositions of lymph chylomicron and serum HDL apo A-I and apo A-IV remains uncertain. We therefore studied the in vivo fractional turnover rate and the tissue sites of catabolism of these preparations, using the model (leupeptin-treated rats) previously described by Van 't Hooft et al. (85).

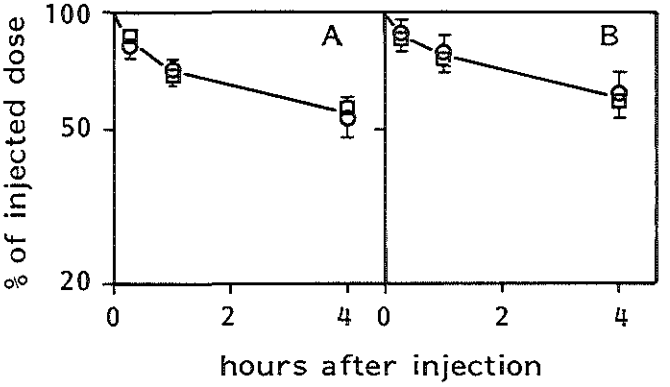

Fig. 5.2. Removal of rat HDL, labeled with ^{125}I-apo A-I isolated from lymph chylomicrons (A) or ^{131}I-apo A-I isolated from rat serum (B) from the blood plasma of control (□) and leupeptin-treated (○) animals. The isolation of rat HDL and the treatment of the rats are described in Experimental Procedures. Results are the mean values ± S.D. from 3 experiments.

Fig. 5.2. shows the serum decay of HDL labeled with ^{125}I-lymph chylomicron apo A-I/^{131}I-serum HDL apo A-I, measured over a period of 4 h after intravenous injection of the labeled HDL. In vivo leupeptin treatment did not have any effect on the serum decay rate of the labeled preparations. No difference was observed in the serum decays of the two labeled apo A-I preparations, indicating that the rates of catabolism of the two isoproteins are identical. It was shown before that during a 4 h experiment only 20% (107) or less (101) of the injected pro apo A-I is converted to the mature protein.

Serum HDL apo A-I is predominantly catabolized in the kidneys and to a lesser extent in the liver (85, 157). The question was raised whether the two apo A-I isoproteins exhibit a different preference for these tissues. The tissue sites of catabolism of both HDL apo A-I preparations was analyzed at various time points after intravenous injection of the labeled HDL in leupeptin-treated and saline-treated animals. No difference in the accumulation of radioactivity was observed between the two apo A-I preparations at any time point in 11 different tissues. Only the liver and the kidneys show a significant leupeptin-dependent accumulation of radioactivity (Table 5.1.). These results indicate that the two apo A-I isoproteins are catabolized at an identical rate by the liver and the kidneys.

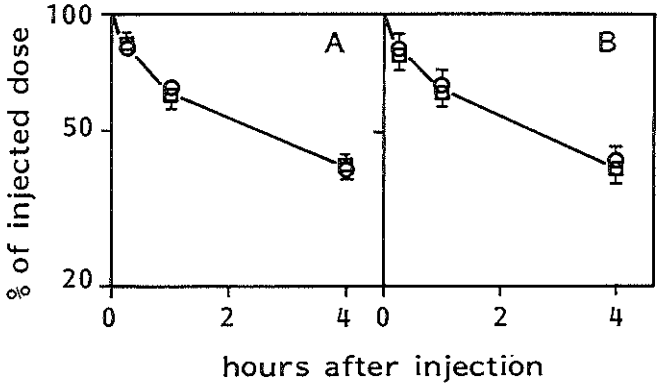

Fig. 5.3. Removal of rat HDL, labeled with ^{125}I-apo A-IV isolated from lymph chylomicrons (A) and ^{131}I-apo A-IV isolated from rat serum HDL (B) from the blood plasma of control (□) and leupeptin-treated (○) animals. The conditions were identical with the experiments described in Fig. 5.2.

Similar studies were conducted with both apo A-IV preparations, incorporated into rat serum HDL. No difference was observed in the serum decay (Fig. 5.3.). However, both apo A-IV preparations were removed from the circulation more rapidly than the two apo A-I preparations (compare Figs. 5.2. and 5.3.). This observation is in agreement with a recent study showing that the fractional turnover rate (measured between 8 and 28 h) was 8.5 h for HDL apo A-IV and 10.2 h for HDL apo A-I (Dallinga-Thie et

al., in press). No differences were observed in the leupeptin-dependent accumulation of radioactivity, measured 4 h after injection of the labeled preparation. It is evident that apo A-IV radioactivity preferentially accumulates in the liver, while apo A-I radioactivity accumulates predominantly in the kidneys (Table 5.1.).

TABLE 5.1.

Leupeptin-dependent accumulation of radioactivity, expressed as % of the injected dose/tissue, measured 4 h after intravenous injection of the labeled HDL preparations*.

	Liver	Kidneys
Lymph chylomicron apo A-IV	6.0±2.9	3.1±0.8
Serum HDL apo A-IV	7.5±3.0	2.9±0.1
Lymph chylomicron apo A-I	2.1±1.7	4.3±0.1
Serum HDL apo A-I	2.4±1.5	4.5±1.3

* Values given are average ± S.D. of 3 experiments.

In conclusion, this study shows that lymph chylomicron and serum HDL apo A-IV have a different isoprotein distribution, as was previously noted for apo A-I. The fractional turnover rates and the tissue sites of catabolism of apo A-I and apo A-IV, isolated from lymph chylomicrons and serum HDL, and reincorporated into serum HDL were identical. These results indicate that the physiological significance, if any, of the pro apo A-I to mature apo A-I conversion and of the posttranslational modification of apo A-IV is not related to the fractional turnover rate of serum HDL or to the catabolism of these HDL proteins in the liver and the kidneys.

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CHAPTER 6

TISSUE SITES OF DEGRADATION OF HIGH DENSITY LIPOPROTEIN APOLIPOPROTEIN A-IV IN THE RAT

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Arteriosclerosis, in press.

ABSTRACT

The in vivo metabolism of high density lipoprotein (HDL), labeled by incorporation of ^{125}I-apolipoprotein (apo) A-IV, was studied in the rat and compared with the metabolism of HDL labeled with ^{131}I-apo A-I. The ^{125}I-apo A-IV labeled HDL was obtained by adding small amounts of radioiodinated apo A-IV to rat serum, followed by separation of the different lipoprotein fractions by chromatography on 6% agarose columns in order to avoid "stripping" of apolipoproteins by ultracentrifugation. Under both in vitro and in vivo conditions the ^{125}I-apo A-IV remained an integral component of HDL and was not exchanged to other lipoproteins, including the "free" apo A-IV fraction. The serum half-life, measured between 8 h and 28 h after intravenous injection of labeled HDL was 8.5 $\pm$ 0.5 h for HDL apo A-IV and 10.2 $\pm$ 0.7 h for HDL apo A-I.

The tissue sites of catabolism of HDL apo A-IV and HDL apo A-I were analyzed in the "leupeptin-model". Only the kidneys and the liver showed a significant leupeptin-dependent accumulation of radioactivity. 4 h after injection of ^{125}I-apo A-IV/^{131}I-apo A-I labeled HDL, 3.5$\pm$1.0% and 8.4$\pm$2.0% of HDL apo A-IV and 4.6$\pm$1.3% and 2.6$\pm$0.6% of the HDL apo A-I were accumulated in a leupeptin-dependent process in kidneys and liver, respectively.

Immunocytochemical studies revealed that the renal localization of apo A-IV was intracellular and confined to the epithelial cells of the proximal tubuli. The amount of intracellularly apo A-IV in rat kidneys was increased in leupeptin-treated animals.

The data suggest that the leupeptin-dependent degradation of HDL apo A-IV is more active in the liver than in the kidneys, while the opposite is observed for HDL apo A-I. These results, as well as the short half-life of HDL apo A-IV, as compared to HDL apo A-I, are compatible with the existence of an apo A-IV-containing HDL subfraction with a relatively fast turnover for which the liver is the major catabolic site.

INTRODUCTION

Considerable evidence in favor of a positive correlation between low levels of high density lipoprotein (HDL)-cholesterol and an increased risk of coronary heart disease and atherosclerosis has been obtained from epidemiological studies (158, 159). However, little is known about the exact physiological function of HDL. Glomset (79) postulated in 1968 that HDL may serve to transport cholesterol from the extrahepatic tissues to the liver ("reverse cholesterol transport"). Since then, several studies showed that in the rat HDL can deliver cholesterol(ester) to the liver and to organs which are involved in the synthesis of steroid hormones (80-83).

Serum apolipoprotein (apo) A-IV is found primarily in HDL and in a "free" apo A-IV fraction (133, 138, 145, 146). Turnover studies in the rat showed that the half-life of iodinated HDL-bound apo A-IV in the circulation is not different from that of total iodinated HDL (133). This study suggested that, upon leaving the lymph compartment and entering the plasma compartment, apo A-IV was transferred to the HDL fraction by way of the "lipoprotein-free" fraction. It has been shown that this transfer of apo A-IV from the "free" fraction to HDL is due to the esterification of free cholesterol by lecithin:cholesterol acyl transferase (144).

Recent studies on the catabolic sites of other HDL apoproteins, apo A-I and apo E, showed that the kidneys play an important role in the degradation of apo A-I (85, 157), as well as apo E (85, 160). In the present paper we studied the degradation sites of HDL apo A-IV, using leupeptin-treated rats (85). Our study shows that the kidneys contribute significantly to the degradation of HDL apo A-IV, although the liver is the major catabolic site.

METHODS

Materials

Sodium [^{125}I]iodine (carrier-free, 350-600 mCi/ml) and sodium [^{131}I]iodine (carrier-free, 40 mCi/ml) were obtained from Amersham International, Amersham, Bucks., Great Britain. Leupeptin was obtained from the Peptide Institute, Osaka, Japan. 6% Agarose was obtained from Bio Rad, Richmond, Va., U.S.A., swine anti-rabbit serum immunoglobulin, conjugated to horse-radish peroxidase was purchased from Dako, Glostrup, Denmark. Rat serum albumin and diaminobenzidine were obtained from Sigma Chemicals, St. Louis, Mo., U.S.A.

Treatment of rats

Male Wistar rats, weighing 350-400 g, maintained on standard laboratory chow and tap water, were used. Where indicated the food was withheld for 20 h. Blood was collected from the animals by aortic puncture under light ether anaesthesia. The blood was kept on ice for 2 h and serum was obtained by low speed centrifugation at 4°C.

Isolation and iodination of apo A-IV and apo A-I

Apo A-IV and apo A-I were isolated from rat serum HDL as described previously (143). After isolation, the pure apolipoprotein fractions were dialysed extensively against 0.05 M Na-phosphate pH 7.8 and stored at -80°C. The purity of apo A-IV and apo A-I was checked by gelelectrophoresis on 12.5% polyacrylamide, containing 0.1% SDS, as described by Cleveland et al. (154).

Radioiodination of apolipoproteins was carried out using the ICl method (161). In short, 0.03 mg of apo A-I or apo A-IV, in a volume of 0.03 ml, was mixed with 0.02 ml 0.1 M glycine-NaOH buffer (pH 10.0), 0.01 ml ^{125}I or ^{131}I (0.5 mCi) and 0.005 ml ICl solution in 2 M NaCl (3.1 nmole or 3.8 nmole for the iodination of apo A-IV and apo A-I, respectively), followed by incubation at room temperature for 5 min. Unreacted ^{125}I or ^{131}I was removed by chromatography on Sephadex G-50 (medium), equilibrated with 0.15 M NaCl, containing 2 mM Na-phosphate (pH 7.4) and 5 mg/ml of

defatted bovine serum albumin. The efficiency of the iodination was 10-20% and the iodine/protein ratio was less than 1 gatom iodine per gmol of protein.

Labeling and separation of lipoproteins by column chromatography

The ^{125}I- and ^{131}I-labeled apolipoproteins were mixed with 2 ml of non-fasted rat serum and incubated for 1 h at 4°C. After addition of sucrose to a final concentration of 10% (w/v), the sample was applied to a 6% agarose column (1.5 x 50 cm) and eluted with 0.15 M NaCl, containing 2 mM Na-phoshpate (pH 7.4), 0.01% NaN_3 and 1 mM EDTA. The flow rate was 3-4 ml/h and fractions of 1 ml were collected. The column was operated at 4°C. Absorbance at 280 nm, ^{125}I- and ^{131}I-radioactivity and total cholesterol were measured in each fraction. HDL fractions, containing ^{125}I-apo A-IV and ^{131}I-apo A-I, were pooled and dialyzed for 18-24 h against Krebs-Henseleit buffer (162). All preparations were immediately used for metabolic studies.

Metabolic studies

After the chow-fed, non-fasted animals were anesthesized with diethylether, one ml of ^{125}I-apo A-IV / ^{131}I-apo A-I-labeled HDL was injected through a femoral vein. Leupeptin was administered intravenously, as described by Van 't Hooft et al.(85), 1 h before the injection of labeled HDL. The rats used for the 4 h decay time point received a second leupeptin injection 2 1/2 h after the first one. Leupeptin was injected in a concentration of 20 mg/kg body weight. All control animals were handled identically, but they received injections of saline instead of leupeptin. At the end of the experiments, the rats were bled from the abdominal aorta. The heart, liver, spleen, kidneys, testes, adrenals and representative sections of muscle, adipose tissue, lung, skin and jejunum were removed. Calculations of the radioactivity present in the organs were made as described previously (85). The radioactivity in serum was measured in 0.25 ml samples and the results were expressed as percentage of the injected radiolabel, assuming a plasma volume of 3.36-3.51% of body weight, dependent on body weight (163).

For "screening", double-labeled HDL was injected in the femoral vein of chow-fed animals. The blood was collected after 1 h and kept on ice for 2 h. The serum was isolated and used directly for the in vivo experiments.

The label and mass distribution of apo A-IV and apo A-I were measured in serum of rats injected with labeled HDL. Serum obtained from control or leupeptin-treated animals was subjected to 6% agarose columnchromatography (column size 2 x 120 cm) to separate the different apo A-IV containing lipoprotein fractions (138). In all samples ^{125}I- and ^{131}I-radioactivity (recoveries 88±3% and 85±5% , respectively), apo A-IV and apo A-I mass (recoveries 99±1% and 98±4%, respectively) and total cholesterol (recovery 96±4%) were determined.

Immunocytochemical methods

The immunocytochemical localization of apo A-IV in rat kidneys was performed as described by Van Ewijk et al. (164). After the kidneys had been perfused in situ with 0.9% NaCl, containing 1 mM EDTA (pH 7.4) at 37°C, they were removed from leupeptin-treated and control rats and embedded immediately in Tissue-tek on specimen stubs frozen on solid carbon dioxide. Frozen sections (5 µ) were cut on a cryostat (Bright Ltd., Huntingdon, Great Britain) and were collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium-potassium sulphate. The tissue was gently fixed by dipping the slide for 1 sec in acetone. These sections were stored at -20°C. All subsequent procedures were performed at room temperature. Before being incubated with antisera, the sections were soaked in 0.9% NaCl (containing 10 mM Na-phosphate (pH 7.4), 5% bovine serum albumin (PBS-BSA) and 0.05% Tween-20) for 30 min in order to remove the embedding medium. Next, the sections were covered with 50 µl of the antibody (1:300 dilution) and incubated for 1 h in a moist chamber. After rinsing with PBS-BSA buffer, the sections were incubated for 1 h with 50 µl swine anti-rabbit serum immunoglobulin conjugated to horse-radish peroxidase (1:40 dilution, containing 1% rat serum). After rinsing in PBS-BSA, the conjugate was visualized by incubation of the sections in diaminobenzidine

(DAB) according to Graham et al. (165). Sections were subsequently incubated in a solution of 0.5% chromium sulphate in 0.9% NaCl in order to improve the staining. After dehydration and fixation, the sections were coverslipped and examined with a microscope.

Chemical analysis

Total cholesterol was measured enzymatically using the CHOD-PAP kit (Boehringer, Mannheim, F.R.G., Cat.No. 310328). Apo A-I and apo A-IV were determined by electroimmunoassay, as described previously (143). Iodine radioactivity was counted in the LKB-Wallac ultrogamma counter (LKB-Wallac, Turku, Finland). The data collected in this study was statistically evaluated using Student's unpaired t-test. Values given are means ± S.D. for 3-6 experiments.

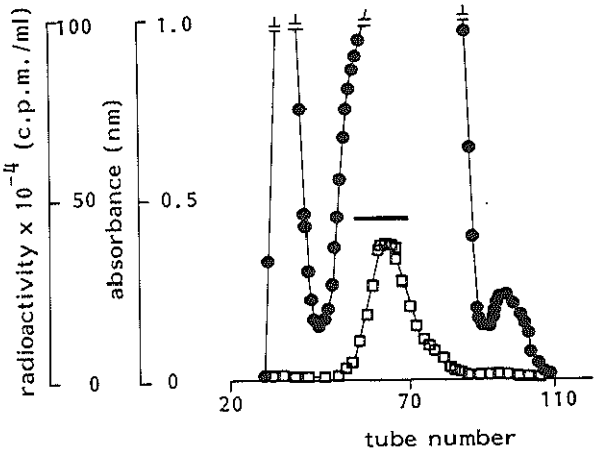


Fig. 6.1. Molecular sieve chromatography of ^{125}I-apo A-IV-labeled serum lipoproteins from rats fed standard laboratory chow.
^{125}I-apo A-IV was incubated with 2 ml of rat serum for 1 h at 4°. The serum lipoproteins were immediately separated on a 6% agarose column (size 1.5 x 50 cm). The absorbance at 280 nm (●), and the distribution ^{125}I-apo A-IV radioactivity (○) are shown. ⟷ indicates the fractions containing 90% of total HDL-cholesterol.

RESULTS

Radioiodinated apo A-IV and apo A-I were incorporated into rat serum lipoproteins and the different lipoprotein fractions were separated on a 6% agarose column. Figure 6.1. shows the in vitro incorporation of ^{125}I-apo A-IV into serum lipoproteins, using a method described for the labelling of rat apo A-I and apo E (85, 160). More than 80% of the label was on particles with the size of typical HDL. Fractions containing the highest level of radioactivity and 90% of total HDL cholesterol (indicated by the horizontal bar) were pooled and immediately used for metabolic studies. In all studies double-labeled apo A-IV/apo A-I HDL preparations were used, containing ^{125}I-apo A-IV and ^{131}I-apo A-I or vice versa.

In control experiments we tested whether the labeled HDL apolipoproteins remained associated with the HDL fraction under various in vivo and in vitro conditions (see below). After re-chromatography of the HDL, labeled apo A-IV and apo A-I remained quantitatively associated with the HDL fraction (not shown). Figure 6.2.A. shows the elution pattern and the distribution of radioactivity on a 6% agarose column of pooled sera obtained from two saline-treated rats killed 4 h after injection of HDL labeled with ^{125}I-apo A-IV and ^{131}I-apo A-I. Figure 6.2.B shows the mass distributions of apo A-IV and apo A-I in the same experiment. In rat serum, apo A-IV was present in HDL as well as in a "free" apo A-IV fraction. In this "free" fraction, 33±3% of the mass of apo A-IV was present, but ^{125}I-apo A-IV could not be detected. The radioactive apolipoproteins were present on HDL particles with the same size as the nonradioactive apolipoproteins. Apo A-IV was present on slightly bigger particles than apo A-I in all sera tested. Serum obtained 3 min or 1 h after injection of the labeled HDL gave essentially the same results as those shown in Figure 6.2. at higher levels of HDL radioactivity. These data suggest that ^{125}I-apo A-IV, once associated with HDL, will not exchange or transfer to other lipoprotein classes, including the "free" apo A-IV fraction, but will remain an integral part of HDL. In another experiment, HDL labeled with ^{125}I-apo A-IV/

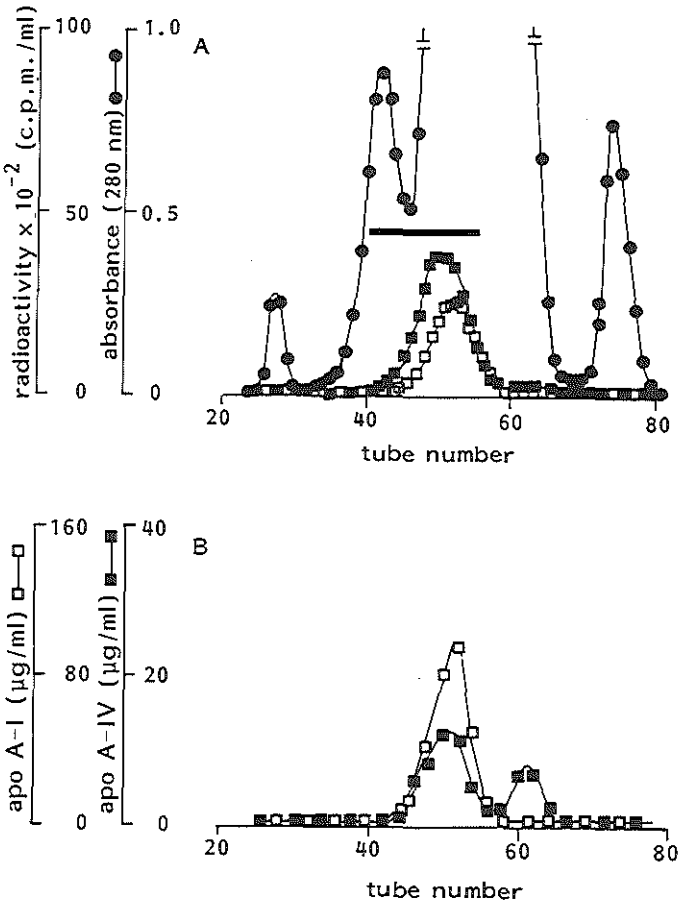


Fig. 6.2. Molecular sieve chromatography on 6% agarose (column size 2 x 120 cm) of serum obtained from saline-treated control rats killed 4 h after the injection of ^{125}I-apo A-IV and ^{131}-I apo A-I HDL.
In Fig. 6.2.A. the absorbance of serum proteins at 280 nm (●) and the distribution of ^{125}I-apo A-IV (■) and ^{131}I-apo A-I (□) label is shown. In Fig. 6.2.B. the distribution of apo A-IV (■) and apo A-I (□) mass, as determined by electroimmunoassay, is given. The second apo A-IV peak has a slightly higher elution volume than ^{125}I-albumin, as was determined in separate experiments (see also ref. 138). This Figure shows a representative experiment out of a series of 6.

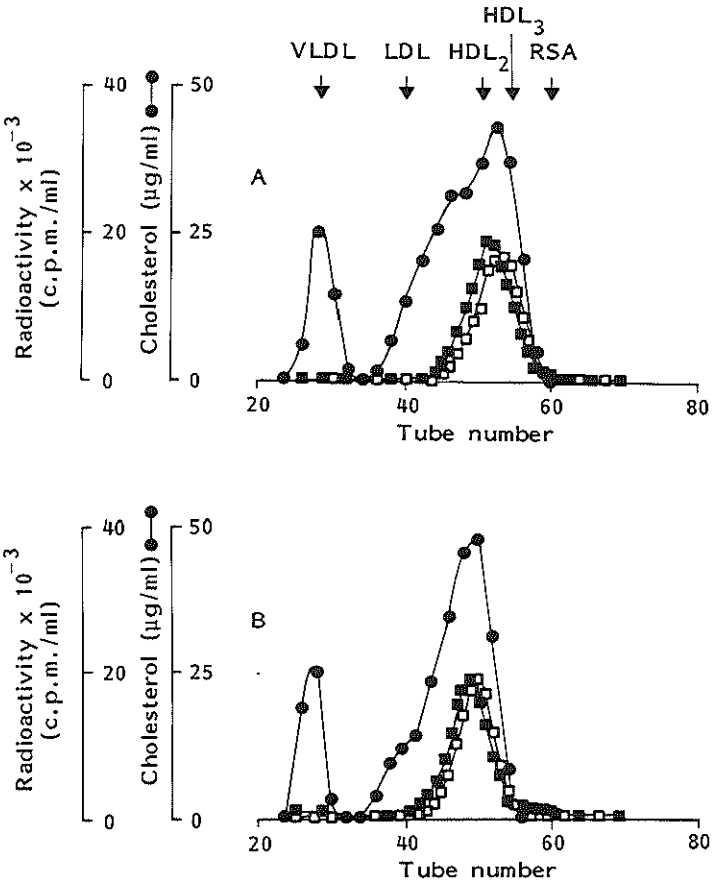


Fig. 6.3. In vitro incubation of ^{125}I-apo A-IV/^{131}I-apo A-I HDL with serum obtained from chow-fed rats followed by chromatograhpy on 6% agarose (column size 2 x 120 cm).
The distribution of ^{125}I-apo A-IV (■), ^{131}I-apo A-I (□) and total cholesterol (●) are shown after 0 h (A) and 4 h (B) of incubation at 37°. VLDL, LDL, HDL_2, HDL_3 and RSA indicate the elution volumes of isolated human lipoproteins and rat serum albumin, which were chromatographed in separate experiments. VLDL and LDL were isolated by sequential ultracentrifugation (2) and washed once. Human HDL_2 and HDL_3 were isolated by rate zonal density gradient ultracentrifugation in a swinging bucket rotor, as described by Groot et al. (56).

^{131}I-apo A-I was incubated with rat serum at 37°C for various periods of time (up to 28 h) and the distribution of radioactivity was subsequently analyzed on 6% agarose columns. Under all conditions ^{125}I-apo A-IV remained quantitativly associated with the HDL fraction (e.g. Fig. 6.3.). In agreement with studies of Delamatre et al. (144), the mass of "free" apo A-IV decreased during in vitro incubation and apo A-IV in the HDL fraction increased.

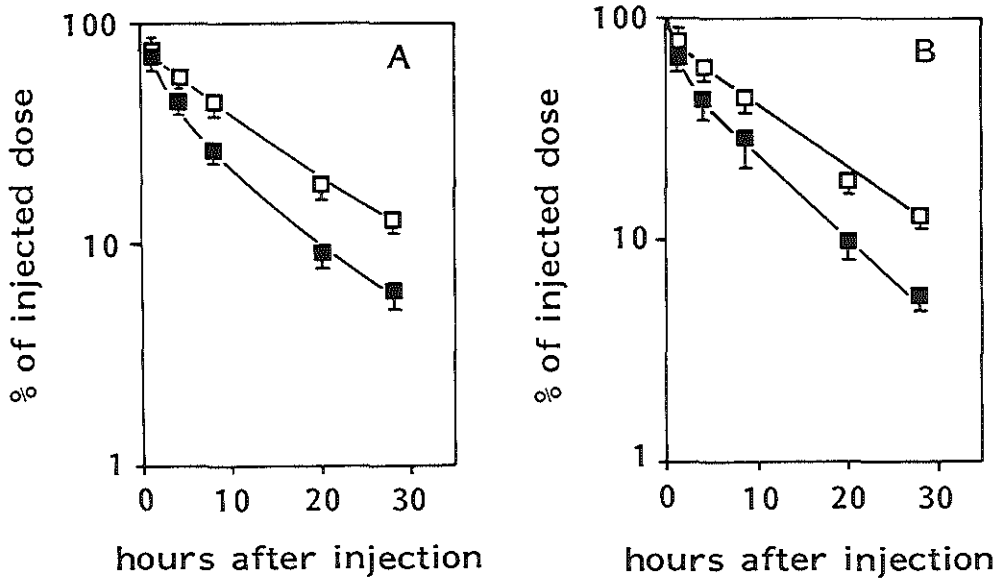


Fig. 6.4. Removal of ^{125}I-apo A-IV HDL (■) and ^{131}I-apo A-I HDL (□) from the serum of chow fed (A) and fasted rats (B).
The isolation of labeled rat HDL is described in "Methods". The HDL preparations were injected intravenously, blood was collected at the indicated timepoints and serum was obtained by low speed ultracentrifugation. All values given, except those measured 1 h after injection, show a statistically significant difference between ^{125}I-apo A-IV and ^{131}I-apo A-I; $P < 0.005$. The graphically determined serum half lifes, measured between 8 and 28 h after intravenous injection of double labeled HDL, were also different ($P < 0.01$) for the two apolipoproteins. Results are means ± S.D. for 4 experiments.

Figure 6.4. shows the serum decay curves of ^{125}I-apo A-IV/^{131}I apo A-I labeled HDL measured over a period of 28 h. In agreement with previously published data (85, 157) the half-life of labeled HDL-apo A-I, measured between 8-28 h after injection of the labeled HDL, was 10.2 $\pm$ 0.7 h. The serum decay of ^{125}I-apo A-IV was faster than the serum decay of ^{131}I-apo A-I. The half-life of ^{125}I-apo A-IV, measured between 8 and 28 h after injection was 8.5 $\pm$ 0.5 h. In vivo "screening" of the labeled HDL had no effect on the serum decay curves. Most experiments were performed in chow-fed rats but, as shown in Figure 6.4.B., no changes in the serum decay of radioactive apo A-IV or apo A-I were induced by fasting the animals for 20 h.

The tissue sites of degradation of HDL-apo A-IV were determined in leupeptin-treated rats according to the methodology described by Van 't Hooft et al. (85). Leupeptin is a tripeptide which inhibits the lysosomal degradation of protein, a process which occurs very fast in the absence of the inhibitor. Radioactivity derived from intravenously injected radioactive proteins therefore accumulates in the lysosomes of tissues of leupeptin-treated animals and actively catabolizes these proteins (85). Figure 6.5. shows that leupeptin treatment has no effect on the serum decay of the radioactive HDL apolipoproteins measured over a period of 4 h after injection of the labeled HDL. Furthermore analyses of sera from leupeptin-treated and saline-treated rats revealed that all radioactivity remained associated with HDL (as shown in Figure 6.2. for saline-treated rats). Also, leupeptin treatment did not change the mass distribution of apo A-IV between HDL and the "free" apo A-IV fraction. The results of these and previous studies (85) indicate that leupeptin treatment does not influence the turnover of HDL-apo A-IV and HDL-apo A-I during the 4 h experiments.

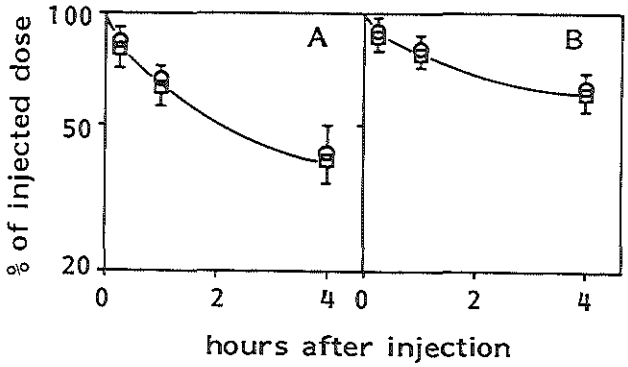


Fig. 6.5. Removal of rat ^{125}I-apo A-IV HDL (A) and rat ^{131}I-apo A-I HDL (B) from the serum of control (□)and leupeptin-treated (○) animals.
The isolation of radioiodinated rat HDL and the treatment of rats are described in "Methods". The labeled HDL preparations were injected intravenously, blood was collected at the indicated time-points and serum was obtained by low speed centrifugation. Results are means ± S.D. for 5 experiments. The values measured 4 h after injection show a statistically significant difference between ^{125}I-apo A-IV (A) and ^{131}I-apo A-I (B); $P < 0.01$.

The tissue radioactivities derived from the ^{125}I-apo A-IV/^{131}I-apo A-I labeled HDL were analyzed in eleven tissues. At different time points after injection of ^{125}I-apo A-IV/^{131}I-apo A-I labeled HDL, the percentage of injected radioactivity present in each tissue was determined. The difference between the tissue radioactivity of saline-treated and leupeptin-treated rats is called the "leupeptin-dependent accumulation" of radioactive apo A-IV or apo A-I. Only the liver and the kidneys showed a significant leupeptin-dependent accumulation of radioactivity (see Figures 6.6. and 6.7.) indicating that these tissues are sites of degradation of HDL apo A-IV and HDL apo A-I. The liver seems to be relatively more important in the uptake and degradation of apo A-IV than in the catabolism of apo A-I. Comparable results were obtained when the double-labeled HDL was "screened" before the experiments.

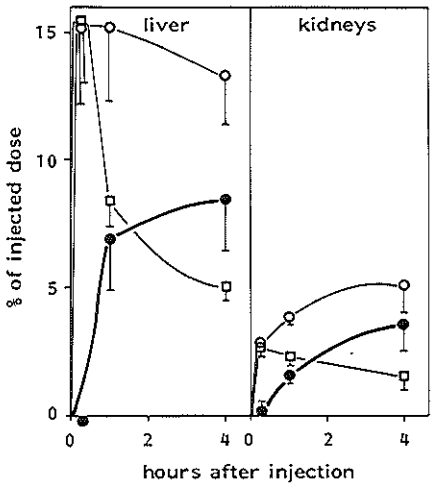


Fig. 6.6. Tissue accumulations of intravenously injected ^{125}I-apo A-IV HDL in the liver and the kidneys of control (□) and leupeptin-treated (○) rats. The isolation of ^{125}I-apo A-IV HDL and the treatment of rats are described in "Methods". At different time-intervals after intravenous injection of labeled HDL, blood was collected and a number of tissues, among others the liver and the kidneys, were excised, weighed and counted for radioiodine. The leupeptin-dependent accumulation (●) in each tissue at each individual time point was calculated as described in "Methods". Values are means ± S.D. for 3 experiments. The leupeptin-dependent accumulations measured 1 h and 4 h after injection showed a statistically significant difference between liver and kidneys; $P < 0.01$.

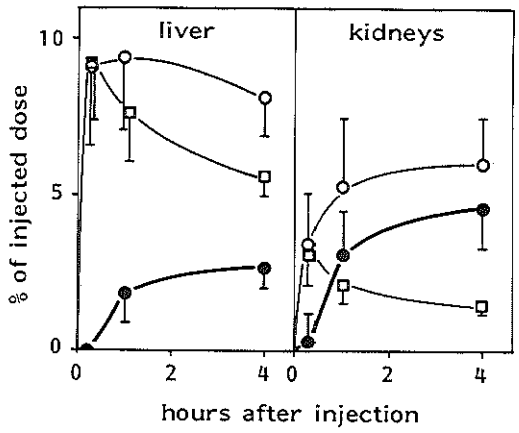


Fig. 6.7. Tissue accumulation of intravenously injected ^{131}I-apo A-I HDL in the liver and the kidneys of control (□) and leupeptin-treated (○) rats. The isolation of ^{131}I-apo A-I HDL and the treatment of rats are described in "Methods". The experiment was performed as in Figure 6.6. The leupeptin-dependent accumulation (●) was calculated as described in "Methods". Values are means ± S.D. for 3 experiments. The leupeptin-dependent accumulation measured 4 h after injection shows a statistically significant difference between liver and kidneys; $P < 0.01$.

Evidence for the intracellular renal localization of apo A-IV was also obtained from immunocytochemical studies by using the horse-radish technique. Figure 6.8.A. shows that apo A-IV is located in the cells of the proximal tubuli of leupeptin-treated rats. No intracellular stain can be detected in cells of the glomeruli. Sections treated with nonimmune rabbit IgG exhibited no staining under conditions identical to those used for visualization and photography of sections treated with anti-rat apo A-IV (see figure 6.8.C.). Kidneys from leupeptin-treated rats have an increased intensity of brightly staining granules in the epithelial cells of proximal tubuli (probably reflecting apolipoprotein accumulated in the lysosomes) when compared with kidneys from saline-treated control rats (Figure 6.8.B.). The kidney sections were also analyzed for apo A-I and apo E with the same technique. Essentially, the same localization was observed for all three HDL apolipoproteins. Also, in the case of apo E and apo A-I the intensity of stain was the highest in leupeptin-treated animals. Minimal staining was present in the lumen of the proximal tubuli.

DISCUSSION

The method of labeling used in the present study opens the possibility of carrying out metabolic studies of HDL specifically labeled in apo A-IV, without disturbing its physiological composition. Using ultracentrifugation, a significant part of the HDL apo A-IV, apo A-I and apo E will be "stripped" and recovered in the fraction d > 1.21 g/ml (87, 88, 138). These changes may result in an altered in vivo metabolic behavior in the rat (152). A study of Funke et al. (166), using canine apo E HDL_C isolated either by ultracentifugation or by column chromatography did not show any difference in metabolic behavior.

In the present work, we intended to study the degradation sites of HDL-apo A-IV. In rat serum, apo A-IV is present on HDL as well as on a particle called "free" apo A-IV, which is smaller than HDL (133, 138, 145, 146). In agreement with our earlier observations (138), it was found that about 1/3 of serum apo A-IV is present as "free" apo A-IV in serum obtained from chow-fed rats. It was therefore important to be sure that no label was present in the "free" apo A-IV fraction. Rechromatography of the labeled lipoproteins, incubations of labeled HDL in vitro with rat serum, or intravenous injection showed that ^{125}I-apo A-IV remained associated with the HDL particle. Analysis of the distribution of ^{125}I-apo A-IV in serum during our in vivo experiments revealed that no label was present in the "free" apo A-IV fraction up to 4 h after injection of labeled HDL-apo A-IV. This indicates that either apo A-IV in HDL is not a precursor of "free" apo A-IV or that the degradation of "free" apo A-IV derived from HDL is very rapid compared to that of HDL-apo A-IV, so that the label cannot be detected in the "free" apo A-IV fraction. The latter possibility seems unlikely since intravenously injected free ^{125}I-apo A-IV showed exactly the same initial rate of disappearance from serum as injected HDL-^{125}I-apo A-IV.

In separate experiments (not shown) it was found that 80 $\pm$ 6% (n=3) and 80 $\pm$ 8% (n=5) of the injected radioactivity of these two preparations respectively, was still present in serum if measured 15 min after injection. No label was present in

fractions containing "free" apo A-IV after fractionation of the sera by gel filtration. In vitro incubations of the HDL preparation labeled by in vitro incorporation of ^{125}I-apo A-IV and ^{131}I-apo A-I again showed that the labels on HDL are very stable (see Figure 6.3.).

Because ^{125}I-apo A-IV remains associated with the HDL particle, it was possible to study the in vivo metabolism of HDL apo A-IV and to compare it with the metabolic behavior of apo A-I, the major apoprotein of rat HDL. As shown in Figure 6.4., the serum decay of HDL apo A-IV was faster than the serum decay of HDL apo A-I. This observation seems to be at variance with data published by Fidge et al. (133), who could not detect any difference in serum tunorver of HDL apo A-IV and rat whole HDL, labeled mainly in apo A-I. It must be stressed that in the present study the turnover of HDL apo A-IV and HDL apo A-I was compared directly by a double labeling approach in order to minimize the effects of other experimental conditions. Also, "screening" of the lipoprotein preparations prior to the metabolic studies did not influence the results. An explanation for the faster rate of metabolism of HDL apo A-IV could be related to the presence of small amounts of triglyceride-rich lipoproteins in serum of non-fasted rats. If there was any exchange of apoA-IV between HDL and triglyceride-rich lipoproteins, this exchange could increase the serum decay of HDL apo A-IV. However, ^{125}I-apo A-IV was not found to be associated under any conditions with the triglyceride-rich lipoprotein fractions. Moreover, the serum removal rate of HDL apo A-IV was identical in 20 h fasted and non-fasted rats. These studies therefore strongly suggest that under our experimental conditions the removal rate of HDL apo A-IV from rat serum is faster than that of HDL-apo A-I, a situation comparable to that in humans (167, 168).

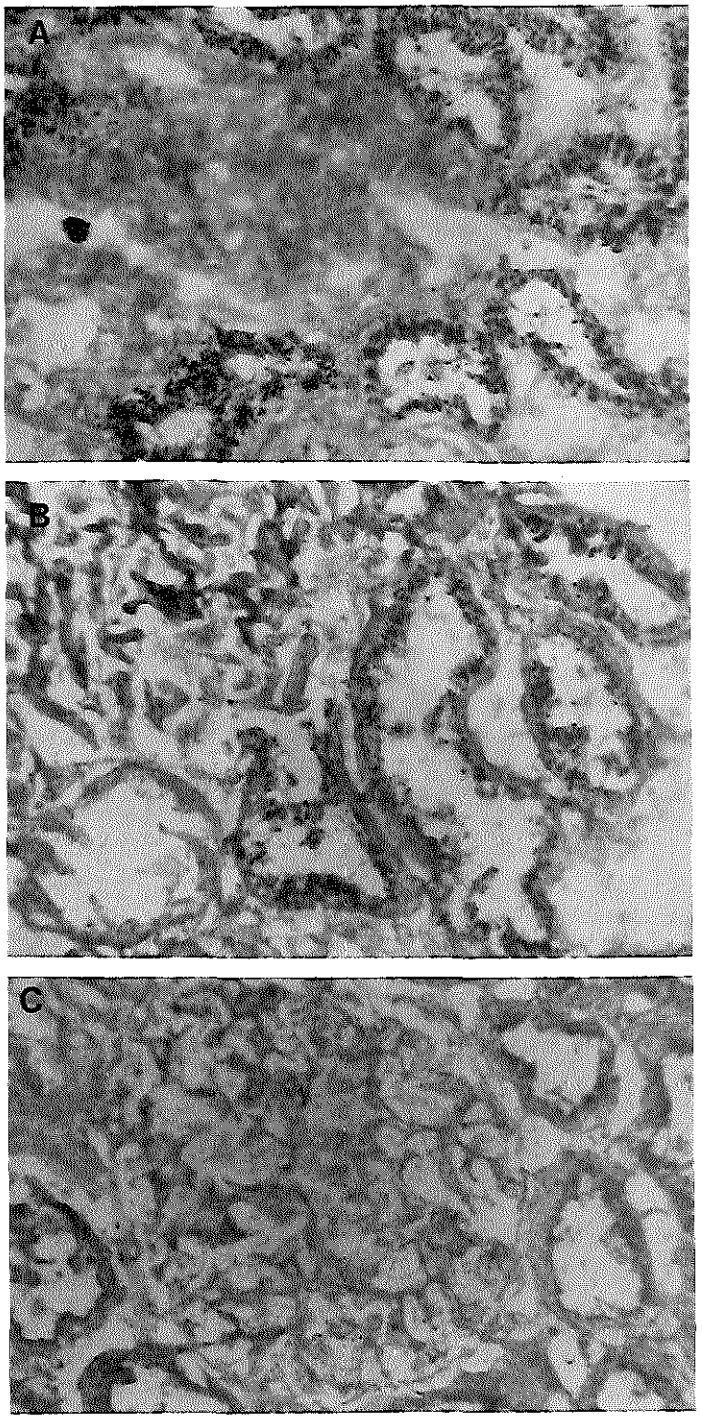
The tissue sites of degradation of HDL apo A-IV were determined using leupeptin-treated rats, as described previously (85). Treatment of rats with leupeptin delays the protein degradation in the lysosomes, causing accumulation of intravenously injected labeled apolipoproteins in those tissues normally involved in the degradation of these proteins. Leupeptin treatment has no effect

on serum levels of lipids and apolipoproteins (85) nor does it influence the serum decay of HDL apolipoproteins measured over a period of 4 h (Figure 6.5.).

Recent studies on the degradation sites of apo A-I either using leupeptin-treated rats (85) or the ^{125}I-tyramine cellobiose label (157), showed that the rat kidneys play an important role in the degradation of HDL apo A-I. Also in dogs (169) and in humans (170, 171) the kidneys are involved in the degradation of plasma HDL. Results of the present study do confirm the role of the rat kidneys in the degradation of HDL apolipoproteins, and extend this role to apo A-IV. The mechanism for the uptake of HDL apolipoproteins by the kidneys is not yet completely understood. Studies on serum HDL cholesteryl ester metabolism have shown that very little is taken up by the kidneys in vivo (81-83), leading to the conclusion that at least part of the HDL apo A-I may be cleared from the circulation separately from the HDL cholesteryl esters.

It was proposed by Glass et al. (157) that a small fraction of "free" apo A-I is rapidly filtered by the glomeruli, followed by endocytosis and degradation of the filtered apo A-I in the epithelial cells of the proximal tubuli. The results of studies on the renal catabolism of HDL in the isolated perfused rat kidney (172) and in microperfused rabbit proximal straight nephron segments (173) are consistent with this hypothesis. The immunochemical localization studies described in this paper show that apo A-IV, as well as apo A-I and apo E (not shown), are present in granules within the cells of the proximal tubuli. Almost no staining was observed in the lumen or on the brushborder membrane of the tubular cells or in the glomeruli. Leupeptin injection resulted in a specific increase of granular staining inside the cells of the tubuli. All these findings support the idea that the degradation of apo A-IV in the kidneys occurs in the lysosomes of the epithelial cells of the promixal tubuli, a conclusion reached earlier for apo A-1 (157).

Fig. 6.8. Localization of apo A-IV in kidneys of leupeptin-treated rats (A) and saline-treated rats (B) by immunocytochemistry (immunoperoxidase staining) as described in "Methods" using rabbit anti-rat apo A-IV (A and B). A control experiment, using non immune rabbit IgG, is shown in C. In all photographs various tubuli can be seen surrounding one glomerulus (x 400).


A
B
C

The results of the present study also suggest an important role of the liver in the uptake and degradation of HDL apo A-IV. this could indicate a role of HDL apo A-IV in "reverse cholesterol transport", as suggested by Delamatre et al. (145). We showed recently (138) that part of the HDL apo A-IV is present on particles which do not contain apo A-I. These apo A-IV particles could have a relatively rapid turnover and be specificaly degraded in the liver. If this hypothesis is true, it would also explain the relatively rapid serum removal rate of apo A-IV, compared with apo A-I (see figure 6.4.). Detailed studies are needed in order to clarify the metabolism of the different apo A-IV containing subfractions and their relationship with cholesterol metabolism.

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CHAPTER 7

IDENTIFICATION AND CHARACTERIZATION OF RAT HIGH DENSITY LIPOPROTEIN SUBCLASSES. ISOLATION BY CHROMATOGRAPHY ON AGAROSE COLUMNS AND SEQUENTIAL IMMUNOPRECIPITATION.

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Journal of Lipid Research, submitted.

SUMMARY

High density lipoproteins (HDL), present in serum of chow-fed rats, were fractionated according to size by chromatography of serum on 6% agarose columns. The distributions of apolipoprotein (apo) A-I, E and A-IV within the HDL size range showed the existence of HDL subclasses with different size and chemical composition. Sequential immunoprecipitations were performed on these HDL fractions, obtained by agarose column chromatography, using specific antisera against apo A-I, apo E and apo A-IV. The resulting precipitates and supernatants were analyzed for cholesteryl esters, unesterified cholesterol, phospholipids, triglycerides and specific apolipoproteins. The following conclusions are drawn from these experiments:

1. 63 ± 3% of the apo E in total HDL is present on a big HDL particle (mol wt 750,000). This apo E-HDL contains apo E as its sole protein constituent. 39 ± 1% of the HDL cholesterol is found in this fraction. The cholesterol:phospholipid ratio is 1:1,1.
2. 69 ± 8% of the apo A-I in total HDL is present on a smaller HDL particle (mol wt 250,000). This apo A-I-HDL has apo A-I as its major protein component and possibly contains minor amounts of apo C's, but neither apo E nor apo A-IV. It contains 39 ± 8% of the total HDL cholesterol and the cholesterol:phospholipid ratio is 1:1,6.
3. 21 ± 8% of the apo A-IV in total HDL is present on an HDL particle (mol wt approximately 250,000), which does not contain any apo E or apo A-I and only 4 ± 1% of the total HDL cholesterol.
4. The remainder of the HDL apolipoprotein and of the total HDL cholesterol is mostly present on two complex lipoproteins: HDL containing both apo A-I and apo A-IV and HDL containing both apo A-I and apo E.

INTRODUCTION

Classification of different serum lipoprotein classes according to their apolipoprotein composition has been described by Alaupovic (174). A distinction was made between socalled simple lipoproteins, containing only one apolipoprotein (apo), and complex lipoproteins, containing more than one apolipoprotein. According to this classification, human HDL can be divided into one simple lipoprotein (Lp A-I), containing only apo A-I, and a complex lipoprotein particle Lp (A-I with A-II), containing both apo A-I and apo A-II (174-178).

Rat HDL subclasses have been described by Weisgraber, Mahley and Assmann (59). Using ultracentrifugation and Geon-Pevikon block electrophoresis, they isolated an HDL fraction (HDL_1), with apo E as its major apolipoprotein, from normal rat serum and a similar HDL fraction (HDL_c) from serum of cholesterol-fed rats. These fractions, however, still contain small amounts of apo A-I. Rat HDL subfractions were also separated by Heparin-Sepharose column chromatography (179). Two HDL subfractions can be obtained using this method: HDL enriched in apo E and HDL enriched in apo A-I.

Evidence for the existence of different HDL subfractions also comes from experiments using agarose column chromatography. If rat serum lipoproteins were separated according to size it was found that the elution profiles of apo E and apo A-I were not identical, as apo E was eluted ahead of apo A-I in the HDL size range (138, 151). This suggests that apo E is enriched on the bigger HDL particles. In order to analyze the different apo A-IV containing fractions in the HDL size range, we recently used specific immunoprecipitations (138). This technique provided evidence for the existence of three types of HDL particles of relatively small size: HDL containing apo A-IV, HDL containing apo A-I and a more complex HDL particle, containing both apo A-I and apo A-IV; none of these three subclasses contained appreciable amounts of apo E. We now report the identification and chemical composition of five distinct rat HDL subclasses, isolated directly from rat serum using 6% agarose column chromato-

graphy and characterized by sequential immunoprecipitation with specific antibodies against apo A-I, apo E and apo A-IV. A major finding is that almost 40% of the HDL cholesterol present in serum from chow-fed rats is carried by a big HDL, containing apo E as its sole protein constituent.

MATERIALS AND METHODS

Isolation of serum HDL

Male Wistar rats, weighing 300-350 g, were maintained on standard laboratory chow and tap water. Blood was collected at 9 AM from fed animals by aortic puncture under light ether anesthesia. The blood was kept on ice for 2 h. Serum was obtained by low speed centrifugation at 4°C and EDTA solution (pH 7.4) was added to a final concentration of 1 mM. Ten ml of pooled serum from 2 rats, containing 10% sucrose (w/v), was applied on a 6% agarose column (2 x 120 cm, Bio Rad, Richmond, USA), equilibrated and eluted with 0.15 M NaCl, containing 2 mM Na-phosphate buffer (pH 7.4), 0.01% NaN_3 and 1 mM EDTA (138). The column was operated at 4°C. Compared to a previous publication (138), we changed the column size and the flow rate. The presently used columns have a diameter of 2 cm, compared to 2,5 cm of the previously used columns. The flow rate applied now is 10 ml/h (previously 15 ml/h). The length of the column still is 120 cm. These changes clearly resulted in an improved separation of high density lipoprotein subclasses present in rat serum.

Standardization of the columns

Several markers were used in order to standardize the agarose columns. Iodinated rat serum albumine, rat serum VLDL and HDL, human LDL, HDL_2 and HDL_3 were used for calibration as shown in Fig. 7.2. These lipoproteins were isolated by way of ultracentrifugation in salt solutions of the appropriate densities. In order to estimate the size of the different lipoprotein fractions, the column was also standardized using a gel filtration standard kit (Bio Rad) containing proteins with known molecular weights, i.e. thyroglobulin (mol wt 670,000), γ-globulin (mol wt

158,000), ovalbumin (mol wt 44,000), myoglobulin (mol wt 17,000) and vitamin B-12 (mol wt 1,350). When the logarithms of these molecular weights were plotted against ve/vo (elution volume/void volume), a linear relationship was found.

Immunoassays

Apo A-I, apo A-IV and apo E concentrations were determined by electroimmunoassay, exactly as described previously (129, 143). In short, proteins present in sera, immunoprecipitates, supernatants, column fractions and standards were routinely precipitated with TCA in the presence of DOC as a carrier. The resolving protein pellets were solubilized in 0,1 ml of 0,5 M NaOH and delipidated with tetramethyl urea. Twenty mM Tris-HCl (pH 8.3), containing 8 M urea was added before the samples were analyzed by immunoelectrophoresis (143).

Quantitative immunoprecipitation

Rabbit anti-apo A-I, anti-apo A-IV and anti-apo E IgG, prepared as described (143), were used for the quantitative immunoprecipitation of apo A-I, apo E and apo A-IV containing lipoproteins. The HDL fraction obtained from rat serum by agarose column chromatography was divided into two pools. Pool A, with HDL fractions containing apo E as their major protein constituent, eluted between 205 and 235 ml and pool B, with HDL fractions containing apo A-I and apo A-IV as major apoprotein components, eluted between 240 and 274 ml (see Fig. 7.1.). Two step immunoprecipitation studies were carried out. In the first step HDL was subjected to precipitation with a combination of two different antibodies (for details see "Results"). The amount of antibody needed for quantitative precipitation of the apoprotein was determined in a separate experiment. Incubations were carried out overnight at 4°C and the immunoprecipitates were collected by low speed centrifugation at 4°C (30 min at 2700 rpm). The resulting supernatant was treated with the third antibody (see "Results") and a second precipitate was isolated, as described above. Apo A-I, apo E and apo A-IV concentrations were measured both in the two precipitates and in the final supernatant. Reco-

veries, as compared to Pool A or Pool B, were 99 ± 10%, 99 ± 9%, 100 ± 9% for apo A-I, apo A-IV and apo E, respectively. The supernatants and the precipitates were extracted according to Bligh and Dyer (149), followed by determination of cholesterol (esters) and phospholipids in the extract. After evaporation of the chloroform phase under a stream of nitrogen, unesterified cholesterol and total phospholipids (141) were determined. Cholesterol was assayed without further treatment using an enzymatic method (CHOD-PAP kit, Boehringer Mannheim, F.R.G. cat. no. 310328) Cholesteryl esters were saponified with alcoholic KOH and, after extraction with petroleum ether, total cholesterol was determined, using the enzymatic method. Cholesteryl esters were expressed as the calculated difference between total cholesterol and unesterified cholesterol. 101 ± 7% of total cholesterol and 99 ± 4% of the phospholipids present in Pool A or Pool B were recovered in the two precipitates. Apo A-I, apo A-IV, apo E, cholesterol and phospholipid were absent from the final supernatant, after correction for small amounts of cholesterol and phospholipid present in the antibody preparations. Triglycerides measured according to Laurell (142) could not be detected in any of the HDL fractions isolated by agarose column chromatography. This is not surprising as it is known that the triglyceride content of total rat HDL is only 0.5%. The assay used is not sensitive enough to measure these small amounts in HDL subfractions obtained by gel filtration.

Aliquots (25 µl) of the eluted HDL subfractions were subjected to electrophoresis on 12,5% SDS-polyacrylamide gels according to Cleveland et al. (154), in order to determine the presence of low molecular weight apolipoproteins (e.g. apo C's) (Fig. 7.3.).

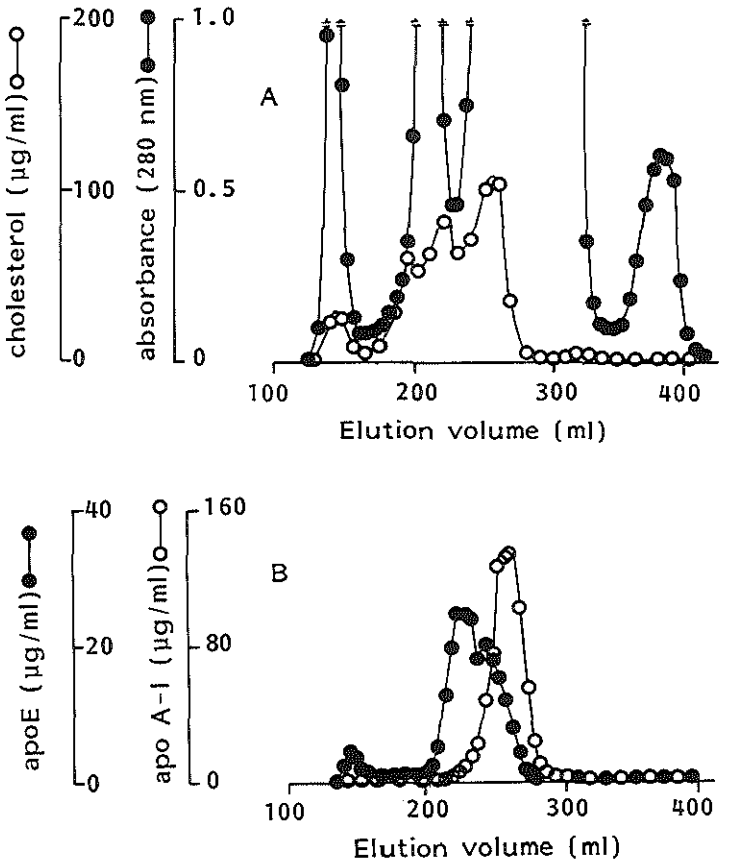


Fig. 7.1. Separation on a column of 6% agarose gel of serum obtained from chow-fed rats. Ten ml of serum were applied to a column of 6% agarose (2 x 120 cm), equilibrated with 0.15 m NaCl, containing 2 mM Na-phosphate pH 7.4, 1 mM EDTA and 0.1% NaN_3, at 4°C. The lipoproteins were eluted with the same medium at a flow rate of 10 ml/hr.

Fig. 7.1.A. shows the absorbance of serum proteins at 280 nm (●) and the distribution of cholesterol (O) in the eluted fractions.

Fig. 7.1.B. shows the elution profiles of apo A-I (O) and apo E (●) mass, as determined by electroimmunoassay.
This figure shows one representative experiment out of a series of four.

RESULTS

Isolation of rat serum lipoproteins by agarose column chromatography

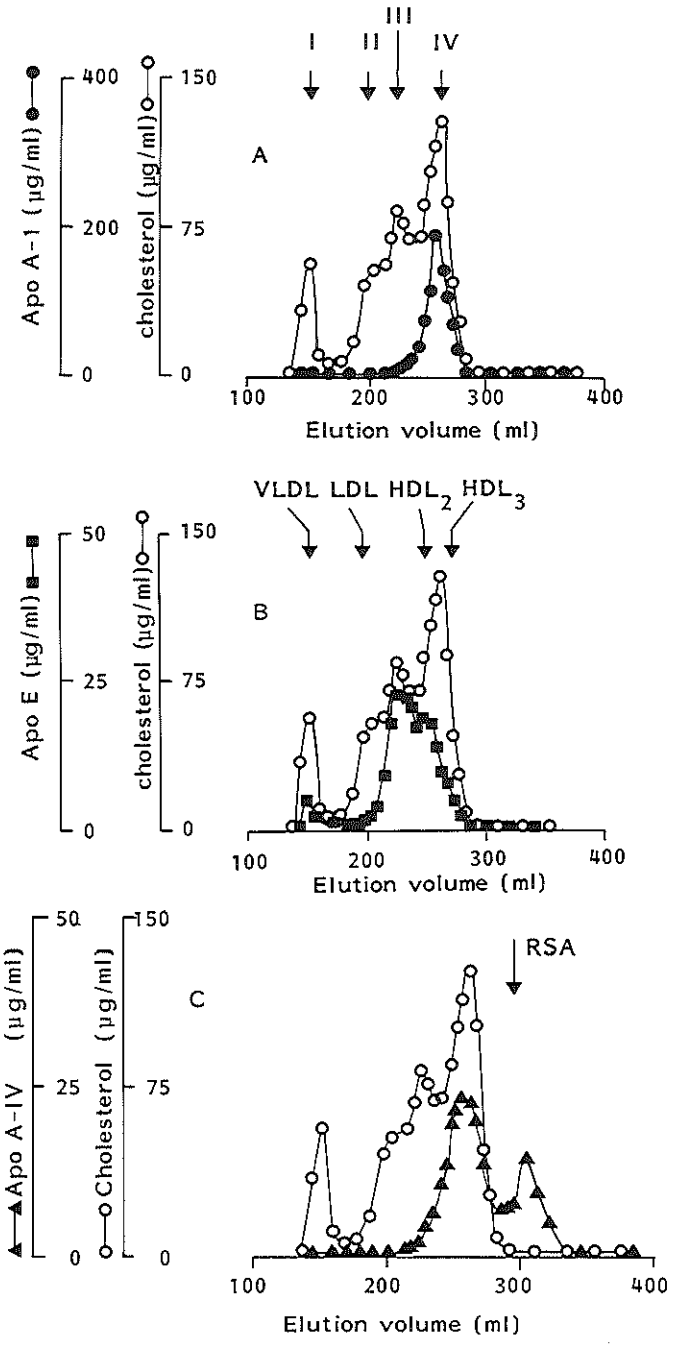
The elution pattern of serum protein and the distribution of cholesterol after gel filtration of rat serum on 6% agarose is presented in Fig. 7.1.A.. The cholesterol profile of another experiment is shown in Fig. 7.2.. All columns were standardized using VLDL, LDL and HDL fractions isolated by density flotation in the ultracentrifuge. Four cholesterol peaks can be distinguished. The cholesterol eluted in the void volume can be ascribed to chylomicrons plus VLDL. The second small cholesterol peak (sometimes in the form of a shoulder) is eluted in the LDL size range (see Fig. 7.2.). This LDL fraction as well as the lipoproteins present in the void volume were not investigated further. Fig. 7.1.A. shows that two additional major cholesterol peaks are eluted between 200 and 280 ml. The peak in absorbance at 280 nm, eluted between 200 and 220 ml, is caused by immunoglobulin M (mol wt 900,000). The elution patterns of apo A-I and apo E are shown in Fig. 7.1.B., Fig. 7.2.A. and B.

Fig. 7.2. Serum obtained from chow-fed rats was chromatographed on a 6% agarose column, exactly as described in the legend to Fig. 7.1.

Fig. 7.2.A. shows the elution profiles of cholesterol (○) and apo A-I (●). I, II, III and IV indicate the elution volumes of the different cholesterol containing fractions, which can be distinguished after chromatography (c.f. Fig. 7.1.). The highest levels of apo A-I are present in fraction IV.

Fig. 7.2.B. shows the elution profiles of cholesterol (○) and apo E (■). VLDL, LDL, HDL_2 and HDL_3 indicated the elution volumes of isolated humans lipoproteins, which were chromatographed in separate experiments. VLDL and LDL were isolated by sequential ultracentrifugation (2), and washed once. Human HDL and HDL_3 were isolated by rate zonal density gradient ultracentrifugation in a swinging bucket rotor, as described by Groot et al. (57). Peaks in the elution profile of apo E are found in fraction I (VLDL + chylomicrons present in void volume), in fraction III (apo E-rich rat HDL, with particle size between that of human LDL and HDL_2) and in HDL particles with a size close to that of human HDL_2.

Fig. 7.2.C. shows the elution profiles of cholesterol (○) and apo A-IV (▲). RSA indicates the elution volume of rat serum albumin which was chromatographed is in a separate experiment.


I
II
III
IV
A
Apo A-1 (µg/ml)
cholesterol (µg/ml)
400
200
0
150
75
0
100
200
300
400
Elution volume (ml)
VLDL LDL HDL2 HDL3
B
Apo E (µg/ml)
cholesterol (µg/ml)
50
25
0
150
75
0
100
200
300
400
Elution volume (ml)
C
RSA
Apo A-IV (µg/ml)
Cholesterol (µg/ml)
50
25
0
150
75
0
100
200
300
400
Elution volume (ml)

Some apo E and very small amounts of apo A-I are present in the void volume (chylomicrons plus VLDL), but the bulk of both apolipoproteins is found in the HDL size range. The elution profiles of apo A-I and apo E in the HDL region were clearly non-identical. Figs. 7.1.B. and 7.2.B. show that a major fraction of the apo E coelutes with the third cholesterol peak, indicating the presence of apo E on big HDL particles. Apo A-I coelutes with the fourth cholesterol peak representing HDL particles with a relatively small size. The elution profile of apo A-IV is different from that of apo A-I (138, 144, 146, Fig. 7.2.C.). As reported before (138), about 65% of the apo A-IV present in serum of chow-fed rats coelutes with apo A-I, while 35% is present as "free" apo A-IV, that elutes from the column clearly after HDL cholesterol and HDL apo A-I. Fig. 7.2.C. shows that "free" apo A-IV is eluted from the 6% agarose columns after rat serum albumin (RSA).

TABLE 7.1.

Apo E-HDl isolated by agarose column chromatograhpy and immunoprecipitation

	Original HDL[1]	Fraction precipitated with anti A-IV and anti A-I	Fraction subsequently precipitated with anti E[2]
Apo A-I	9.0± 3.1[3]	9.1±3.4.	0.8± 0.3
Apo A-IV	4.7± 0.2	4.5±0.6	0.3± 0.5
Apo E	19.8± 2.3	3.3±0.4	16.2± 0.6
Cholesterol	78.5±17.2	19.1±2.6	57.4± 4.7[4]
Phospholipids	81.5±23.5	23.1±8.1	65.4±21.3

[1] Fractions containing the bulk of apo E were pooled (e.g. Fig. 7.1.: 205-235 ml).

[2] Apo A-I, apo A-IV and apo E were not detectable in the final supernatant.

[3] Results are expressed as µg/ml column fraction. Values are mean ± S.D. (n=3).

[4] 34,9±2,5% of the total cholesterol was present as unesterified cholesterol.

The distributions of apo A-I and apo E, shown in Fig. 7.1.B. and in Fig. 7.2.A. and B, already provide strong evidence in favour of the existence of various HDL subclasses in normal rat serum.

Identification and characterization of HDL subclasses by sequential immunoprecipitation

HDL, isolated by molecular sieve chromatography on 6% agarose, was divided into two pools based on their apolipoprotein composition: Pool A, eluted between 205-235 ml, containing the bulk of the apo E and pool B, eluting between 240-274 ml, containing the bulk of apo A-I and apo A-IV. Both pools were subjected to a series of immunoprecipitations, using two antibodies in the first step and one antibody in the second precipitation step.

In order to determine the "molecular weight" of lipoprotein particles, the column was calibrated with a mixture of pure protein markers with known molecular weights. A calibration curve can be constructed by plotting elution volume/void volume versus the logarithm of molecular weight. The molecular weights of the lipoproteins carrying the bulk of the cholesterol, present in pool A or pool B, can be roughly estimated using this standard curve. Apo E-HDL and apo A-I-HDL (see below) represent more than 70% of the cholesterol present in pool A and pool B, respectively. However, the determination of the molecular weights of the other HDL subfractions cannot be performed in the same way because they represent only minor fractions of the cholesterol present in pool B. When pool A was treated with a combination of antibodies against apo A-I and apo A-IV (Table 7.1.), only 18% of the apo E was coprecipitated, together with 100% of the apo A-I and apo A-IV. The resulting supernatant was subsequently treated with anti-apo E IgG (Table 7.1.). This treatment results in the quantitative recovery of apo E in this second precipitate. This apo E-HDL has a particle weight of about 750,000. Because of our inability to measure rat C apolipoproteins by means of electroimmunoassay due to a lack of proper antisera, we analyzed all original column fractions as well as the immunoprecipitates on SDS polyacrylamide gel electrophoresis. However, because the immunoprecipitates contain big amounts of IgG in addition to the

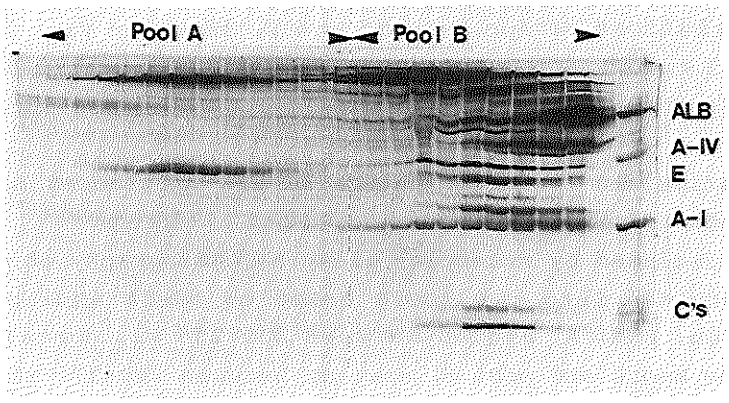


Fig. 7.3. Localization of C apolipoproteins in Bio-gel A-5m gel-filtered serum. Aliquots (25 µl) of column fractions were analyzed on 12,5% polyacrylamide gel electroforesis. Pool A eluted between 205 and 235 ml whereas Pool B eluted between 240 and 274 ml as indicated in Figs. 7.1. and 7.2..

apolipoproteins, it was not possible to visualize the apolipoproteins present in the immunoprecipitates by this electrophoresis technique. The gels of the original HDL pools (pool A and pool B) shown in Fig. 7.3., reveal that pool A, containing the bulk of the HDL apo E does not contain any C apolipoproteins, while pool B, containing the bulk of HDL apo A-I and HDL apo A-IV does contain the low molecular weight C apolipoproteins. We therefore conclude that apo E-HDL is free of C apolipoproteins. The C apolipoproteins may be present on apo A-I-HDL, on apo A-IV-HDL, as well as on any of the more complex HDL subfractions (see below). Specific immunoassays for apo C's are needed to substantiate this point. On the basis of the data shown in Table 1, it is possible to calculate the following chemical composition for apo E-HDL: 12% protein, 47% phospholipids and 36% cholesterol (34,9% of this cholesterol is in the unesterified form). The protein content is calculated using the amount of apo E determined by quantitative electroimmunoassay.

Another simple lipoprotein complex was isolated from the column fractions containing the bulk of the apo A-I (Pool B), by a first precipitation with a combination of anti-apo E and anti-apo A-IV IgG's (Table 7.2.). Only 23% of the apo A-I was coprecipitated in this step. This indicated that only 23% of the apo A-I is present on complex HDL particles containing also apo E and/or apo A-IV. The remaining 77% of the apo A-I was subsequently precipitated, from the supernatant of the first precipitation step, with anti-apo A-I IgG. Neither apo E nor apo A-IV could be detected in this second precipitate. We propose to name this simple lipid-protein complex apo A-I-HDL because it carries apo A-I as its only major apolipoprotein. However, it cannot be excluded that apo C molecules are also present (see above).

TABLE 7.2.

Apo A-I-HDL isolated by agarose column chromatography and immunoprecipitation.

	Original HDL[1]	Fraction precipitated with anti A-IV and anti E	Fraction subsequently precipitated with anti A-I[2]
Apo A-I	103.1±15.8[3]	23.8±7.2	73.3±13.1
Apo A-IV	24.3± 1.8	23.7±3.0	0.9± 0.8
Apo E	12.2± 0.2	11.5±0.9	0.6± 1.0
Cholesterol	86.9± 5.4	37.1±4.3	56.2± 5.3[4]
Phospholipids	127.7±18.3	47.8±7.3	80.5/95.4

[1] Fractions containing the bulk of apo A-I and apo A-IV were pooled (e.g. Fig. 7.1.: 240-274 ml).

[2] Apo A-I, apo A-IV and apo E were not detectable in the final supernatant.

[3] Results are expressed as µg/ml column fraction. Values are mean ± S.D. (n=3).

[4] 18,4±2,6% of the total cholesterol was present as unesterified cholesterol.

TABLE 7.3.

Apo A-IV-HDL was isolated by agarose column chromatography and immunoprecipitation

	Original HDL[1]	Fraction precipitated with anti A-I and anti E	Fraction subsequently precipitated with anti A-IV[2]
Apo A-I	103.1±15.8[3]	97.6±13.5	0.9±0.2
Apo A-IV	24.3± 1.8	19.0± 2.7	5.1±1.8
Apo E	12.2± 0.2	10.7± 0.6	0.6±0.6
Cholesterol	86.9± 5.4	81.0± 6.2	2.4±1.1[4]
Phospholipid	127.7±18.3	125.3±16.0	3.2±3.6

[1] Fractions containing the bulk of apo A-I and apo A-IV were pooled (e.g. Fig. 7.1.: 240-274 ml).

[2] Apo A-I, apo A-IV and apo E were not detectable in the final supernatant.

[3] Results are expressed as ug/ml column fraction. Values are mean ± S.D. (n=3).

[4] 72% of the total cholesterol was present as unesterified cholesterol.

The composition of apo A-I-HDL resembles that of total rat HDL. Its chemical composition is 34% protein, 26% cholesterol (of which 18,4% is in the unesterified form) and 40% phospholipids. Apo A-I-HDL is relatively small (particle weight approximately 250,000).

A third simple lipid-protein complex can also be isolated from the column fractions containing the bulk of the apo A-I (Pool B). 21% of the apo A-IV in this pooled fraction is not precipitated by a combination of anti-apo A-I and anti-apo E IgG's (Table 7.3.). Subsequently apo A-IV-HDL can be quantitatively precipitated with anti-apo A-IV. Apo A-IV-HDL is a protein-rich HDL particle probably with a relatively small size (approximately 250,000). From the data presented in Table 3 it can be calculated that it contains 54% protein, 20% cholesterol (only 38% of which is in the form of cholesteryl esters) and 26% phos-

pholipids. The remaining 79% of the apo A-IV in Pool B is present on a lipoprotein particle together with apo A-I. This can be concluded because earlier experiments (138) permitted us to exclude that apo A-IV and apo E in Pool B are present on one and the same HDL particle.

TABLE 7.4.

Distribution of total cholesterol, apo A-I, apo E and apo A-IV among the different HDL subclasses, identified by specific immunoprecipitation.

	Cholesterol	Apo A-I	Apo E	Apo A-IV
Simple lipoproteins				
Apo E-HDL	39± 1%[1]	-	63±3%	-
Apo A-I-HDL	39± 8%	69±8%	-	-
Apo A-IV-HDL	4± 1%	-	-	21±8%
Complex lipoproteins				
HDL with apo A-I and apo E	18±10%	31±8%	37±3%	-
HDL with apo A-I			-	79±8%

[1] Values are means ± S.D. (n=3).

Table 7.4. summarizes the results of the present study. We calculated the fraction of the total HDL cholesterol present in the various HDL subclasses, assuming that the 2 pooled column fractions together comprise total rat HDL. In fact it can be calculated that these 2 pools contain more than 90% of total HDL cholesterol. Apo A-I-HDL and apo E-HDL each accounted for as much as 39% of total HDL cholesterol and therefore both are major HDL subclasses. 69% of the total HDL apo A-I and 63% of the total HDL apo E are present on apo A-I-HDL and apo E-HDL, respectively. Apo A-IV-HDL is a minor HDL subclass accounting for only 4% of the total HDL cholesterol. Only a relatively small fraction (about 18%) of the total HDL cholesterol is present on complex HDL

particles. It can be calculated on the basis of the data shown in Tables 7.1.-7.3. and earlier experiments (138), that these complex HDL subfractions will consist mostly of 1) HDL containing both apo A-I and apo A-IV and 2) HDL containing both apo E and apo A-I.

DISCUSSION

In the present study data are presented in favor of the existence of five different HDL subclasses in rat serum. The evidence was obtained by using a combination of gel filtration and specific immunoprecipitation. In a previous study (138), preliminary evidence was obtained suggesting the existence of HDL subfractions, containing either only apo A-I or only apo A-IV. The present experiments were designed to supplement and extend these findings.

An important observation in the present report is that the separation on 6% agarose columns of rat serum lipoproteins in the LDL/HDL size range is improved, compared to our earlier data (138). Two major HDL subfractions can be easily distinguished now, just by measuring cholesterol in the eluted fractions. This is due to the use of a different column size and a lower flow rate. The four peaks in the cholesterol profiles were obtained using five different sera and two different columns, indicating that the column chromatography technique is reproducible.

Apolipoproteins were determined by electroimmunoassay, both in the precipitates and in the final supernatants resulting after immunoprecipitation. The precipitates already contained antibody and therefore the theoretical possibility existed for underestimation of the apolipoprotein concentrations in the immunoprecipitates. The recoveries of apo A-IV, apo E and apo A-I were, however, 99 $\pm$ 9%, 100 $\pm$ 9% and 99 $\pm$ 10% respectively (Tables 7.1.-7.3.). This indicates that the antibodies present in the precipitates did not interfere with the apolipoprotein assays. It can be expected that the high concentrations of urea (8M) used for the sample preparation cause a dissociation of the apolipoprotein-IgG complexes.

The existence of HDL subclasses in rat serum is already known for some time. Weisgraber, Mahley and Assmann (59) isolated an apo E-rich HDL (HDL_1) from serum of normal rats and another apo E-rich HDL (HDL_c) from serum of cholesterol-fed rats. These apo E-rich lipoproteins also contained small amounts of apo A-I. Heparin-Sepharose column chromatography (179) has also been used successfully for the isolation of apo E-poor and apo E-rich HDL fractions from rat serum. It should be noted, however, that all these procedures require prior ultracentrifugal isolation of HDL (59, 179). Because ultracentrifugation results in "stripping" of apo A-I and especially apo E from serum HDL (60, 86-88, 138, 151), it is important to avoid the ultracentrifuge during the isolation of specific HDL subfractions. The data presented in this paper shows that a combination of gel filtration and immunoprecipitation may be very useful.

A major conclusion from the present experiments is that, even in serum from rats fed normal chow (which is free of cholesterol), 63% of the total HDL apo E is present on an HDL particle which has apo E as its sole apolipoprotein constituent (apo E-HDL). It should be noted that more than 80% of rat serum total cholesterol is present in the HDL fraction. Keeping this in mind, it is evident that the quantitative importance of apo E-HDL is endorsed by the fact that as much as 39% of the total HDL-cholesterol is present in this HDL subclass. HDL particles containing apo E as there sole apolipoprotein (apo $E\text{-}HDL_c$) have also been isolated by Mahley et al. (181) from serum obtained from cholesterol-fed dogs. However, a comparison of the composition of both particles reveals several differences. Canine apo $E\text{-}HDL_c$ (isolated from the density range 1.006-1.02 g/ml) is a particle with a weight of $3,6 \times 10^6$ dalton, which is even larger than both human and canine LDL (182). On 6% agarose columns, therefore, these particles elute before the LDL peak (see Fig. 7.2.B.). Rat apo E-HDL, on the other hand, clearly elutes after the LDL fraction and is therefore smaller in size (mol. wt. approximately 750,000). It is calculated that canine apo $E\text{-}HDL_c$ contains sixteen apo E molecules per particle (180). In contrast, it can be calculated from our data, assuming a molecular weight of 34,000

for apo E and taking into account the fact that 12% of rat apo E-HDL is apo E protein as measured by electroimmunoassay (Table 7.1.), that only three apo E molecules are present on an average rat apo E-HDL.

Very low levels of LDL are present in rat serum. Consequently HDL is the major cholesterol-carrying lipoprotein in this species. We suggest that apo E-HDL, isolated from normal rat serum, plays an important role in the transport of plasma cholesterol to the liver. Several arguments in favor of this hypothesis can be given. Firstly, in vivo studies using HDL specifically labeled in apo E (85, 160) have also shown that the liver is the major degradation site for HDL apo E, while the kidneys are most active in HDL apo A-I degradation (85, 157, 160). These in vivo turnover studies support the notion that apo A-I and apo E are not always together on the same HDL particle and that the metabolic pathways of HDL apo E and HDL apo A-I are not identical. Secondly, we observed that apo E-HDL does not contain C peptides. Studies of Van Berkel et al. (184) and Windler, Chao and Havel (185) have shown that C peptides inhibit the hepatic uptake of apo E containing lipoproteins. The absence of C peptides on apo E-HDL is therefore in favor of a role for apo E-HDL in reverse cholesterol transport. Thirdly, studies with HDL_c, isolated from plasma of cholesterol-fed dogs, which has apo E as its sole apolipoprotein component, binds with a very high affinity to hepatic lipoprotein receptors (186, 187). The high affinity interaction between HDL_c and the B/E receptor was shown to be caused by multiple interactions between four sites on the receptor protein and four apo E molecules present on the HDL_c particle (180). A second hepatic receptor probably exists for the binding and uptake of chylomicron remnants. This receptor also interacts with canine HDL_c (188). These observations strongly suggest that HDL_c is capable of delivering serum cholesterol to the liver from where it can be excreted in the bile or degraded to bile acids. A similar mechanism can be expected to operate for the apo E-HDL present in serum of normal rats, as described in the present study.

The second major HDL subclass described in the present paper, does not have any apo E or apo A-IV, but contains mostly apo A-I (apo A-I-HDL). 69% of the total HDL apo A-I and 39% of the total HDL cholesterol are present in this subclass. Apo A-I-HDL has a particle weight of about 250,000 ; the chemical composition resembles that of typical rat HDL. Approximately three apo A-I molecules can be accomodated on one apo A-I-HDL particle.

Apo A-IV is the third major apolipoprotein present in rat HDL (91, 138, 142). In a previous publication (138), we already suggested that a small part of the apo A-IV present in rat HDL was probably located on a protein-rich subclass, containing neither apo E nor apo A-I. The data of the present study provide direct evidence in favor of the existence of this HDL subclass, that was called apo A-IV-HDL. However, the major part of the total HDL apo A-IV (79%) is present on an HDL particle containing apo A-I as well as apo A-IV, but no apo E. Calculations (assuming a particle weight of 250,000) suggest that two molecules of apo A-IV and 1 molecule of apo A-I can be accommodated on this complex lipoprotein. Metabolic studies with HDL, specifically labeled in apo A-IV or in apo A-I, have shown that the serum half-life of HDL apo A-IV is much shorter than that of HDL apo A-I; the liver is the major catabolic site of HDL apo A-IV and only a minor fraction of total HDL apo A-IV is degraded in the kidneys (Dallinga-Thie, Van 't Hooft and Van Tol, Arteriosclerosis, in press). Our hypothesis is that apo A-IV-HDL will have a relatively rapid turnover, compared to the apo A-IV present on the more complex HDL subclass, containing both apo A-IV and apo A-I, due to a specific degradation of apo A-IV-HDL in the liver. This would indicate a role of apo A-IV-HDL in reverse cholesterol transport. Earlier studies of Delamatre et al. (144) have already implicated a role of apo A-IV in reverse cholesterol transport, based on the observation that active lecithin-cholesterol acyltransferase causes a redistribution of apo A-IV between "free" apo A-IV and HDL apo A-IV. Our observation that the cholesterol present on apo A-IV-HDL is mainly (72%) unesterified cholesterol, supports the idea of a specific role of this HDL subfraction in cholesterol transport. The low fraction of esterified cholesterol

is consistent with a location of apo A-IV-HDL at the start of overall reverse cholesterol transport as well as with a relatively rapid turnover of this HDL subfraction.

Finally, we suggest the existence of a second complex HDL subclass, containing both apo E and apo A-I. Thirtyseven % of the apo E present in total rat HDL, isolated from chow-fed rats, was found to be present in combination with apo A-I. At the moment it is impossible to determine the exact chemical composition of this complex HDL subclass.

In conclusion, the data presented in the present paper draw a new picture of rat HDL. Five subclasses can be distinguished, including three relatively simple lipid protein complexes containing only one major HDL apolipoprotein each (apo A-I-HDL, apo A-IV-HDL and apo E-HDL), and two complex lipoproteins, containing either both apo A-I and apo A-IV or apo E as well as apo A-I. Apo C's are not present on apo E-HDL, but may be present on one or more of the other HDL subclasses.

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CHAPTER 8

GENERAL DISCUSSION POINTS

ISOLATION PROCEDURES FOR HDL

Lipoproteins can be separated by ultracentrifugal flotation using a variety of preparative (2, 3) or analytical (189) techniques. This fractionation procedure, however, was shown to cause structural changes and may result in the loss of apolipoproteins from the particle surface. Since these proteins govern many of the functional properties of the lipoproteins, it is not inconceivable that their metabolic behaviour is affected by this isolation procedure (152).

After ultracentrifugal isolation of lipoproteins from both human and rat serum, apolipoproteins A-I and E were recovered in the lipoprotein-deficient ($d > 1.21$ g/ml) fraction. It was shown that up to 40% of the apo E, derived either from VLDL or the HDL fraction, was "stripped" from the lipoprotein particles during a standard isolation of HDL lipoproteins by sequential ultracentrifugation (60, 151, 152, 193). Mahley et al. (60) showed that losses of apo E were smaller using the 40 rotor instead of the 50.Ti rotor stressing, the complexity of this problem. We showed that 11-22% of serum apo E was recovered in the $d > 1.21$ g/ml infranatant after only one ultracentrifugal step using the 40.3 rotor (Chapter 4).

Apo A-I, the major apolipoprotein of both human and rat HDL, was also found in the lipoprotein-deficient ($d > 1.21$ g/ml) fraction after ultracentrifugation. Values up to 50% of the total human serum apo A-I concentration were reported to be recovered in this fraction (190). Elegant studies by Kunitake et al. (88), designed to examine the effects of temperature, rotor configuration and tube size on the losses of apo A-I from HDL caused by ultracentrifugation, have shown that repeated ultracentrifugation leads to the loss of up to 35% of the HDL apo A-I. The underlying cause is complex and probably attributable to combined effects of high ionic strength and high shear forces. In our studies 9% of rat serum apo A-I was recovered in the $d > 1.21$ g/ml fraction after one ultracentrifugal spin (Chapter 4). Similar results were obtained by Fainaru et al. (151).

Other ultracentrifugal isolation methods, including separa-

tion of lipoproteins in a salt gradient or isolation by means of rate zonal ultracentrifugation, have also been used (52, 54, 56, 189, 191). Both methods, however, also result in severe losses of apo A-I (10-15%, (54, 56)) and especially apo E (40%, (191)). It can therefore be concluded that ultracentrifugal procedures for isolation of lipoproteins must be used with caution.

Molecular sieve chromatography, using agarose beads, provides a method for separating lipoproteins on a preparative scale without exposure to ultracentrifugal forces or high salt concentrations. Using 6% agarose columns, a separation according to size can be obtained between triglyceride-rich lipoproteins, LDL, HDL and possible "free" apolipoproteins if present. Fig. 8.1. represents the elution pattern of cholesterol, apo A-I, apo E and apo A-IV in rat serum, fractionated on a 6% agarose column. A minor cholesterol peak elutes in the void volume and represents chylomicrons and/or VLDL. The major cholesterol peak is largely found in particles eluting in the size range corresponding to HDL, since LDL cholesterol in the rat is very low (192). Two distinct cholesterol peaks are observed in the HDL fraction. Both contain apo A-I and apo E, but these apolipoproteins are not evenly distributed across the HDL-cholesterol spectrum. Apo E is associated with bigger HDL particles than apo A-I (Chapter 4 and 7, Fig. 8.1.). The same observations were reported for human serum HDL (193-195). Free apo A-I and apo E were not generated by this gel filtration method, supporting the view that the free apo E or apo A-I, appearing in the bottom fraction of density > 1.21 g/ml after ultracentrifugation, are an artefact due to ultracentrifugation

Apo A-IV was first recognized as a component of rat HDL (91, 108). It was later also isolated and characterized in human serum in a "free" fraction, unassociated with the major lipoproteins (110, 111, 147, 153), and as a constituent of human lymph chylomicrons (147, 153, 196). In rat serum a substantial portion of apo A-IV (up to 50%) can be found in the d > 1.21 g/ml bottom fraction (133, 145, 146, 197, 198, Chapter 4), while the remainder circulates on HDL. It is important to realize that the precise distribution of apo A-IV among the lipoprotein classes is

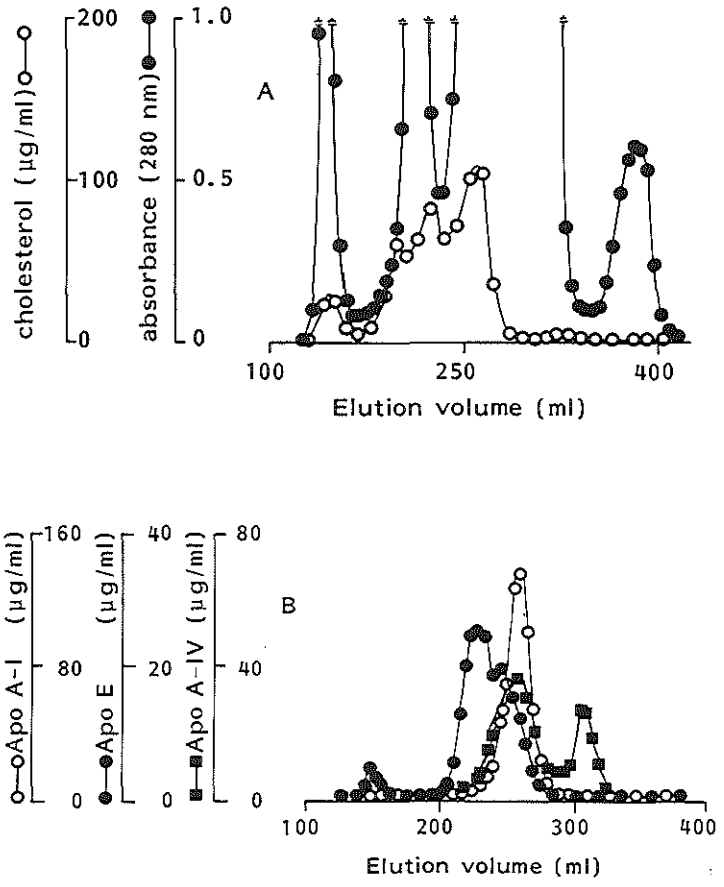

Fig. 8.1. Separation on a column of 6% agarose gel of serum from chow-fed rats. Ten ml of serum was applied to a column of 6% agarose (2.0 x 120 cm), equilibrated with 0.15 M NaCl containing 2 mM Na-phosphate pH 7.4, 1 mM EDTA and 0.01% NaN_3, at 4°C.

difficult to assess owing to the ease with which apo A-IV is removed from lipoproteins during ultracentrifugation. Almost all apo A-IV present in the d < 1.006 g/ml fraction from non-fasted human plasma can be removed by repeated ultracentrifugation (111, 153).

Molecular sieve chromatography of human plasma leads to the recovery of about 25% of total plasma apo A-IV in the HDL region, indicating that, also in human plasma, this protein is a component of HDL (147, 199). The remaining 75% is present in a "free" form. Also in the rat part of the apo A-IV appears in a fraction that elutes from 6% agarose gels after the main (i.e. HDL) cholesterol peak (Fig. 8.1., 145). Immunoprecipitation studies (Chapter 4) have shown that only small amounts of cholesterol and phospholipid coelute with it. Therefore this fraction was named "free" apo A-IV. In fasted rat serum only 9% of the total apo A-IV concentration is present as "free" apo A-IV. In contrast, after one ultracentrifugal isolation step at d = 1.21 g/ml, 35% of the total serum apo A-IV is found in the d > 1.21 g/ml bottom fraction. This indicates that not only apo A-I and apo E but also apo A-IV is stripped from HDL during ultracentrifugation. For these reasons gel filtration is the isolation method of choice, especially if the HDL will be used for metabolic studies in vivo.

THE ROLE OF THE INTESTINE IN APOLIPOPROTEIN A-IV METABOLISM

The small intestine plays an important role in the synthesis of apolipoproteins and the assembly of intact lipoproteins. During active lipid absorption, triglycerides are packaged into chylomicron particles, which serve as vehicles for triglyceride transport through the intestinal lymph into the plasma compartment. Our knowledge of the intestinal contribution to the synthesis of the individual apolipoproteins is incomplete. However it is clear, from a variety of studies involving trace radiolabeling, immunocytochemistry or mRNA quantitation, that both apo A-I and apo A-IV are derived from this source (11, 13, 96-103, 114-116). Although apo A-I and apo A-IV share a remarkable degree of sequence homology, they are different gene products and are

subject to independent regulation. As mentioned in the introduction, apo A-I is synthesized as a propeptide which is cotranslationally cleaved into mature apo A-I. Apo A-IV, however, lacks the prosegment and is directly secreted in its mature form. A second difference between apo A-I and apo A-IV concerns the effect of dietary influences on the regulation of their intestinal synthesis. Several reports have shown that active triglyceride absorption has either no influence at all or affects only modestly the rate of intestinal apo A-I synthesis (13, 95, 96, 200, 201). In distinction, apo A-IV synthesis increases substantially in this circumstance. Studies by Krause et al. (98), using ethinylestradiol treated rats as a model, revealed that an increase of apo A-IV transport rates in mesenteric lymph during triglyceride absorption is a reflection of increased synthesis. More direct evidence for increased apo A-IV synthesis was given by Gordon et al. (115) and Pessah et al. (202). They reported a 4-fold increase in intestinal mRNA levels. The results from our study (Chapter 3) are in agreement with the abovementioned observations and suggest that apo A-IV synthesis is likely to be regulated by triglyceride absorption. Therefore, apo A-IV may have an important function in the assembly of large triglyceride-rich chylomicron particles.

Characterization of lymph lipoprotein particles, isolated from mesenteric lymph of rats infused with either glucose or a triglyceride emulsion, have been described in Chapter 3. Lymph was fractionated by molecular sieve chromatography on agarose columns. Again differences in the behaviour of apo A-IV and apo A-I were observed. Apo A-I in mesenteric lymph is found only in association with chylomicrons and HDL. No "free" apo A-I can be detected. Apo A-IV, on the other hand, occurs in a lipoprotein-bound and in a "free" form. During the triglyceride absorption an increase in apo A-IV output was observed in all lymph lipoprotein fractions, including "free" apo A-IV, while for apo A-I an increase in output was only seen in the large chylomicrons fraction. Apo A-I output into lymph HDL was not changed by lipid infusion.

Evidence for intestinal HDL production in the rat came first

from studies using the isolated perfused intestine (9, 203). The amount of HDL produced in this system was very small. In several other studies it was found that, in lymph from either glucose infused (96) or lipid infused (67) rats, about 85% of the apo A-I was present in d < 1.006 g/ml infranatant, representing lymph HDL. Furthermore it was found that mesenteric lymph contains discoidal HDL with a chemical composition strongly different from that of serum HDL. In agreement with studies of Imaizumi et al. (12) we could detect only very small amounts of lymph HDL, using molecular sieve chromatography as the isolation procedure (Chapter 3). Certainly the apolipoprotein composition was different from that of total serum HDL, in keeping with the intestinal origin of this particle. No apo E could be detected. On the basis of our data it is evident that lymph HDL is a quantitatively unimportant precursor of serum HDL. Intestinally synthesized apo A-I and apo A-IV are largely transported into the plasma compartment, while packaged on chylomicron particles.

"FREE" APOLIPOPROTEIN A-IV

The synthesis and secretion of apo A-IV by the enterocytes of the small intestine are significantly increased during lipid absorption. The nascent protein combines with lipid in the Golgi apparatus of these cells (204) and is subsequently secreted into the mesenteric lymph. Apo A-IV is associated mostly with triglyceride-rich lipoprotein particles and in addition is present as "free" apo A-IV (Chapter 3). Entrance of the chylomicron triglycerides into the serum compartment, followed by hydrolysis of the triglycerides by lipoprotein lipase (LPL), was associated with a rapid transfer of apo A-IV and apo A-I to the HDL fraction (17, 19, 133, 150). Characterization of serum chylomicron particles shows that this transfer occurs very rapidly because circulating chylomicron particles do not contain any apo A-I or apo A-IV. Elegant studies by Weinberg et al. (205) have shown that, at least in man, apo A-IV was released from triglyceride-rich lipo-

proteins during incubation with either human HDL_2 or pure apo C-III. Using modified HDL_2 particles, which do not contain C apolipoproteins, the dissocation of apo A-IV was less affected. This suggests that the primary mechanism of apo A-IV dissociation from lymph chylomicrons upon entering the plasma compartment depends on the transfer of C apolipoproteins from HDL to the chylomicron particles. It is not clear if all apo A-IV, dissociated from chylomicrons, is transferred directly to HDL. Studies by Fidge (133) support the view that the pool of "free" apo A-IV acts as an intermediate. Upon dissociation from the chylomicrons, some of the apo A-IV may be rapidly released into the "free" apo A-IV fraction ($d > 1.21$ g/ml) and subsequently transferred to HDL. This could explain the origin of "free" apo A-IV in rat serum. However, in this study (133), the apo A-IV containing fractions were separated by ultracentrifugation, making the results difficult to interpret. Direct injection of free radiolabeled apo A-IV intravenously into rats revealed that within 5 minutes almost all radioactivity was in HDL, as demonstrated using gel filtration for lipoprotein fractionation. Consequently, there may be direct transfer to HDL without the intermediacy of "free" apo A-IV.

As discussed above, part of the apo A-IV in mesenteric lymph was present as "free" apo A-IV during glucose infusion. This amount of "free" apo A-IV significantly increased during triglyceride absorption. Because most of the mesenteric lymph, produced during our observation period, is recovered extracorporally, as measured by the recovery of triglycerides in the lymph, it is suggested that this increased "free" apo A-IV flux is of intestinal origin. This concept is supported by the observation that, compared to fasting, administration of standard chow to rats produces an eight-fold increase in the serum "free" apo A-IV concentration (Chapter 4). Our data therefore suggest that the increase in "free" apo A-IV in serum after feeding may be partially explained by an increased influx of "free" apo A-IV from the lymph into the plasma compartment.

In addition to stimulatory effects of diet (138, 145), plasma "free" apo A-IV levels also increase during aging, but in this situation total plasma apo A-IV is unaffected (146). Redis-

tribution of the protein between its "free" and lipoprotein-bound pools is also affected by LCAT activity; when LCAT activity is inhibited by DTNB, the proportion of "free" apo A-IV in plasma rises dramatically (144). No changes occur in the concentrations of "free" apo A-I or apo E. Evidence for a role of apo A-IV in LCAT activation has come from studies by Steinmetz et al. (206) and Chen et al. (207) in which human apo A-IV was incubated with different phospholipid substrates. Under these conditions apo A-IV has a stimulating effect on LCAT, however, the measured factor of stimulation is about 25% of that found for apo A-I. The stimulation of LCAT by apo A-I is believed to be a function of its multiple 22 amino acid amphiphatic segments (94, 208, 209). A comparison of the amino acid sequences of apo A-I and apo A-IV revealed that apo A-IV also contains 13 tandem repetitions of a 22 amino acid segment (109). It has been proposed that apo A-I and apo A-IV have evolved from a divergence of a common ancestral gene (94). Because of the close similarity of the structure of both apolipoproteins, it has been suggested that apo A-IV could compensate for functional abnormalities in apo A-I.

CATABOLISM OF HDL APOLIPOPROTEIN A-IV

Apo A-I in rat serum is mainly associated with particles of the size of HDL. The metabolic properties of HDL apo A-I were extensively studied using different methods: i.e. O-(4-diazo-3-[^{125}I]iodobenzoyl)sucrose (^{125}I-DIBS) label (160), ^{125}I-tyramine-cellobiose (157) or the leupeptin model (85). The main purpose of all these methods is to delay the release of apolipoprotein degradation products from lysosomes in order to locate those tissues involved in apolipoprotein catabolism. ^{125}I-DIBS and ^{125}I-tyramine cellobiose remain trapped intralysosomally following uptake and degradation of the protein moiety and provide a semi-quantitative index of the extent to which each tissue is involved in the catabolic process. Leupeptin, on the other hand, is a competitive inhibitor of lysosomal cathepsin activities (210). When injected in vivo, this agent is known to suppress lysosomal cathepsin activity, thereby diminishing the ability of

the cell to degrade internalized (radiolabeled) proteins in the lysosomal compartment (85). It must be stressed, however, that only a partial blockade of catabolism of serum proteins can be obtained.

Several studies have been published now, employing these different techniques in order to locate catabolic sites of HDL apolipoproteins A-I and E. The most striking outcome of these investigations was that not only the liver, but also the kidneys seem to play a role in the degradation of both apolipoproteins (85, 157, 149, 160). However, there is a difference in the relative contributions of the liver and the kidneys in the catabolism of HDL apo A-I and apo E. The amount of apo A-I degraded in renal tissue is higher than in the liver, whereas for apo E the opposite was observed (160). At this point it was of interest to know whether HDL apo A-IV behaves identical to apo A-I.

The catabolic sites of apo A-IV were therefore determined in non-fasted rats, using the leupeptin model. Purified apo A-IV, isolated either from chylomicrons (Chapter 5) or from HDL (Chapter 6), was labeled with ^{125}I and reincorporated into HDL by incubation with serum. The labeled HDL was then isolated by molecular sieve chromatography on 6% agarose. Because the catabolic sites of apo A-I had been extensively studied, this protein labeled with ^{131}I was used as control material in all studies. This way it was possible to make a direct comparison of the metabolic behaviour of apo A-I and apo A-IV in the same animal. Because apo A-IV in rat serum is present both in HDL as well as in a "free" form, control experiments are required in order to check whether the label remains associated with HDL throughout the whole experimental period. It was clear from a variety of studies that both ^{125}I apo A-IV and ^{131}I apo A-I remained associated with HDL (Fig. 6.2. and 6.3.) following its reintroduction into serum, both in vivo and in vitro. Comparing the mass and the radiolabel distribution after separation of the serum lipoproteins on 6% agarose, it can be seen that the HDL elution patterns are similar. Therefore it seems reasonable to conclude that HDL preparations, labeled as described above, maintain their normal physiological behaviour. Results from the in vivo studies showed

that up to 4 h after injection of the labeled HDL preparation all radioactivity remained associated with HDL. No ^{125}I-apo A-IV could be detected in the "free" apo A-IV fraction. This indicates that either HDL apo A-IV is not a precursor of "free" apo A-IV or that "free" apo A-IV has a very rapid turnover rate in comparison to HDL apo A-IV. However, ^{125}I apo A-IV, injected directly into the circulation of rats has an initial rate of disappearance similar to that of HDL apo A-IV and in fact becomes rapidly incorporated into this fraction within 15 minutes. There is no evidence for rapid serum removal of the "free" protein in this situation. In summary, radiolabeled apo A-IV, once incorporated into HDL, remains associated with the particle under various in vivo and in vitro conditions, as was also reported by Ghiselli et al. (211) and Sherrill et al. (212). The approach described above therefore seems useful for the examination of the catabolic sites of HDL apo A-IV.

Studies on the metabolic fate of HDL apo A-IV were first described by Fidge (133). In these experiments they found no difference between the turnover rates of labeled apo A-IV, incorporated into HDL, and of the labeled intact HDL which mainly traced the fate of its apo A-I moiety. In contrast, we observed a significantly faster turnover rate for HDL apo A-IV (8.5 h) than for HDL specifically labeled with apo A-I (10.2 h). Because all these studies were performed in non-fasted rats, it is arguable that an exchange of apo A-IV between HDL and triglyceride-rich particles could account for the observed differences. However, the turnover rates of HDL apo A-IV in 20 h food-deprived rats or in non-fasted rats were identical so that under all our experimental conditions the serum decays of ^{125}I-apo A-IV are faster than of ^{131}I-apo A-I. The same observation was made in man (167, 211).

We also observed marked differences in metabolic behaviour between apo A-IV and apo A-I, which related to the degradation of both proteins (Table 8.1.). Clearly the majority of HDL apo A-I is degraded in the kidneys. In contrast, the most important organ involved in HDL apo A-IV degradation is the liver. Further information about the cellular localization of the degradation sites

of both apo A-IV and apo A-I has been obtained using cytochemical techniques. In the kidneys specific apolipoprotein containing granules were observed within the cells of the proximal tubules,

TABLE 8.1.

Tissue accumulation of iodinated HDL apolipoproteins due to inhibition of lysosomal proteases by leupeptin. Leupeptin-dependent accumulation as % of injected dose measured 4 h after injection[1]

	Liver	Kidneys
^{125}I-apo A-IV	8.4±2.0	3.5±1.0
^{131}I-apo A-I	2.6±0.6	4.6±1.3

[1] Values obtained from ref. 37.

while the cells of the glomeruli did not show any staining (Fig. 6.8). Leupeptin injection markedly increased specific granular staining inside the tubular cells, supporting the idea that degradation of both apo A-I and apo A-IV occurs in the lysosomal compartment of the epithelial cells of the proximal tubules. Little is known about the mechanism involved in the uptake of HDL apolipoproteins in the kidneys. Several suggestions have been proposed. Pittman et al. (213) implied that it resulted from tubular reabsorption and degradation of filtered free plasma apo A-I, unassociated with intact HDL. In line with such a mechanism is the observation that HDL cholesteryl esters are preferentially degraded in the liver (81-84). So, at least part of the apo A-I is cleared from the circulation independently of HDL cholesteryl esters. Studies using isolated perfused kidneys suggest that HDL can be reabsorbed as a unit by the proximal nephron via a mechanism involving endocytosis at the luminal membrane, followed by proteolysis in lysosomes (172, 173). Alternatively HDL may be bound to an HDL receptor present on kidney membranes (214), followed by the preferential uptake of HDL apolipoproteins. All these proposals require a more detailed investigation in order to

clarify the mechanism by which the kidneys are able to take up and degrade HDL apolipoproteins.

Immunocytochemical localization studies in our laboratory, showed that the hepatic parenchymal cells are involved in the degradation of HDL apo A-IV as indicated by a leupeptin-dependent increase in granular staining in this celltype. Earlier studies of Rachmilewitz et al. (215) already demonstrated the presence of intravenously injected ^{125}I-HDL in these cells using autoradiographic techniques. Also evidence for a specific HDL receptor in rat liver parenchymal cells has been obtained from binding studies (216-218). In all these studies, however, total labeled HDL preparations were used, which mainly follow the fate of apo A-I. As stated below not all apo A-IV present in rat HDL is located on subfractions together with apo A-I. Further experiments are required to determine the binding properties to membrane receptors of these various subfractions.

In summary it is clear that HDL apo A-IV and apo A-I have different metabolic fates. In order to find an explanation for the observed differences, the various carriers of apo A-IV in serum were determined using specific immunoprecipitation techniques (Chapters 4 and 7). From these studies it is concluded that apo A-IV is present on two different populations of particles within the HDL size range : firstly, HDL particles containing apo A-IV but no apo A-I or apo E (apo A-IV-HDL) and secondly particles containing both apo A-IV and apo A-I but no apo E (apo A-I + A-IV)-HDL. About 70% of the total apo A-IV present in HDL, is associated with the latter group of particles. It can be speculated that the apo A-IV HDL particles have a relatively rapid turnover and are specifically degraded in the liver. If this hypothesis is true this could also explain the more rapid turnover of apo A-IV, compared to apo A-I. It is conceivable that these apo A-IV-HDL particles play a role in the process of "reverse cholesterol transport", a possibility which is further substantiated by the finding that in peripheral lymph of cholesterol-fed dogs an increased amount of apo A-IV containing particles has been reported (219, 220).

In addition to the abovementionend experiments, a short

study was designed in order to determine whether the isoprotein compositions of apo A-I and apo A-IV isolated either from chylomicrons or from HDL, has any effect upon in vivo metabolic behavior (Chapter 5). Following incorporation of chylomicron apo A-IV and HDL apo A-IV or chylomicron apo A-I and HDL apo A-I into serum HDL, no differences in their turnover rates or tissue uptakes could be observed. From these studies it may be reasonable to conclude that the in vivo conversion of pro apo A-I to mature apo A-I or the posttranslational modification of apo A-IV does not influence their metabolic behavior to any great extent.

CHARACTERIZATION OF HDL SUBCLASSES IN RAT SERUM

Lipoprotein subclasses can be defined on the terms of their specific apolipoprotein composition. Alaupovic (174) introduced the term "simple lipoproteins", by which he meant those lipoprotein particles which only contain one apolipoprotein species (i.e. LDL). On the other hand "complex lipoproteins" contain more than one apolipoprotein species per particle. Using specific antibodies raised against the apolipoproteins, it is possible to separate these simple and complex lipoproteins. The following subclasses have been isolated from human plasma: Lp A-I Lp(A-I + A-II) (175-178) and Lp E (183). Chapter 7 describes an analysis of rat HDL using specific immunoprecipitations. We were able to distinguish 3 relatively simple lipoproteins i.e. apo E-HDL, apo A-I-HDL and apo A-IV-HDL and two more complex lipoprotein particles: HDL containing both apo E and apo A-I and HDL containing both apo A-IV and apo A-I.

A disadvantage of the immunoprecipitation method is that only the "simple" lipoprotein complexes can be isolated and completely analyzed. The composition of complex lipoproteins can be indirectly predicted from the results of the immunoprecipitation studies presented in Chapter 4 and 7. Based upon the fact that no apo E precipitates with anti apo A-IV in a single immunoprecipitation (Chapter 4), the conclusion is warranted that apo A-IV and apo E are not present on the same particle. Table 8.2. summarizes the size, chemical composition and calculated apolipo-

TABLE 8.2.

Characteristics and percentage chemical composition of the simple HDL complexes[1]

	Apo E HDL	apo A-I HDL	apo A-IV HDL
Molecular weight	750,000	250,000	250,000
Chemical composition			
total cholesterol	36%	26%	20%
(% free)	(35%)	(18%)	(72%)
phospholipids	47%	40%	26%
triglycerides	---	---	---
protein	12%	34%	54%
Number of apolipoproteins per molecule	3 apo E	3 apo A-I (apo C)	3 apo A-IV (apo C)

[1] Values obtained from experiments described in chapter 7.

proteins per particle for the three relatively simple lipoprotein complexes. A few comments are necessary in this context. First the protein content is taken as that amount of specific apolipoprotein measured by electroimmunoassay. Secondly, these values are used to calculate the number of apolipoprotein present on one particle on the basis of the following molecular weights: apo E 34,000; apo A-I 27,000 and apo A-IV 46,000. The "molecular weights" of the particles were determined by standardizing the agarose column with proteins of known molecular weights. Thirdly, because no antibody against C apolipoproteins was available, the presence of these small molecular weight apolipoproteins was predicted by analyzing the original column fractions on SDS-polyacrylamide gelelectrophoresis (Fig. 7.3.). No C apolipoproteins were present on apo E-HDL whereas the presence of apo C's on the other two relatively simple lipoproteins, as well as on the complex lipoproteins, cannot be excluded.

From Chapter 7, Table 7.4., it can be concluded that apo A-I-HDL and apo E-HDL account for 80% of the total HDL cholesterol. This has an important impact for our understanding of HDL metabolism. It is known that apo E particles can be rapidly removed by the liver, either through the apo B,E receptor or the apo E receptor (21, 22). Because the rat maintains very low levels of LDL it is conceivable that apo E-HDL compensates for the absence of LDL by functioning in the transport of cholesterol to the liver. Apo A-IV-HDL could have a similar function. The role of apo A-I-HDL remains unclear, but it is possible that this HDL particle is taken up preferentially by the kidneys. However, more studies are required in order to elucidate the mechanism and physiological impact of this process.

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CURRICULUM VITAE

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