High Density Lipoprotein Metabolism in Health and Disease

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IN THE NAME OF ALLAH, THE BENEFICENT
THE MERCIFUL

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SUMMARY

High density lipoprotein increasingly attracts the attention of investigators working in the atherosclerosis field due to the reported negative correlation found between its plasma levels and the incidence of ischaemic heart disease. However, the present classification of HDL depends on the flotation properties of its subfractions, but such fractions may not represent distinct biological entities. This project sets out to subfractionate HDL according to its apoprotein content rather than its flotation density, and to investigate the metabolism of these subfractions under a variety of conditions. Moreover, the effects of two hypolipidaemic drugs on HDL metabolism are studied in a group of hypertriglyceridaemic patients. HDL is prepared from normolipidaemic females and subfractionated by means of immunoaffinity chromatography. The physical and chemical properties of the isolated subfractions are studied and three methods for their quantitation presented. The distribution of these subfractions in plasma and in HDL density subclasses is studied in the normal state, during nicotinic acid and probucol therapy. Mathematical analysis of the tracer decay data is also discussed. The results of these studies are summarised as follows:

1. HDL can be subfractionated into two metabolically distinct types of particles, i.e., those containing primarily apo-AI without apo-AII and those with both apoproteins. Both species are detectable and have been quantified in fresh plasma and in HDL density subclasses. It is found that HDL_2 from normal young females contain a higher proportion of the (AI)HDL particles (71%) than HDL_3 (43%). Moreover, it is demonstrated that minimal exchange of A apoproteins seems to occur between the two types of particles.

2. The kinetic behaviour of (AI)HDL is, generally, similar to that of total HDL. On the other hand, their apoproteins show inconsistent deviations in the locations of their catabolism. Evidence for intravascular and extravascular site of catabolism is discussed.

3. Nicotinic acid is shown to have a reciprocal effect on (AI)HDL and (AI/AII)HDL. It raised the plasma levels of the former by 20% ($p<0.05$) and lowered those of the latter by 19.5% ($p<0.05$). A similar effect was observed on HDL_2 and HDL_3 concentrations. The former rose by 45% ($p<0.01$) and the latter fell by 28% ($p<0.01$) during treatment with the drug. The rise in the former was attributable in its entirety to an increase of its (AI)HDL content (20%, $p<0.01$).

4. Probucol, unlike nicotinic acid, lowers the plasma concentration of (AI)HDL by 22% ($p<0.01$) and of HDL_2 by 58% ($p<0.05$) but it has no consistent effect on either (AI/AII)HDL or HDL_3. The drop in HDL_2 level is

mainly a result of a reduction in its (AI)HDL content. In hypertriglyceridaemia the drug reduced the plasma levels of apo-AI, apo-AII and HDL. The decrease in the latter is due mainly to a fall in the HDL_3 level. This was associated with a reduction in the plasma level of apo-AII due to a fall in its rate of synthesis. Moreover, the treatment with the drug affected the composition of HDL where its cholesterol content was decreased with a concomitant increase in the protein content.

5. Bezafibrate shows little influence on HDL metabolism.

6. Examination of three mathematical approaches for tracer data analysis, i.e., curve peeling (Matthews), multicompartmental (Berman) and graphical integration (Nosslin) methods, showed that the same value of the total fractional catabolic rate of HDL can be obtained by any of these procedures. In addition, calculation of the extravascular catabolic rate can be done by the last two methods which produced similar results.

ABBREVIATIONS

apo-:	apoprotein
CETP:	cholesteryl ester transfer protein
d:	density
EC:	esterified cholesterol
Ex.V.:	extravascular
$F_{1.20}$:	flotation rate coefficient at a background density of 1.2 kg/l.
FC:	free cholesterol
FCR:	fractional catabolic rate (pools/day)
HDL:	high density lipoprotein
HL:	hepatic lipase
IDL:	intermediate density lipoprotein
I.V.:	intravascular
LCAT:	lecithin:cholesterol acyl transferase
LDL:	low density lipoprotein
LpL:	lipoprotein lipase
M_{app}:	apparent molecular weight
NIRS:	non-immune rabbit serum
PAGE:	polyacrylamide gel electrophoresis
PL:	phospholipid
r:	correlation coefficient
$t_{1/2}$:	half life
TG:	triglyceride
VLDL:	very low density lipoprotein

SECTION 1: INTRODUCTION

The following introduction provides a background to the structure, function and metabolism of high density lipoproteins (HDL).

Initially consideration is given to the epidemiology of HDL (especially with regard to its readily measured cholesterol component) since it is from the findings in this field that much of the current interest in the lipoprotein sprang. However, any detailed investigation of HDL must take into account its unique structural and physico-chemical properties and these are presented in some depth in later sections to be followed by a discussion of what is known of the metabolism of this important lipoprotein.

1.1 Cholesterol in Human Plasma:

Cholesterol in plasma is not found in a free form but it is transported by lipid-protein complexes known as lipoproteins. These are classified according to their flotation properties into four main density fractions: chylomicrons d<0.95 kg/l, very low density lipoproteins (VLDL) d 0.95-1.006 kg/l, low density lipoproteins (LDL) d 1.006-1.063 kg/l and high density lipoproteins (HDL) d 1.063-1.210 kg/l.

In plasma from healthy fasting adults a large proportion of the cholesterol (about 60%) is carried by LDL mainly in the form of cholesteryl ester; little cholesterol is associated with VLDL and the rest, some 0.9-1.8 mmol/l, is associated with HDL.

The distribution of cholesterol among the lipoprotein classes is not constant, even in normal subjects, but varies with age and sex (Fig. 1). The LDL-cholesterol level shows a slight decrease in childhood, but at adolescence it tends to rise in both sexes and continues to do so up to the age of 60 when it declines again. HDL-cholesterol is similar in both sexes during childhood; in females it remains constant up to the age of 25 and thereafter rises gradually to a peak at 60 years. In males the concentration of HDL-cholesterol in the plasma drops sharply following puberty and remains constant and lower than the female value up to the age of 50 where it rises again.

VLDL-cholesterol tends to rise gradually in both sexes up to the sixth decade then drops slightly afterwards (1).

1.2 Epidemiology of HDL Cholesterol

1.2.1: Plasma total cholesterol has been accepted for many years as a risk factor of ischaemic heart disease (IHD), but now it is thought to be less predictive than LDL- or HDL-cholesterol (Table 1) (2,3). The former (LDL-cholesterol) shows a strong positive correlation with the incidence of IHD in the age group 20-50 years (4), but its predictive capabilities become less in people beyond this age since LDL-cholesterol levels plateau and then decline after the fifth decade.

Among the various lipid risk factors examined by the extensive Framingham study (2), HDL-cholesterol was found to have the strongest relationship to IHD. This epidemi-

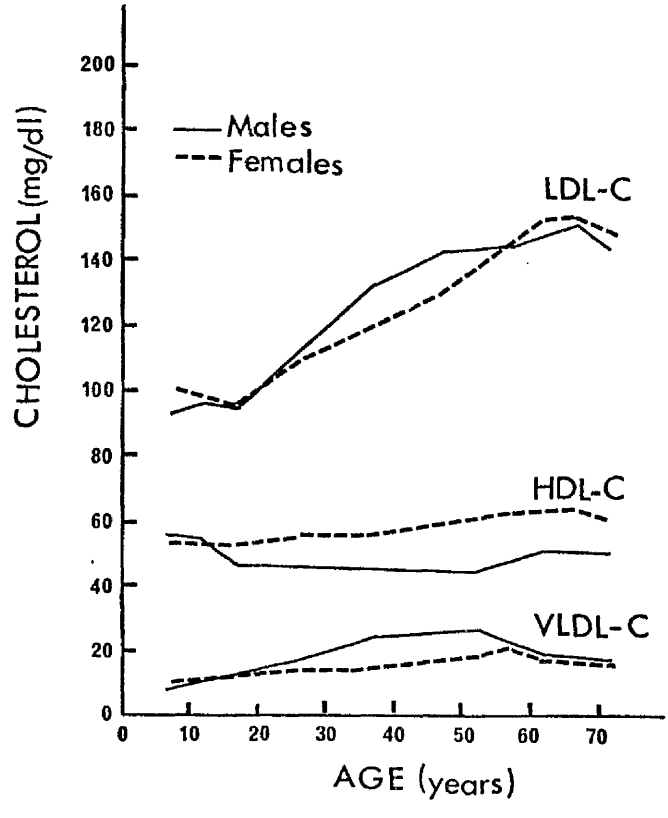


Figure 1. Plasma LDL-, HDL- and VLDL-cholesterol levels.

Data derived from the Lipid Research Clinics Program (1).

Table 1: IHD prediction from plasma lipids - the Framingham study.

Lipid	Likelihood ratio	
	Men	Women
Triglyceride	0.51	9.52[b]
Total-cholesterol	1.98	2.26
LDL-cholesterol	4.39[a]	4.54[b]
HDL-cholesterol	14.03[c]	21.21[c]

a. $p<0.05$
b. $p<0.01$
c. $p<0.001$

This table compares the lipids predictive capabilities of IHD in terms of their likelihood ratios. It is clear that HDL-cholesterol is more predictive than either LDL- or total cholesterol.

ological study showed that there exists a strong negative correlation between the cholesterol contained in this lipoprotein fraction and IHD which has generated much interest in the structure and function of HDL in recent years. The Framingham population was aged 50 and over at the time of recruitment and may not therefore be representative of the population at large. Subsequently, however, HDL-cholesterol has been found in other investigations to be a negative risk factor for IHD at all ages in both sexes (2,3,5) and in different races (2,3,5,6,7). The high independent predictive value of HDL-cholesterol concentrations for clinical IHD was further exemplified by results from the Trømsö and Framingham (2) studies. The first was retrospective and showed that persons who developed clinical IHD during the two years of the trial had significantly lower HDL-cholesterol concentrations than the matched control subjects who remained free of clinically manifest disease. The prospective, long term Framingham study established that, in persons who had initially been free of clinical IHD, the incidence of IHD was increased in those with lower HDL-cholesterol concentrations. Supportive evidence has also been gathered in two other studies (5,8) which established that patients with clinically manifest IHD tend to have low HDL-cholesterol concentrations independently of other lipoproteins; and in addition the presence of atherosclerotic lesions in the cerebral (9,10,11) and femoral (12) vessels has been found to be associated with low HDL-cholesterol levels.

Angiographic studies (13,15) have provided evidence that the correlation between HDL-cholesterol concentrations and coronary risk reflects an underlying relationship to coronary atherosclerosis. It has been found that significantly low levels of HDL-cholesterol were associated with high coronary atherosclerosis scores which reflect the number and severity of lesions in major coronary arteries.

In addition a strong negative correlation exists between HDL-cholesterol concentration and the whole body cholesterol pool, whereas no correlation was observed between body cholesterol and total plasma cholesterol, triglyceride, VLDL- or LDL-cholesterol (16,17).

From the above mentioned evidence it is clear that HDL can be considered as an antiatherogenic factor. However, it must be borne in mind that it in isolation does not give the whole picture since subjects with extremely low levels of HDL-cholesterol are not prone to atherosclerosis when they also have low LDL-cholesterol concentrations.

1.2.2: Factors associated with low HDL-cholesterol levels:

other IHD risk factors have been reported to be associated with reduced concentrations of HDL-cholesterol (Table 2). Among these are the following:

Table 2: HDL-cholesterol and risk factors

Factor	HDL-cholesterol - Percentage of normal value
Normal	100%
FH heterozygote	84%
FH homozygote	60%
Hypertriglyceridaemia (type IV)	65%
Cigarette smoking	86%
Obesity	86%
Physical inactivity	93%
Diabetes	89%

1. Hyper-LDL-cholesterolaemia:

An inverse correlation has been reported between LDL-cholesterol and HDL-cholesterol concentrations in both men and women (7,16,18,19). Patients with primary type II hyperlipoproteinaemia, especially homozygous familial hypercholesterolaemia, tend to have lower HDL-cholesterol concentrations than healthy controls (16,20,21).

2. Hypertriglyceridaemia:

The risk of developing IHD has been found to rise as the fasting plasma triglyceride concentration increases (7,20,22); moreover, a negative correlation between HDL-cholesterol and the concentration of this sterol in VLDL (the main carrier of triglyceride in fasting normal plasma) has been reported (18,23-25) especially in types IV- (23,26) and V-hyperlipoproteinaemia (23). In fact it has been postulated that the risk factor status of triglyceride may be due to its association with low HDL-cholesterol levels.

3. Tobacco smoking:

A strong negative correlation has been found between HDL-cholesterol concentrations and the amount of tobacco smoked (3,7,18,27). Smoking of 40 cigarettes daily lowers the HDL-cholesterol up to 16%. This is reversed if the subject abstains (3).

4. Other risk factors:

Similarly, low levels of HDL-cholesterol have been reported in association with obesity (2,3,18,27) diabetes mellitus (2,28), high blood pressure (7) and physical inactivity (23). Males have been found to have lower HDL-cholesterol concentrations than females of the same age group (20,29).

5. Family history of low HDL-cholesterol:

The study of the predictive power of HDL-cholesterol for future IHD has been extended to investigate its concentration in first degree relatives of patients with IHD. Significantly lower concentrations of HDL-cholesterol have been found in asymptomatic first degree relatives of patients with IHD (30,31), which may explain, in part, the well known fact that IHD appears to cluster in families.

The inverse relation of HDL-cholesterol concentration and risk factors can be expressed more strongly when a cumulative risk factor rating is established for each subject. This rating has been calculated (3) based on relative weight, diastolic blood pressure, cigarette smoking, total cholesterol and exercise. A score of 0, 1 or 2 is allocated for each of these parameters, and the sum of these individual scores is taken as the cumulative rating. A highly significant inverse relation is found between the decrease in HDL-cholesterol level and the increase in risk score.

6. Genetic factors:

Many genetic disorders have been reported to have an effect on HDL-metabolism and, consequently, they are associated with low levels of HDL-cholesterol. These are:

a) Tangier disease: In this disorder there is a near absence of normal HDL from plasma and an accumulation of cholesteryl ester in many tissues throughout the body. Its clinical features are splenomegaly, enlarged orange tonsils, relapsing neuropathy and corneal opacities (34,35).

Premature atherosclerosis is not a manifestation of this disease possibly due to the normal or reduced levels of LDL-cholesterol in the patients' plasma (36). However, the extremely low levels of HDL-cholesterol have been shown to be a result of greatly increased catabolic rates of HDL apoproteins with moderately reduced synthesis rates (37). Moreover, Schaefer and Brewer (33) have reported an impaired conversion of chylomicrons to HDL in Tangier disease patients probably due to alterations in the amino acid composition of the apo-$AI_{Tangier}$ (32).

b) Lecithin-cholesterol acyl transferase deficiency (LCAT): The clinical features of this disease are corneal opacity, anaemia, proteinuria and nephropathy (38,39). It is characterised by the

absence of LCAT, the major cholesterol esterifying enzyme (Figure 7), from plasma. This results in a low concentration of esterified cholesterol in plasma and the presence of high concentrations of free cholesterol (38).

All lipoprotein fractions are affected and have abnormal structures and compositions (40,42), but the most severely altered fraction is HDL. The plasma concentration of this particle is very low and its morphology is changed so that it appears as discoidal bilayers which tend to form rouleaux. The bilayer discs comprise phospholipid sheets containing some cholesterol and stabilised by the HDL apoproteins (41,42). They can be converted into normal spherical HDL particles if they are incubated with purified LCAT preparations (42).

c) Fish eye disease: This condition is characterised by severe corneal opacities causing impaired vision, hypertriglyceridaemia and very low levels of HDL-cholesterol. Relatives of patients also have reduced HDL-cholesterol levels but they remain asymptomatic. In spite of the extremely low HDL-cholesterol levels in this disease, it is not apparently characterised by premature atherosclerosis (43,44).

d) Milano-AI-apoprotein disease: Patients with this condition have moderate hypertriglyceridaemia and very low levels of HDL-cholesterol.

LCAT and lipoprotein lipase activities are normal. Moreover, no clinical signs of atherosclerosis have been reported either in overt cases or in their blood relations.

Indeed, despite their hypertriglyceridaemia and low HDL-cholesterol levels the patients themselves and their relatives were healthy (45).

The condition derives from the presence of an abnormal apo-AI, designated apo-AI_{Milano}, in the HDL class which differs from the normal apo-AI by two amino acid residues, cysteine and isoleucine. These residues are not present in the amino acid sequence of normal apo-AI.

However, this variant form of apo-AI has physical properties similar to the normal apo-AI in that it has the same electrophoretic mobility on SDS and a molecular weight of 28,000; moreover, it reacts with monospecific apo-AI antiserum. The presence of cysteine residues (2 mol/mol AI) causes the formation of intermolecular disulphide bonds which account for the presence of apo-AI dimers (molecular weight = 55,000) as well as the apo (AI-AII) complex (molecular weight = 35,000) in the HDL fraction of patients with this disease (46).

1.2.3: Factors associated with increased HDL-cholesterol levels:

A number of conditions have been identified as being associated with increased concentrations of HDL-cholesterol. Among these are the following:

1. Race: Some ethnic groups tend to have higher HDL-cholesterol levels than the reported normal average. Most notable of these groups are the Greenland Eskimos (47), the Jamaican hill farmers (7) and Black Americans (48).

2. Other factors: Several studies have shown that regular exercise (49-51), oestrogen administration (52) and reductions in body weight (53) are associated with increased concentrations of HDL-cholesterol.

Other agents have been reported to induce an increase in HDL-cholesterol concentrations such as the exposure to chlorinated hydrocarbon pesticides (54), phenytoin therapy (55) and excessive alcohol intake (56). The increase caused by the latter may be a result of excessive lipolysis of VLDL, and the formed HDL is probably abnormal (57,58).

3. Hyper-alpha-lipoproteinaemia: This rare disorder is characterised by longevity and apparent absence of significant atherosclerosis (59-63).

1.3 Heterogeneity of HDL

All the studies mentioned above consider HDL as a homogeneous class of lipoprotein; but in fact it has been known from early investigations that HDL is a heterogeneous group of particles which can be divided into many subclasses depending on the method of separation. Each of these subfractions may have differing functions and possibly different antiatherogenic potential. For example, it has been reported that the increase in HDL levels in runners is mainly due to the increase in the HDL_2 subfraction (d 1.063-1.125 kg/l) levels, while no significant difference in HDL_3 (d 1.125-1.21 kg/l) levels has been observed (49). In some cases of familial hyper-alpha-lipoproteinaemia a gross increase in HDL_2 accounts for most of the rise in HDL concentration (63). On the other hand, IHD patients have significantly lower HDL_2 levels than controls, the difference in HDL_3 levels being less pronounced (64). Furthermore in patients with angiographically defined coronary artery disease, a strong negative correlation has been reported between coronary scores and HDL_2-cholesterol concentration. The correlation between total HDL cholesterol concentration and coronary score is much weaker (15).

The question arises as to which subclass of HDL is the effective candidate as the antiatherogenic factor. To answer this we must separate and examine individual sub-population of particles.

1.3.1 Separation of total HDL from plasma.

1) Total HDL can be separated from plasma by preparative ultracentrifugation (65,66) at a flotation density of 1.063-1.21 kg/l. This is achieved by raising plasma density to 1.063 kg/l and centrifuging at 100,000 g for 20 hours. The upper layer which contains VLDL and LDL fractions is removed. The density of the infranatant is raised to 1.21 kg/l and recentrifuged; HDL is separated as the top layer.

2) Agarose column chromatography has been used to prepare total HDL. Lipoproteins are separated from plasma at d 1.225 kg/l by preparative ultracentrifugation, then the lipoprotein fraction is applied to an agarose gel chromatography column (4-6% w/v). Three peaks are obtained: peak I, II and III corresponding to VLDL, LDL and HDL fractions, respectively (67).

3) A precipitation technique has been used to separate total HDL from plasma. In this method the apo-B containing lipoproteins, ie VLDL and LDL, are precipitated from plasma by addition of a polyanion, eg heparin, and a divalent cation, eg Mn^{++}, to the plasma. The mixture is then incubated at 4°C for 30 minutes then centrifuged at low speed to separate the precipitated complex. Thereafter the supernatant is adjusted to a solution density of 1.23 kg/l and recentrifuged in a fixed-angle rotor for 22 hr at 105,000 g to isolate the HDL (68,69).

1.3.2 Subfractionation of HDL

1) Preparative ultracentrifugation:

Preparative ultracentrifugation can be used to fractionate total HDL into two main subclasses, ie HDL_2 and HDL_3, in two ways:

a) The density of the first infranatant, obtained after the separation of VLDL and LDL, is adjusted to d 1.125 kg/l then subjected to centrifugation under the same conditions used to separate total HDL. The top third of the tube, after completion of centrifugation, contains the HDL_2 fraction. The infranatant in the lower two thirds of the tube is then adjusted to d 1.21 kg/l and subjected to centrifugation under the same conditions; HDL_3 is separated from the top third of the tube (65).

b) Alternatively the washed total HDL fraction prepared in the preparative ultracentrifugation method, is dialysed against a NaBr solution of d 1.11 kg/l then centrifuged for 40 hours at 140,000 g and 16°C. During this time, HDL distributes over the entire length of the tube with two concentration maxima, one in the top third of the tube which contains HDL_2 and the other in the bottom two thirds of the tube which contains HDL_3 (66).

2) Analytical Ultracentrifugation: this technique has been considered as the most reliable tool for studying the purity and distribution of HDL, it also provides a

mean for the quantitation of its density subfractions (66). The Schlieren pattern of HDL shows two main subfractions, one between flotation coefficients $F_{1.20}$ 0-3.5 and the other between $F_{1.20}$ 3.5-9 corresponding to HDL_3 and HDL_2 respectively (Fig. 2). Anderson et al. have resolved the Schlieren pattern of HDL, by computer-fitted peak analysis, into three main subfractions called HDL_3 ($F_{1.20}$ 0-3.4), HDL_{2a} ($F_{1.20}$ 1.5-5) and HDL_{2b} ($F_{1.20}$ 3-8) (70,71).

3) Rate Zonal Ultracentrifugation: this method separates HDL into its two main subfractions HDL_2 and HDL_3 directly from plasma in one centrifugation step. This is done by forming a non-linear density gradient from d 1.00 to 1.4 kg/l in the zonal rotor.

The sample solution, adjusted to d 1.4 Kg/l, is injected into the rotor and centrifuged. During ultracentrifugation, lipoproteins of similar densities band in zones under the influence of the centrifugal field. At the end of the run, the rotor effluent is passed through a flow cell where its absorbance is monitored continuously at 280 nm and recorded on a chart. The elution pattern has two well separated maxima corresponding to HDL_2 and HDL_3 (72,73) (Fig. 3).

Quantitation of these HDL_2 and HDL_3 subfractions can be made from the elution pattern by appropriate integration of the area under the curve. This value together

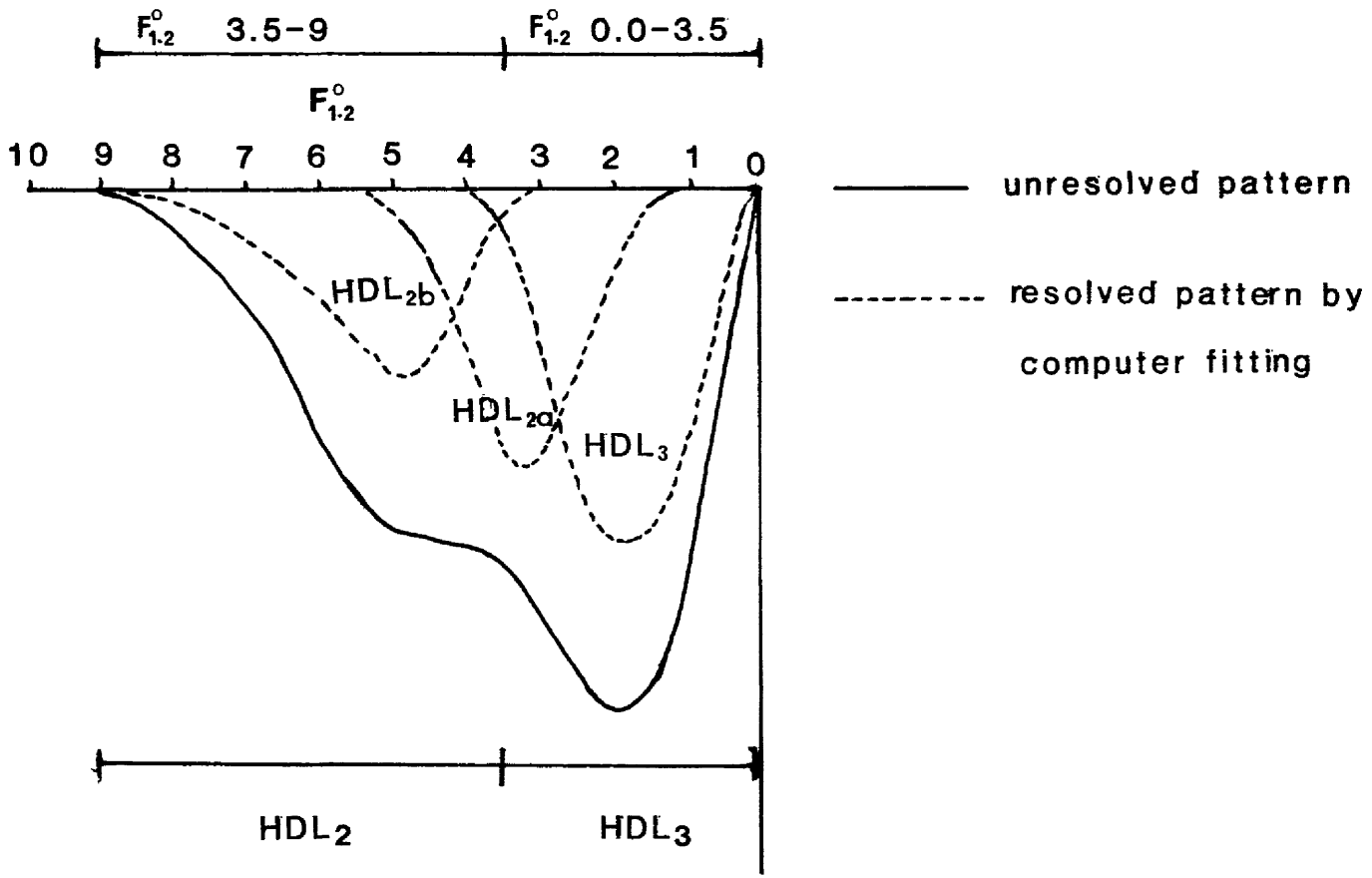


Figure 2. Analytical Ultracentrifuge pattern of HDL.

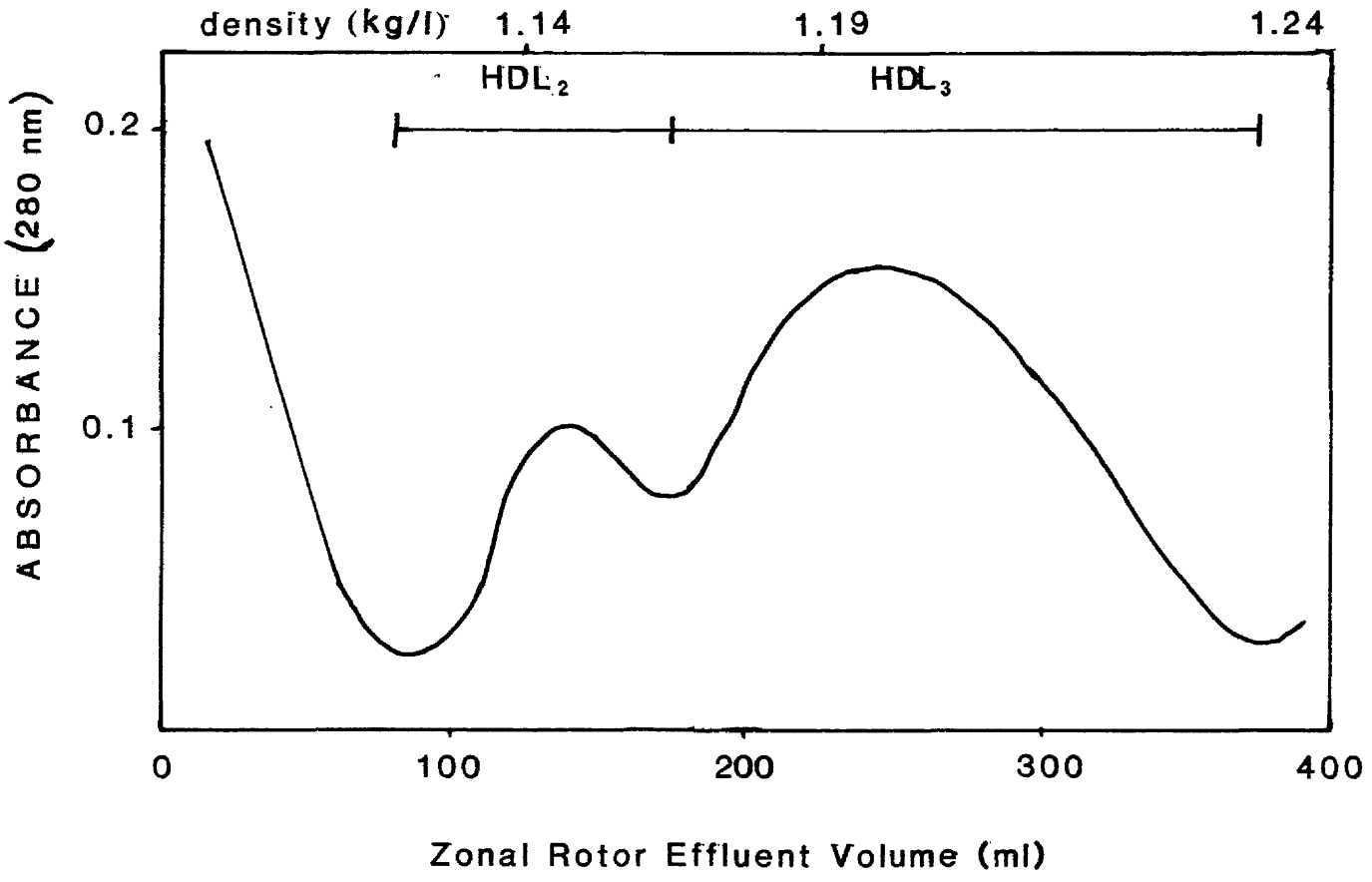


Figure 3. Rate zonal ultracentrifugation pattern of HDL.

with knowledge of the chemical composition and the extinction coefficients of these two subfractions (29) can provide an estimate of lipoprotein concentration.

4) Heparin-Sepharose affinity chromatography has been used to subfractionate HDL into two main subfractions: HDL without apo-E and HDL with apo-E (Fig. 4)(74).The latter has a cell receptor binding capability *in vitro*.

Other methods eg isoelectric focusing (75) and hydroxyapatite column chromatography (76) have been shown to subfractionate HDL into many small fractions of different compositions.

1.4 Properties of HDL

1.4.1: HDL Composition

Human high density lipoproteins lie in the density range 1.063-1.21 kg/l and can be fractionated in the preparative ultracentrifuge, into two major components, HDL_2 (d 1.063-1.125 kg/l) and HDL_3 (d 1.125-1.210 kg/l).

These subfractions consist of protein in the form of different apoproteins and lipids, comprising free and esterified cholesterol, phospholipids, and a small amount of triglycerides. Their chemical composition may differ depending on the method of isolation; in addition, a difference in composition has been reported between males and females. Typical compositions in the sexes are shown in Table 3 (30).

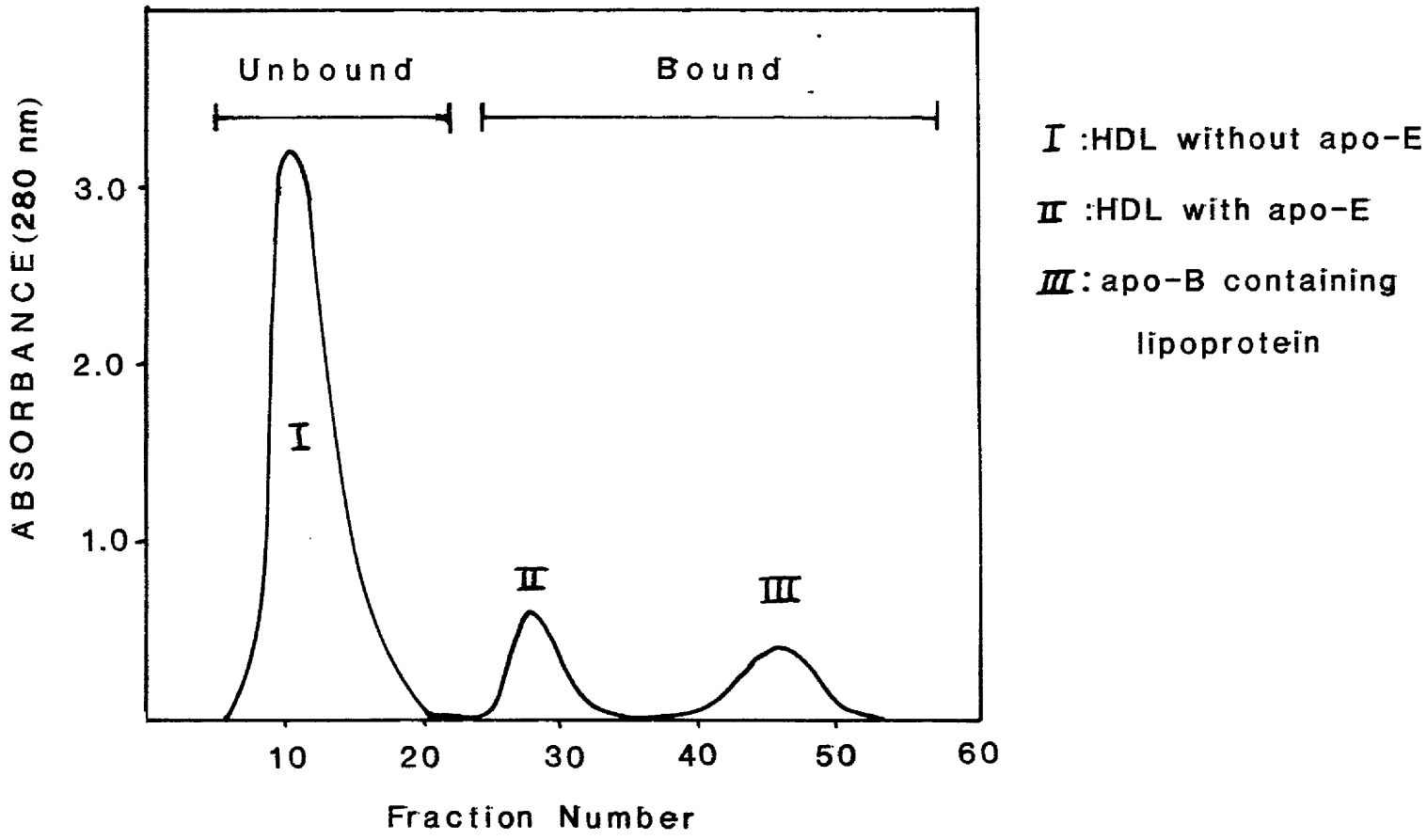


Figure 4. Heparin-Sepharose affinity column chromatography of human HDL.

Table 3: HDL Subfraction Compositions in Normals

		% Cholesterol		% Triglyceride	% Phospholipid	% Protein	apo-AI/ apo-AII ratio
		Free	Esterified				
HDL_2	males	6.7	18.6	3.6	26.4	44.7	4.7
	females	6.0	17.8	2.6	29.6	44.0	7.1
	p<	.02					.01
HDL_3	males	2.8	15.7	2.1	16.3	53.1	2.6
	females	3.4	16.7	2.0	24.9	53.0	2.9
	p<	.05					

Males: n = 13 Females: n = 12

Data taken from reference 29.

1.4.2: HDL Apoproteins

The major apoproteins of HDL are apo-AI and apo-AII, together comprising 90% of the HDL apoprotein. Other minor apoproteins present in the HDL density class include apo-CI, apo-CII, apo-CIII, apo-D, apo-E and apo-F. Apo-C's and apo-E are also found to a large extend in the VLDL density class and are considered primarily as VLDL apoproteins.

These apoproteins have different molecular weights and they differ in their amino acid compositions. The approximate molecular weights of these apoproteins are documented in Table 4 (66).

The minor apoproteins of HDL D and F are poorly investigated and little is known about their distribution among other lipoprotein density classes.

Analysis of the distribution of individual apoproteins in HDL subfractions, HDL_2 and HDL_3, isolated by rate zonal ultracentrifugation (Table 4) shows that apo-AI and apo-AII are the major constituents of both subfractions. It is generally found that the ratio of apo-AI to apo-AII is higher in HDL_2 than in HDL_3.

1.4.3: Properties of HDL Apoproteins

i) Structure of Apo-AI

Apo-AI is a single polypeptide chain of 245 amino acids, and is devoid of cysteine, cystine, isoleucine and carbohydrate (77). It has limited water solubility between pH 4.0 and 7.0, but it is readily soluble in

Table 4: Apo-HDL molecular weights

apoprotein	Molecular weight	% (wt/wt) of total apolipoprotein in	
		HDL_2	HDL_3
AI	28300	89.4	73.0
AII	17400	6.1	20.4
CI	7000	1.3	1.5
CII	9500	0.5	0.3
CIII	8700	2.7	3.1
D	20000	0.2	1.7
E	39000		
F	27000		
apo-AI/apo-AII ratio		14.7	3.6

Data taken from reference 66.

buffers containing 8M urea, 3M guanidine hydrochloride, 0.1% sodium dodecyl sulphate (SDS), or 1-2M acetic acid (66).

In solution, apo-AI undergoes self-association forming dimers, tetramers and octamers depending on the concentration of the protein (78) and on the ionic strength of the solution; the higher the ionic strength the greater the association (79,80). Moreover, the rotor speed has been reported to have an effect on the self-association of apo-AI during determination of the molecular weight by sedimentation equilibrium technique; lower speeds enhance association (80).

Circular dicroism studies (81) have shown that apo-AI in native HDL has a highly helical structure in which 70% of the protein is present as an alpha-helix, while the rest is in the form of a beta-sheet (5-15%) or is disordered (15-20%). Delipidation decreases the helical content by about 20% with a corresponding increase in the disordered structure. However, upon isolation of apo-AI from HDL in 6M urea and dialysis, apo-AI has been found to contain 55% alpha-helix, 8% beta-structure, and 37% disordered structure (77).

ii) Structure of Apo-AII

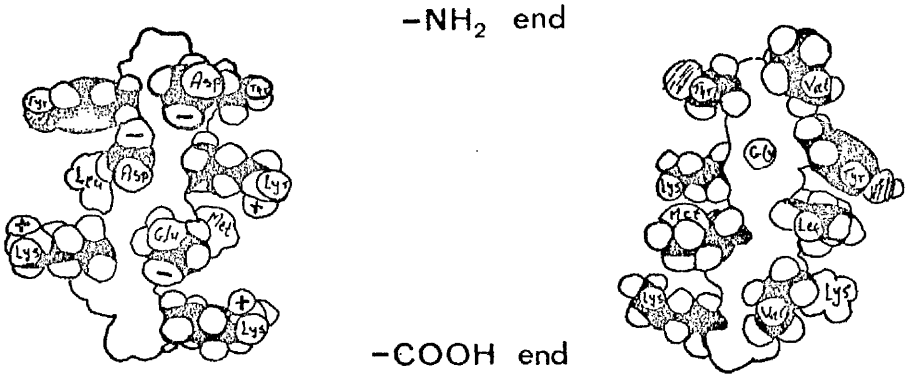
Apo-AII contains two identical polypeptide chains of 77 amino acids each, linked by a symmetrical sulphide bridge at cystine-6. The molecule is devoid of histidine, arginine, tryptophan, and carbohydrate. In solution, apo-AII, like AI, undergoes self-association, which has been described by a monomer-dimer-trimer step

association (82). Studies of its secondary structure using circular dicroism shows that apo-AII, after isolation from HDL in 6M urea and dialysis, contains 35% alpha-helix, 13% beta-structure, and 52% disordered structure (81). It has an apparently less ordered structure than apo-AI, since apo-AI showed 55% alpha-helical structure and 15-20% disordered structure under the same conditions.

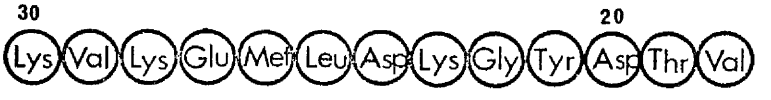
iii) Amphiphilicity of apo-AI and apo-AII

The helical structures present in these apoproteins have been shown to have amphipathic properties in that they contain two clearly defined faces, one polar, the other hydrophobic. This amphiphilicity arises from the constituent amino acids residues being arranged in such a way that those with non-polar groups appear on one side of the helix, while amino acids with polar groups are all present on the other side (Fig.5). The polar (charged) amino acids have been found to be located in the following manner: the negatively charged residues glutamic acid (Glu) and aspartic acid (Asp) invariably occur in a strip along the centre of the polar face, while the positively charged residues, lysine (Lys) and arginine (Arg) are located on the lateral edge of the polar face, alternating from side to side (83,84).

The alpha-helix so constructed locates the positive charged residues at the periphery so that their relatively apolar side chains are available for hydrophobic



A. alpha-helical structure of the peptide segment B.



B. Amphipathic peptide segment from apo-AII

Figure 5. Amphiphilicity of apo-AII. Diagram A shows the building of the helical segment B by the CPK-space-filling model. The polar face represents one side of the helix where its axis is oriented parallel to the plane of the page and the N-terminal end towards its top. The non-polar face shows the other side of the helix when it is rotated around its axis by 180°. Phospholipids and cholesterol polar ends interact with the polar face and neutral lipids with the non-polar face.

interactions with the non-polar acyl chains of phospholipids and the polar heads of these phospholipids interact with the polar surface of the helix (83,85).

This amphiphilicity explains the ability of these apoproteins to bind lipids, ie phospholipids and cholesterol, and interact with non-polar lipids ie cholesteryl ester and triglycerides, to form stable psuedomicellar lipoprotein complexes. Cholesteryl esters alone do not bind significantly to HDL apoproteins, whereas phospholipids bind in large quantities. Moreover, while delipidation of HDL decreases the helical content of its apoproteins by 20%, reconstitution of apo-HDL with phospholipids and cholesteryl esters restores all the initial helical content of the apoproteins. Reconstitution with phospholipids alone does not restore all the initial helical content (81,86). This evidence shows that both phospholipids and cholesteryl esters are necessary for the complete reorganisation of the secondary and tertiary structure of HDL.

Combination of apo-AI and apo-AII with lipids leads to the formation of lipid protein complexes which can be separated as lipoproteins by density gradient ultracentrifugation. Thus, when phospholipid, cholesterol and cholesteryl ester are combined with these apoproteins they form lipoprotein complexes similar in their physical properties to native HDL molecules (81,87).

iv) Interaction between apo-AI and apo-AII

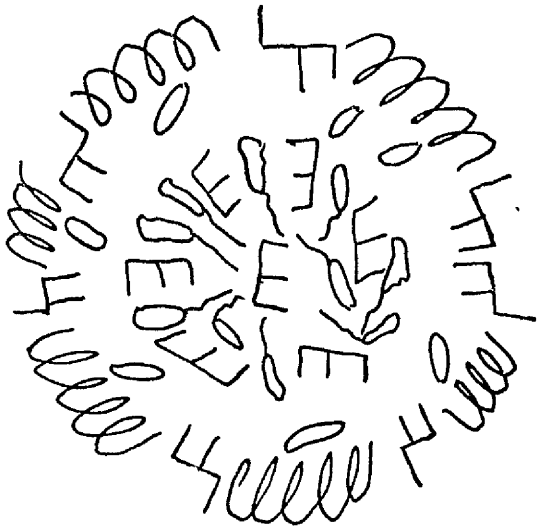
It has been reported (88) that when apo-AI and apo-AII are present together in solution in a molar ratio of 1:1, they behave as a single molecular species. This molecular species has a molecular weight of 50,000, (similar to the sum of the molecular weights of the two apoproteins which equals 46,300) and shows a single boundary during sedimentation equilibrium. Evidence for an association between the two major HDL proteins was also obtained from detergent binding studies. The number of sites available to bind SDS was found to be less than the additive number of binding sites of individual apoproteins. This decrease in the number of binding sites led to the suggestion that some of these sites are involved in the association between apo-AI and apo-AII. Apo-AI has been reported to bind less lipids than apo-AII and the presence of apo-AII with apo-AI increases the binding capacity of apo-AI (77,85). Such protein-protein interactions may have important implications in the substructure of HDL as discussed in this thesis.

1.4.4: Structure of HDL

The study of recombinant particles of apoproteins AI and AII with lipids contributed to the understanding of the structure and physical properties of native HDL particles. These studies have shown that an HDL particle, similar to native HDL in its physical properties, can be reconstituted from individual lipid components.

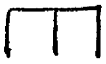
The major lipid-protein interaction in HDL is between the phospholipid and the apoproteins. As mentioned earlier, cholesterol alone binds to apo-AI or apo-AII much less than phospholipid (81,86,89,90). In addition the recombination of neutral lipids to these apoproteins occur only in the presence of phospholipid (81). The conformational stability of these apoproteins increase on interaction with lipids (81) since the alpha-helical structure increases on such interaction.

Plasma HDL can be envisaged as a spherical particle where the polar lipids and apoproteins are on the surface and the apolar lipids in the core (Fig. 6). The presence of the apoproteins on the surface of HDL particle has been confirmed by X-ray scattering studies. The scattering patterns can be explained by a model in which an electron deficient core (containing cholesteryl ester) is surrounded by an electron dense shell consisting of apoproteins and polar head groups of phospholipid and cholesterol (85,91). The thermal behaviour of cholesterol ester core does not show a liquid crystalline transition between 0-60°C which indicates that cholesteryl ester in the HDL core exists in an unorganised phase; while cholesteryl ester isolated from HDL shows reversible liquid crystalline transitions between 20-40°C. X-ray scattering studies of HDL support the view that there is an unorganised phase in the HDL core. This unorganised core has been attributed to either: a) a small core size which is inadequate to accommodate organised structures of cholesteryl ester (91) or b) the



Apoprotein

Phospholipid



Triglyceride

Cholesterol

Cholesteryl ester

Figure 6. Structure of spherical HDL.

presence of an interaction between cholesteryl ester molecules and protein-phospholipid surface of HDL, which prevents the formation of an organised lipid phase (91,92).

1.4.5: Stability of HDL particle

HDL molecules are not rigid entities of fixed composition, but rather comprise a wide spectrum of particles varying in size, flotation density, and molecular weight. A dynamic relationship exists between these particles, and protein exchange may take place among them.

In the HDL particle some apo-AI is less tightly bound than the apo-AII, so that repeated ultracentrifugation releases about half of the former protein. Moreover, apo-AI can be dissociated from HDL on exposure to low concentrations of guanidine hydrochloride. On the other hand, high concentrations of the latter release apo-AII from HDL accompanied by more disruption of the lipoprotein particle (91). This release of apo-AI from the HDL particle has been confirmed by thermal disruption studies using differential scanning calorimetry (92,93). HDL can be denatured irreversibly when heated slowly between 60-90°C where a double-peak endotherm is obtained. The first component of the endotherm corresponds to a selective release of apo-AI from HDL, and the second component corresponds to a more generalised disruption of lipoprotein structure with the release of cholesteryl ester and apo-AII. In each treatment, ie exposure to guanidine hydrochloride and heating, the

remaining residue of the HDL particle, after the release of apo-AI, retains the spherical structure of HDL, but is larger in size. This suggests that the lipoprotein particles fuse after they have been depleted of their emulsifying apo-AI. Generalised disruption of HDL by high temperatures leads to the separation of cholesteryl ester domains and the formation of phospholipid vesicles and discs (91). The enthalpy of denaturation of apo-AI in the HDL particle is only slightly greater than that of lipid free apo-AI, suggesting that apo-AI in spherical HDL is only marginally more stable than in its lipid-free form. Thus, it is probable that an equilibrium exists between apo-AI in spherical HDL and small amounts of apo-AI free in solution. In the presence of acceptors for apo-AI (eg phospholipid vesicles), this free apo-AI will bind to the acceptors, leading to further dissociation of apo-AI from spherical HDL and, eventually, destabilisation of the spherical HDL particle (91,93).

Incubation of HDL at 37°C with turbid suspensions of dimyristoyl lecithin (DML) decreases the turbidity as a function of the amount of HDL added till it becomes clear. Discoidal bilayer phospholipid apoprotein complexes are formed which indicates that the phospholipid multi-lamellar liposomes are broken down and solubilised by the released apo-AI (93).

Similarly, incubation of HDL_2 or HDL_3 with dimyristoyl lecithin leads to a decrease in their density, due to the loss of apo-AI, and an increase in their size which may be attributed to a process involving

fusion of apo-AI depleted lipoproteins. The formed dimyristoyl lecithin-apo-AI complexes are discoidal and contain traces of the HDL lipid and apo-AII (93,94). This incorporation of phospholipid and protein into HDL particles is thought to occur in vivo.

The phenomenon of the release of apo-AI from HDL particles by physicochemical means has been extended to study the displacement of apo-AI by apo-AII in human and canine HDL (95,96). Canine HDL contains primarily apo-AI and no apo-AII, and each HDL particle contains three apo-AI molecules. Displacement studies have shown that apo-AII can displace apo-AI from HDL in steps, and two molecules of apo-AII displace one molecule of apo-AI; the process can replace all the three apo-AI molecules by six apo-AII molecules. All of the hybrid particles retained the physicochemical properties of native HDL. This displacement process is rapid and independent of temperature, ionic strength, and apo-AII initial concentration (ie variation in its state of association). The ability of HDL to accommodate the extra mass of protein derived from the interchange of apo-AII with apo-AI (replacement of about 28,000 daltons of apo-AI by 35,000 daltons of apo-AII) has been attributed to the fact that apo-AII has a smaller limiting area per amino acid than apo-AI (95).

The surface properties of apo-AI and apo-AII have been studied at air-water interface where these apoproteins readily form stable monolayers. Different kinetic processes are responsible for the formation of these monolayers; apo-AI forms an adsorbed monolayer by a

diffusion-controlled process in which apo-AI molecules at the interface are in equilibrium with those in solution through rapid adsorption and desorption. On the other hand, apo-AII adsorbs to the interface irreversibly with a rate limiting step that presumably involves a conformational change.

From these differences in surface properties of apo-AI and apo-AII, the substitution of apo-AII for apo-AI in HDL can be interpreted as a combination of displacement, due to the relatively high surface affinity of apo-AII, and its ability to irreversibly occupy the surface left by the desorbed apo-AI molecules. According to this concept, any transfer of apo-AII from one particle to another would be expected to occur via surface collisions and not through a fluid phase, the latter being likely to occur in the case of apo-AI exchange (95).

1.5 Origin and Catabolism of HDL

1.5.1: HDL structure in vivo

Plasma HDL exists normally as spherical particles composed of an apolar core of cholesteryl ester and triglyceride which is stabilised by a shell of phospholipid and apoproteins (Fig. 7). It is believed that this form of HDL is the product of a series of intravascular metabolic processes on the initially secreted particle. There is good evidence that the precursors of this mature HDL are bilayer discs (nascent HDL) composed primarily of phospholipid, free cholesterol and apo-

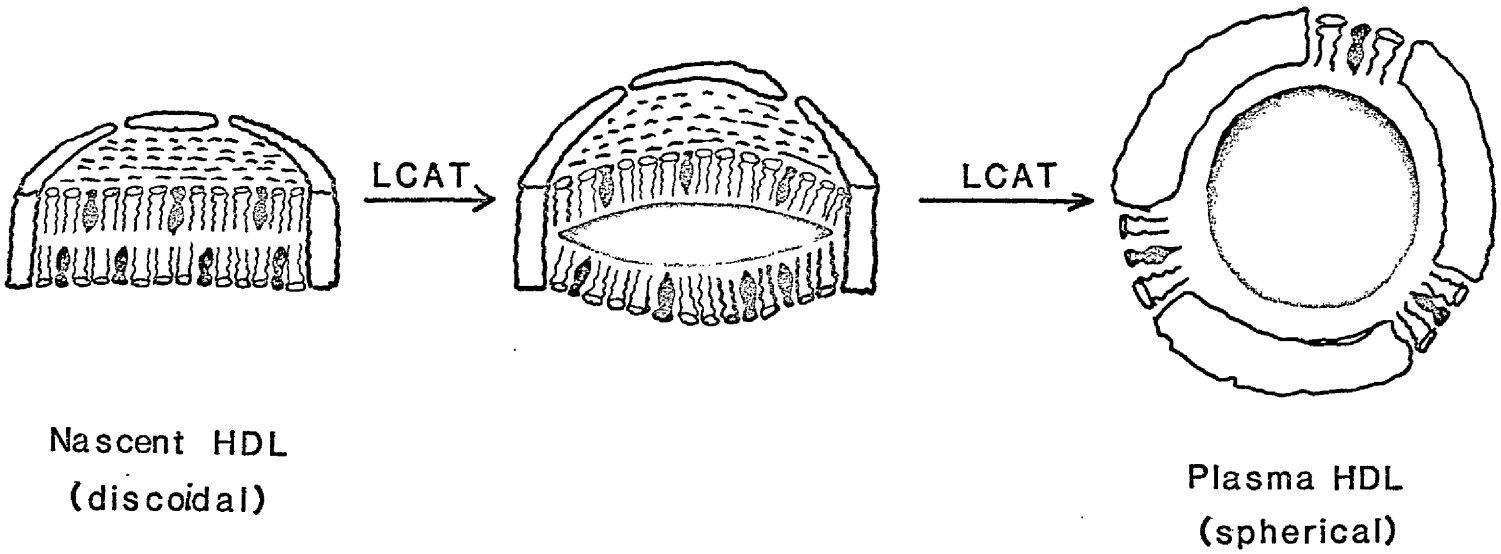


Figure 7. HDL formation from nascent bilayer discs.

protein with little cholesteryl ester, the apoprotein encircling the edges of the lipid bilayer. Nascent HDL particles are transformed into mature (spherical) HDL by the action of the enzyme lecithin:cholesterol acyl transferase (LCAT) which esterifies cholesterol by transferring a fatty acid group from lecithin to cholesterol with the formation of lysolecithin (Fig. 8) and the newly formed cholesteryl ester migrates to the interior (core) of the HDL particle.

It has been reported that nascent HDL is a good substrate for the LCAT reaction (90) since it contains apo-AI which is known to activate the enzyme (91).

Patients with inherited (familial) (42) and acquired (cholestasis (97,98) and alcoholic liver disease (98,99)) LCAT deficiency have discoidal HDL particles rich in phospholipid, unesterified cholesterol, and apo-E (4nm thick and 15-20 nm in diameter) which tend to form rouleaux structures. When these discoidal particles are purified from the patients' plasma and incubated with normal plasma or purified LCAT, they are transformed into spherical particles similar to the normal HDL (9-11 nm diameter) (42). Moreover, clinical improvement of the acquired LCAT deficiency is associated with a return of the discoidal HDL to the normal spherical HDL (99). Similar discoidal structures are formed when apo-HDL is recombined with phospholipid-cholesterol mixtures, which upon the action of LCAT are transformed into spherical particles resembling the native HDL in size and shape (100).

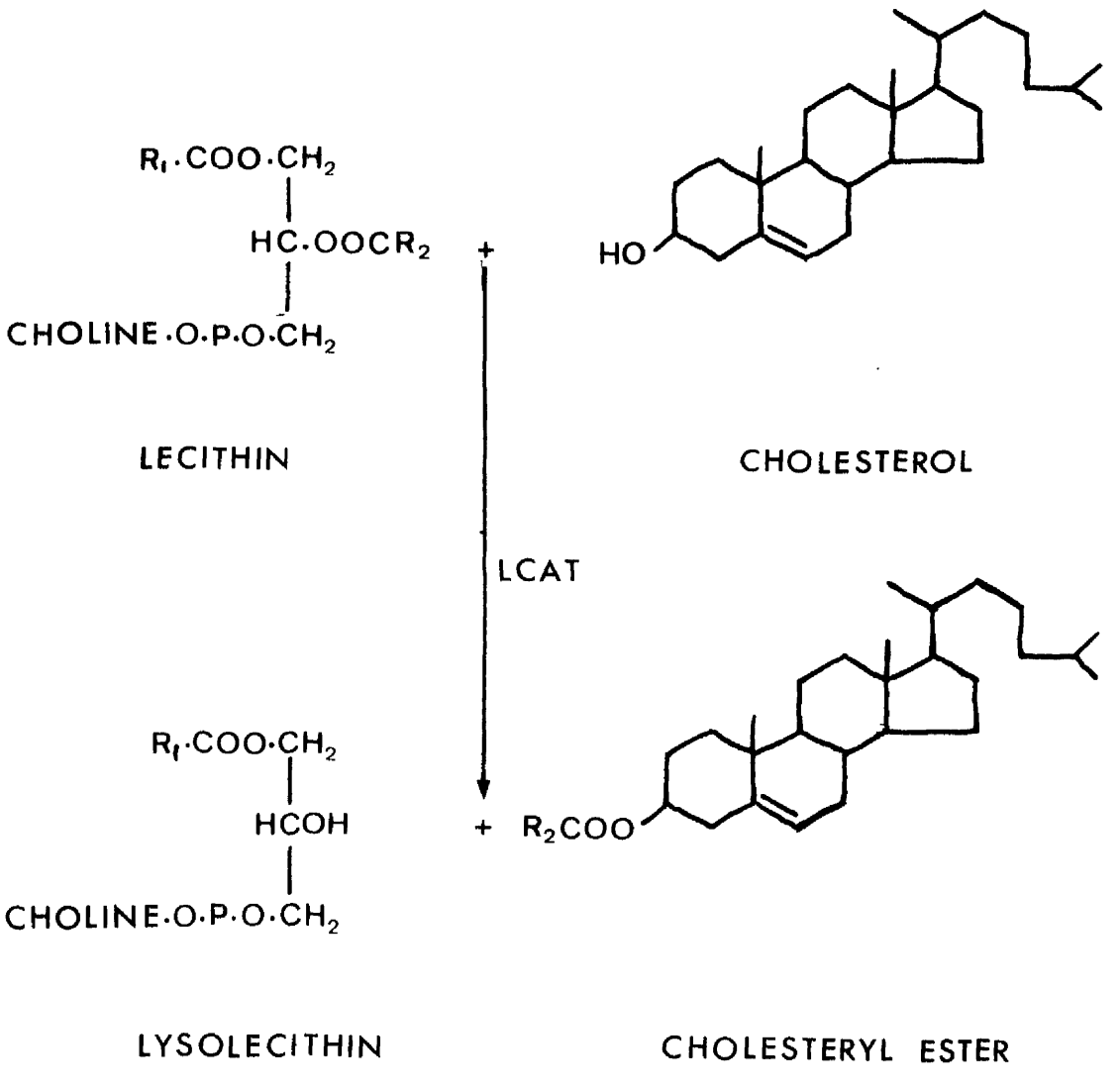


Figure 8. The LCAT reaction.

1.5.2: Sources of HDL

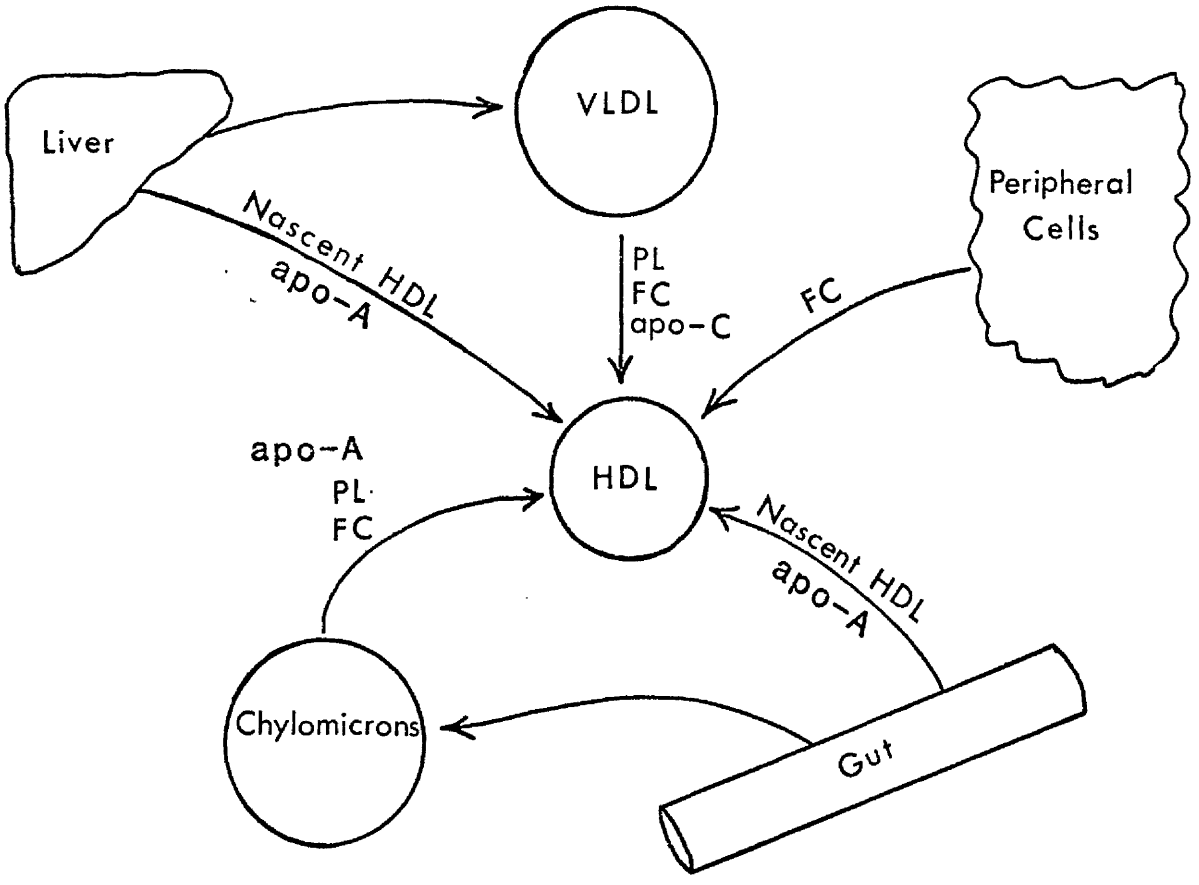
Nascent HDL is derived from hepatic and intestinal secretion, as well as from lipolysis of triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins (Fig. 9)).

1) Hepatic secretion: Perfusion experiments performed on rat liver using either the non-recirculating (101) or recirculating (102) technique, have shown that the liver can synthesise nascent discoidal HDL particles rich in phospholipid and poor in cholesteryl ester with apo-E as the major apoprotein; in contrast to the circulating HDL in which apo-AI is the major apoprotein. A similar pattern has been observed in perfusates of orotic acid-fatty livers, which secrete very little VLDL, ruling out the possibility that HDL is produced from lipolysis of VLDL in the perfusate (101).

Rat liver has been found to secrete 44% of the total plasma apo-AI and almost all of its apo-E (104).

Liver secretion of HDL does not account for all the HDL present in circulation. Hepatectomised animals still incorporate radioactive amino acids into plasma HDL suggesting a nonhepatic source of HDL apoproteins (103).

2) Intestinal secretion: Discoidal nascent HDL particles have been found in mesenteric lymph from fasting rats. These particles are enriched in phospholipid and deficient in cholesteryl ester when compared to serum HDL. Mesenteric lymph from fat-fed rats also contains discoidal HDL, but the ratio of phospholipid to chol-



PL : phospholipid

FC : free cholesterol

Figure 9. Origin of HDL components.

esteryl ester is higher than in the fasting lymph, and the discoidal HDL in fatty lymph accounts for 30% of lymph HDL compared to 50% in fasting lymph (105). Fasting lymph HDL is rich in apo-AI and deficient in apo-E, in contrast to hepatic HDL (105).Bile diversion in the rat, which depletes intestinal epithelial cells and mesenteric lymph of triglyceride-rich lipoproteins, resulted in sustained mesenteric apo-AI and HDL output (106), which may indicate that intestinal apo-AI secretion is responsive to other stimuli as well as to triglyceride absorption.

Human intestine secretes significant amounts of HDL apoproteins, apo-AI and apo-AII. Cultured human intestinal mucosa has been found capable of secreting newly synthesised apo-AI and the secretion is enhanced three fold in the presence of micellar lipid solutions (107). Immunofluorescence techniques (108) applied to intestinal biopsy specimens have shown that fat ingestion promotes apo-AI synthesis in the gut in agreement with the observation *in vivo* that the apo-AI and apo-AII content of mesenteric lymph from chyluric subjects has been shown to increase after a fatty meal. The amount of apo-AI and apo-AII secreted by the intestine can account for 50 and 33% of the calculated daily synthetic rate of apo-AI and apo-AII, respectively (109).

Data on the secretion of HDL from human liver has been more difficult to obtain. Turner *et al*. (110) have isolated HDL_2 from human hepatic vein where it has been found to contain a mixture of spherical and discoidal

particles. The latter were similar to the discoidal HDL particles present in the peripheral blood of patients with LCAT deficiency. In contrast, HDL_2 isolated from hepatic artery and HDL_3 isolated from both hepatic vein and artery, were composed of spherical particles, identical to normal plasma HDL.

In addition to the difference in apoprotein composition between hepatic and intestinal nascent HDL, the ratio of phospholipid to cholesteryl ester is higher in the intestinal HDL (105). It is not known whether the different compositions of hepatic and intestinal nascent HDL reflect differing functions.

3) Formation of HDL from chylomicrons and VLDL: Chylomicrons and VLDL, derived from the small intestine and the liver, respectively, function as transporters of triglyceride from sites of synthesis to tissues requiring them for storage or for metabolic fuel. They consist of particles containing a core of mainly triglyceride which is stabilised by a monolayer surface film of phospholipid, apoprotein, and a small amount of cholesterol. These triglyceride-rich particles are partially degraded in skeletal muscle, adipose tissue and other tissues by lipoprotein lipase (LPL), an enzyme on the capillary endothelium. Lipoprotein lipase selectively hydrolyses the lipoprotein triglycerides forming partial glycerides and fatty acids that are partly taken up by the tissues and partly removed by albumin. As triglyceride is removed, the chylomicron shrinks and is released back into the circulation as a remnant particle containing the

cholesteryl ester and residual triglyceride that is rapidly assimilated by the liver (111). Fresh chylomicrons isolated from thoracic duct (112) or intestinal (108) lymph contain apo-AI and apo-AII while plasma chylomicrons contain very little or no such apoproteins which indicate the loss of apoproteins from chylomicrons. Subsequently, these apoproteins are found on HDL. The major portion of VLDL and chylomicrons apo-C and phospholipids (113,114) are transferred to HDL during lipolysis (Fig. 10). This metabolic link between the triglyceride rich lipoproteins and HDL may go some way to explaining the reciprocal relationship observed in their plasma concentrations (114).

Lipolysis of chylomicrons and VLDL leads to selective removal of triglyceride which causes shrinkage of the lipoprotein core. This in turn causes redundancy of the polar surface constituents-phospholipid, unesterified cholesterol and apoproteins. The decrease in the core volume increases the lateral pressure on the surface of the particle causing the surface monolayer to fold into bilayers that protrude from the particle (Fig. 11) (90). Such folds of bilayer membrane may be seen arising from the chylomicron surface when treated with lipase in vitro (115). Some of the bilayer may dissociate as discoidal micelles containing apo-A and apo-C in addition to the phospholipid and cholesterol. But since the amount of the apoproteins is not enough to convert all the bilayer to discs, most of the bilayer fragments may split from the particle surface as larger sheets that can form

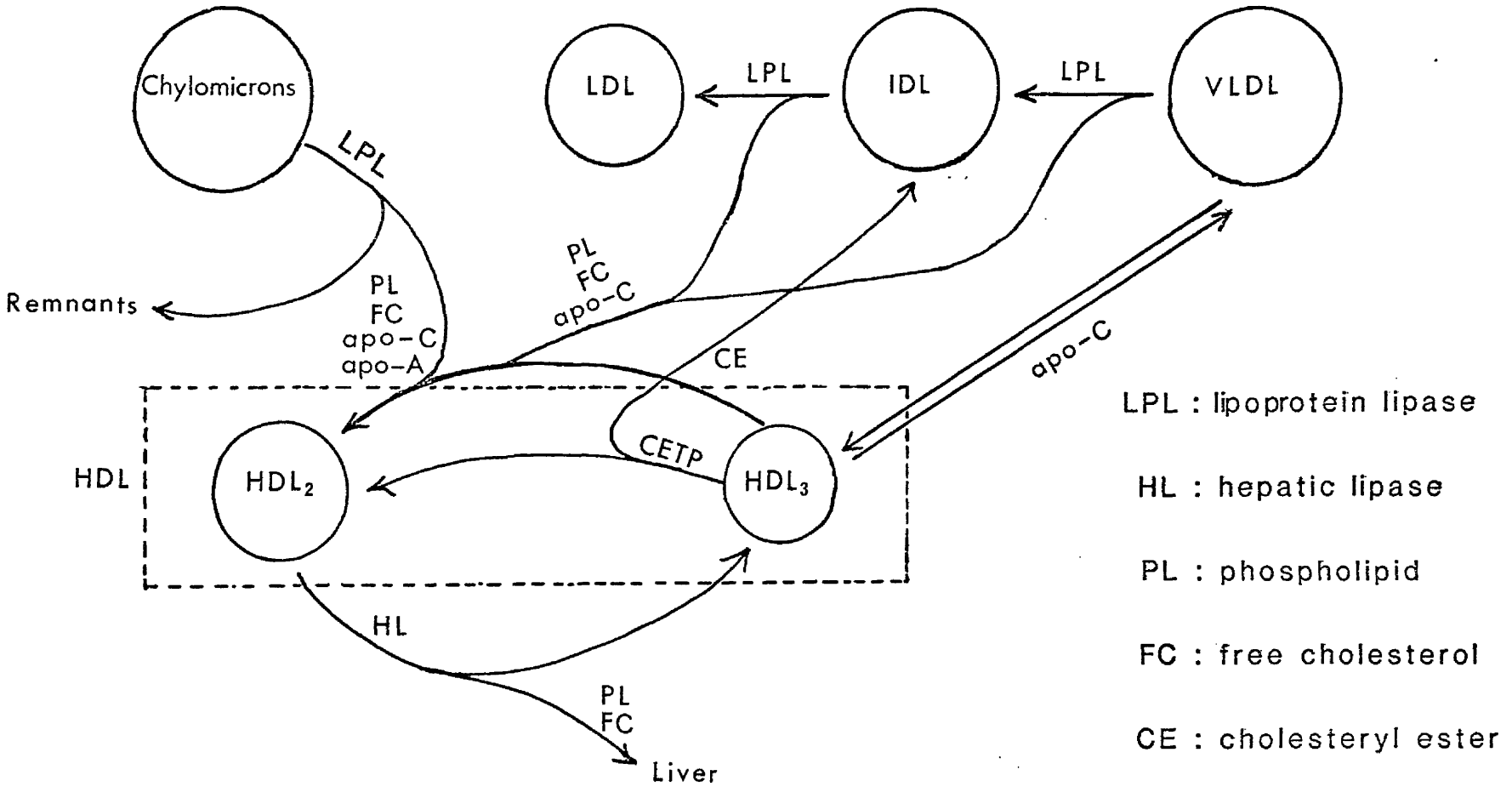


Figure 10. Metabolism of HDL.

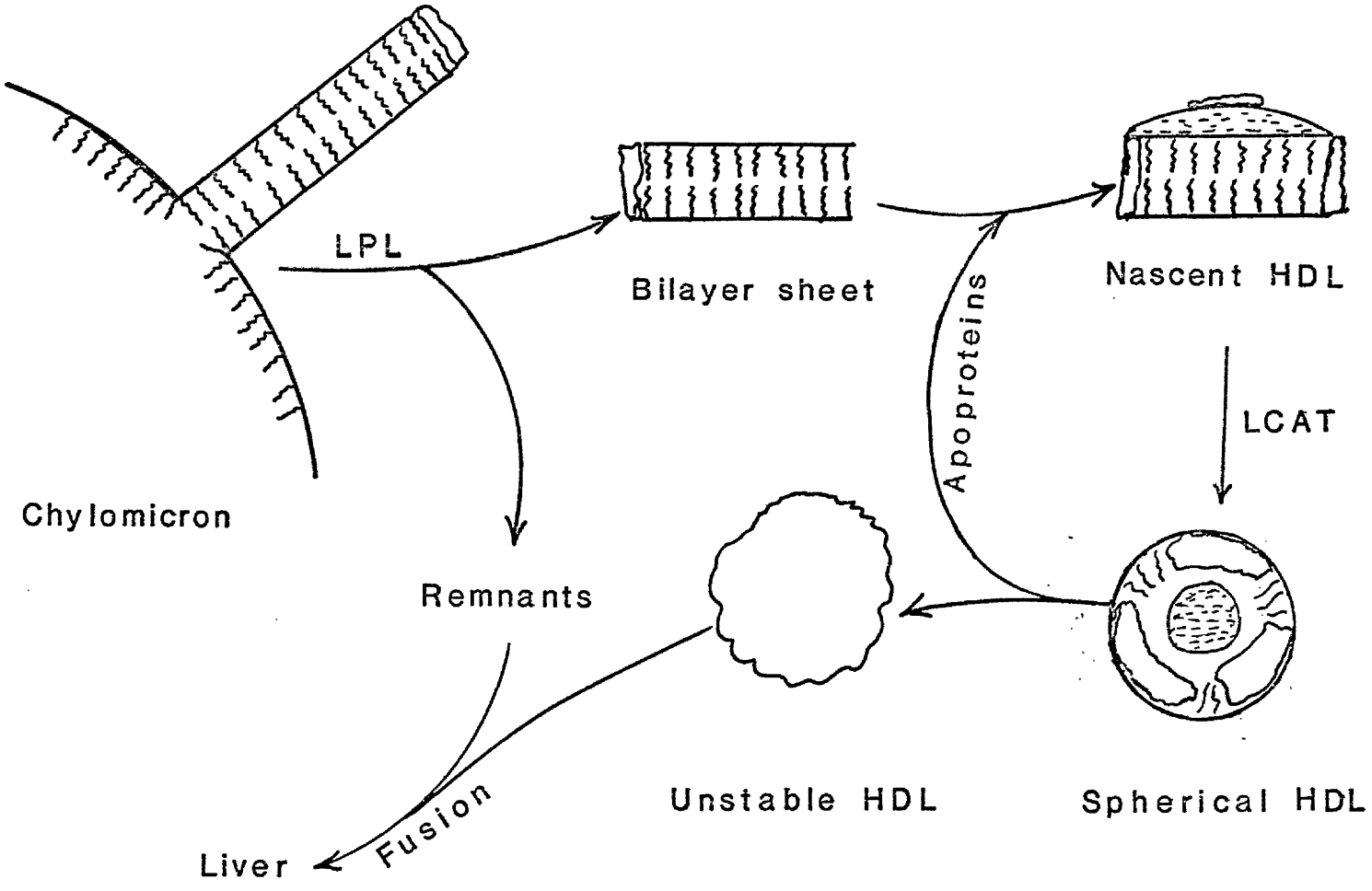


Figure 11. Formation of HDL from lypolysis products of chylomicrons. After Tall and Small (90).

phospholipid vesicles. These sheets and vesicles are converted into discs by capturing apoproteins from circulating HDL which becomes thermodynamically unstable and could fuse with the chylomicron remnants which are then avidly taken up by the liver (116). The vesicles or discs formed may be converted to spherical HDL secondary to the action of LCAT, or be inserted directly into HDL_3 producing HDL_2 (90).

1.5.3: Catabolism of HDL

HDL components are catabolised, at least in part, independently of the removal of HDL particles from the circulation. It has been demonstrated by kinetic studies that the cholesterol ester of HDL has a higher turnover rate than HDL apoprotein-A (117), which may reflect their different catabolic fates.

The liver has been considered as the major catabolic site of HDL. However, there is increasing evidence that other organs and tissues contribute significantly to HDL catabolism (118,119). In the rat, it has been shown that the liver and small intestine are the major sites of uptake of intravenously administered radioiodinated HDL. Up to 10% and 3.5% of injected dose has been recovered after 6 hr in the liver and ileum, respectively (120, 121). Moreover, the adrenal gland in the rat and monkey (122) and the kidney in the dog (123) have been shown to bind radioiodinated HDL.

A particular subfraction of HDL, HDLc, which is rich in apoprotein E, may have a distinct role different from the bulk of non-E containing HDL. As discussed above,

such particles can be isolated from normal plasma HDL_2 or induced by cholesterol feeding in animals and internalised and degraded by cell surface receptors of human fibroblasts. This binding capacity of HDLc has been abolished on modification of the arginine residues of apo-E by cyclohexanedione (124). Thus this HDL fraction may be removed predominantly by receptor-mediated processes.

1.5.4: HDL apoprotein turnover studies

Individual molecules of proteins in the body do not serve the organism for life but are continually being degraded and replaced by newly synthesised molecules, and it is the balance of these two processes which determines the actual body pool of a protein at any time. Thus the biological system can be considered as an open system in a steady state.

The different structural roles of apo-AI and apo-AII and their different stabilities in HDL as well as their different interaction modes with other lipoproteins and lipid bilayers may indicate different catabolic rates. When human radioiodinated HDL was injected into rats, it was found that its apo-AI was removed from the circulation more slowly than apo-AII (121).

Moreover, metabolic studies in man suggest disparate removal rates of apo-AI and apo-AII in normo-lipidaemia (125-127), hypertriglyceridaemia (126,127) and in

homozygous Tangier disease (125). In contrast, Blum et al. (128) have reported identical apo-AI and apo-AII removal rates in normal controls, after carbohydrate diet and on nicotinic acid therapy.

Differences in the fractional removal rates of apo-AI and apo-AII reported by different workers may be attributed to the different methodologies used.

1.5.5: The role of lipases in HDL metabolism: As mentioned before, the action of lipoprotein lipase leads to the formation of HDL_2 particles by transferring cholesterol and phospholipid from chylomicrons and VLDL to the HDL fractions (mostly HDL_3) resulting in a decrease in their density. On the other hand, hepatic lipase hydrolyses HDL_2 phospholipids resulting in a decrease in its lipid content and an increase in its density, which may lead either to its catabolism in the liver or its conversion to HDL_3 and subsequent return to the circulation. A significant negative correlation has been reported between the HDL_2-cholesterol concentration and hepatic lipase activity in normal subjects. It is noteworthy that females have higher lipoprotein lipase and lower post heparin plasma hepatic lipase activities than males, compatible with the observation that females have higher HDL_2 levels than males. These observations may explain the role of the lipases in regulating HDL metabolism where lipoprotein lipase regulates the

conversion of HDL_3 to HDL_2 and hepatic lipase removes cholesterol and phospholipid from HDL_2, converting it back to HDL_3 (Fig. 10).

1.6 Functions of HDL

HDL plays an important role in lipoprotein metabolism which may explain its function as an anti-atherogenic factor. The main functions of HDL are:

1) It is the principal substrate for plasma LCAT, the enzyme responsible for esterification of most of the cholesterol in plasma (130). Apo-AI in HDL is the main activator of the enzyme LCAT (131). In contrast, lighter density classes of HDL (132) inhibit the action of LCAT. Moreover, it has been reported that apo-AII, incorporated into lecithin-cholesterol vesicles, has no effect on LCAT activity, but when it is added to vesicles incorporating apo-AI, it inhibits the LCAT activity via the displacement of apo-AI from the surface of the vesicle, and the LCAT activity is proportional to the number of apo-AI molecules in the vesicle (133).

2) It provides a reservoir for C-apoproteins which are lipase activators. Apo-CII is the principal activator of lipoprotein lipase (134).

3) It has an essential role in the remodelling of triglyceride rich lipoproteins during the rapid catabolism by lipoprotein lipase. This is necessary in order to preserve structural integrity and promote efficient lipolysis. During hydrolysis of triglycerides from the

core of chylomicrons and VLDL, progressive loss of surface components, ie, phospholipid, unesterified cholesterol and apoproteins takes place. These components are transferred to HDL where the phospholipid is hydrolysed and cholesterol esterified by the action of LCAT. The cholesterol ester produced is transferred back to VLDL. The remnants of chylomicrons and VLDL, after the removal of part of the surface components and hydrolysis of most of the core triglycerides, are taken up and catabolised by the liver (90,135).

4) It can remove and incorporate cholesterol from cultured cells.

It has been shown that HDL or recombinant HDL particles (made from apo-HDL and phospholipid) remove cholesterol from ascites cell cultures (136,137). Moreover, the presence of HDL, which acts as cholesterol acceptor, in the culture medium promotes cytoplasmic cholesterol-ester hydrolysis in macrophages (138).

5) It may also decrease the atherogenic process caused by LDL by: a) inhibiting the cell injury which occurs when LDL is incubated together with endothelial cells from human umbilical cord veins (139); and b) partially inhibiting the uptake and degradation of LDL by cultured cells, eg porcine arterial smooth muscle cells (140) and human fibroblasts (141).

SECTION 2: METHODOLOGY

2.1 Preparation of HDL:

Venous blood was collected from human subjects after a 14 hours fast into 0.01% Na_2EDTA and the plasma, separated by low speed centrifugation at 4°C, used to isolate HDL (65). Plasma was raised to a density of 1.063 kg/l by adding solid KBr (0.0834 gm/ml) and transferred to the appropriate ultracentrifugation tube where it was overlayered with a solution of d 1.063 kg/l and subjected to centrifugation at 100,000 g and 15°C for 20 hours. The upper layer, which contains VLDL and LDL, was removed and the infranatant density was raised to 1.21 kg/l by adding solid KBr (0.236 gm/ml) and then overlayered with 1.21 kg/l density solution and centrifuged at 145,000 g and 15°C for 24 hours. The supernatant now containing HDL, was washed once by recentrifugation at d 1.21 kg/l for a further 24 hours and then dialysed against 0.15M NaCl/0.01% Na_2EDTA buffer, pH 7.0. HDL prepared by this method was free from albumin as detected by double immuno-diffusion.

2.2 HDL Subfractionation:

HDL was subfractionated into its main subclasses HDL_2 and HDL_3 by means of rate zonal ultracentrifugation (72,73). Plasma was adjusted to a density of 1.4 kg/l by the addition of solid KBr (0.532 gm/ml) and a known volume applied to the periphery of a zonal rotor (Ti14, Beckman, Palo Alto, C.A.) which had previously been loaded with a discontinuous gradient of NaBr solution of density 1.0-1.4 kg/l. The gradient was formed using an

LKB gradient maker (Ultrograd 11300, LKB, Bromma 1, Sweden) which pumped solutions of 1.0 kg/l and 1.4 kg/l in appropriate proportions into the rotor which was spinning at 3,500 rpm. Application of the sample was followed by a cushion of 20-40 ml of the higher density solution to ensure that the entire sample was within the rotor. The rotor was capped, accelerated to 41,000 rpm (125,000 g at the periphery) and maintained at this speed at 15°C for 21 hours. At the end of the run, the rotor was decelerated to 3,500 rpm and its contents displaced by pumping high density solution into the periphery at a rate of 21 ml/minute. The rotor effluent was monitored by continuous measurement of absorbance at 280 nm in an absorbance monitor (ISCO Model UA-5, ISCO, Lincoln, Nebraska) and collected into 14.0 ml fractions. The peak fractions corresponding to HDL_2 and HDL_3 were pooled, dialysed exhaustively against 0.15M NaCl/0.01% Na_2EDTA, pH 7.0, and concentrated by pressure filtration in a Diaflo cell (Amicon Corp., Lexington, Mass. 02173).

2.3 Quantitation of HDL_2 and HDL_3 from the zonal pattern:

The area under the absorption curve from the zonal elution profile was integrated and used to calculate the plasma concentration of these subfractions from the knowledge of the extinction coefficients of HDL_2 and HDL_3 (0.6 and 0.86, respectively) and their chemical composition (29).

2.4 Antibody Preparation:

Antibodies were raised in New Zealand white rabbits against purified (142) human apo-AI and apo-AII. A solution containing 1.0 mg of antigen was sonicated with an equal volume of Freund's complete adjuvant and injected into multiple subcutaneous sites of the rabbit. Three additional injections of similar protein content, mixed with Freund's incomplete adjuvant, were given at 3 week intervals. Ten days after the last injection, blood was withdrawn and serum was separated and stored at -20°C.

The antibodies were checked against purified apo-AI and apo-AII in an Ouchterlony plate and were found to be monospecific. They did not react with any of the other apolipoproteins B, E or C or with albumin.

2.5 Radiolabelling of HDL:

HDL or its subfractions were labelled with either ^{125}I or ^{131}I by the iodine monochloride technique of McFarlane (143). 100 nmol of HDL protein in 1.5 ml of 1.0 M glycine buffer, pH 10.0, was mixed with 2.0 mCi of radioiodide in the form of Na *I (purchased from Amersham International Plc, White Lion Road, Amersham, Buckingham-shire, England HP7 9LL), then 200 nmol of ICl were added and mixed thoroughly and immediately the iodinated protein was separated from unbound radioiodide by gel filtration through a 1.0 x 25 cm G10 Sephadex column. The elution buffer contained 0.1M Tris HCl, 0.15M NaCl and 0.01% Na_2EDTA, pH 8.6. Labelling efficiency determined

by paper electrophoresis was approximately 50%. Less than 1% of the radioactivity was associated with lipid and greater than 97% was precipitable in 5% trichloroacetic acid.

2.6 Preparation of the antibody gamma globulin fraction:

Each 1.0 ml of antiserum was mixed with 180 mg of anhydrous sodium sulphate and rotated end-over-end for 30 minutes at room temperature. The suspension was then centrifuged at 3,000 rpm for 20 minutes at room temperature and the supernatant was discarded. The precipitate was washed twice with a double volume of 18% sodium sulphate solution and dissolved in a volume of 0.15M NaCl designed to restore it to its original concentration. This gamma globulin fraction was then dialysed against 0.15M NaCl/0.01% Na_2EDTA (144).

2.7 Preparation of anti-apo-AII immunoaffinity column:

1) The gamma globulin fraction of anti-apo-AII was prepared and conjugated to CNBr activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as follows: 1.0 gm of the Sepharose powder was washed with 200 ml 0.01M HCl over a period of 15 minutes in a glass filter then mixed with 1.0 ml of anti-apo-AII gamma globulin, which had previously been diluted with 0.4 ml of a buffer solution containing 0.35M $NaHCO_3$ and 1.75M NaCl. The mixture was transferred to a tube and rotated end-over-end for 2 hours at room temperature.

2) The suspension was then spread on a glass filter and washed once with 5 ml bicarbonate buffer (0.1M $NaHCO_3$/0.5M NaCl, pH 8.0) to remove unbound material.

3) The remaining active groups on the gel were reacted with 1.0M ethanolamine at pH 8.0 for 2 hours.

4) The non-covalently adsorbed protein was removed by three washing cycles, each cycle consisting of a wash at pH 4.0 (0.1M AcONa/1.0M NaCl) followed by a wash at pH 8.0 (0.1M borate/1.0M NaCl). More than 95% of the starting protein was coupled to the gel.

5) The antibody coupled to sepharose was packed in an appropriate column and primed with buffer A (0.2M Tris HCl/0.5M NaCl), pH 8.0.

2.8 Preparation of (apo-AI)HDL:

HDL or one of its subfractions was applied to anti-apo-AII immunoaffinity column and eluted with the buffer A, pH 8.0. 1.0 ml fractions were collected, and their absorbance measured at 280 nm. When absorbance was plotted versus fraction number, a single peak was obtained and the appropriate fractions were pooled and dialysed against 0.15M NaCl/0.01% Na_2EDTA, pH 7.0.

The amount of (AI)HDL in HDL or its subfractions could be quantitated from the protein content of the applied material and of the eluate, giving the percentage of (AI)HDL in the starting material. The percentage remaining represents the amount of protein in (AI/AII)-HDL particles in the starting material.

Urea solutions used in the following experiments were prepared from highly purified urea grade (ARISTAR, BDH Chemicals Ltd, Poole, England). These solutions were passed through an ion exchange chromatography column before use to prevent protein carbamylation by cyanate.

2.9 HDL Delipidation:

1) Delipidation with 1,1,4,4-tetramethyl urea (TMU) (145).

Equal volumes of HDL solution and TMU were mixed and incubated for 30 minutes at room temperature, then centrifuged at 10,000 rpm for 15 minutes. The dissociated lipids formed a pellet on the top of the solution and the apoproteins were solubilised in the clear infranatant.

2) Delipidation with organic solvents (81).

HDL was extracted with a mixture of ethanol:diethyl ether; 1:3 (V/V) at room temperature for 30 minutes then centrifuged and the organic phase decanted. The extraction was repeated with diethyl ether, the delipidated HDL was dried at room temperature and then dissolved in 8M urea.

2.10 Separation of HDL apo-AI and apo-AII in PAGE:

Polyacrylamide gel columns, prepared in 0.4 x 6 cm tubes, comprised a 0.5 cm concentrating gel of large-pore size (2.5% polyacrylamide) and a 5.0 cm separating gel of smaller pore size (7.0% polyacrylamide) (146). All gel solutions were prepared in 8M urea as follows:

The separating gel was prepared from

1.0 ml of 8M urea

1.0 ml of 36.6 gm Tris base + 0.23ml "TEMED" (N,N,N',N'-tetramethyethylenediamine) (BDH) in 152 ml 8M urea + 48 ml 1M HCl, pH 8.9

2.0 ml of 28.0 gm acrylamide monomer (BDH) + 0.735 gm N,N'-methylene-bis-acrylamide (BDH) in 100 ml 8M urea

4.0 ml of 0.14% ammonium persulphate

1.0 ml of this mixture (freshly prepared) was placed in each tube, which had been sealed at the bottom with parafilm, and overlayered with water to give a flat surface and exclude air. These were left to polymerise at room temperature for 30 minutes after which the water was decanted and the tube surface blotted dry. Then 0.1 ml of the concentrating gel was added, overlayered with water and left to polymerise under a fluorescent light source for 10 minutes.

The concentrating gel solution was prepared from:

1.0 ml of 5.98 gm Tris base + 0.46 ml TEMED in 52 ml 8M urea + 48 ml 1M HCl, pH 6.7

2.0 ml of 20.0 gm acrylamide monomer + 5.0 gm N,N'-methylene-bis-acrylamide in 200 ml 8M urea

1.0 ml of 8.0 mg riboflavin (BDH) in 100 ml 8M urea

4.0 ml of 40% sucrose in 8M urea.

Apoprotein samples (100 µl) were mixed with 10 µl of bromophenol blue as a dye marker, placed on top of the gel column and overlayered with the electrophoresis buffer (6.0 gm Tris base, 28.8 gm glycine/l, pH 8.3).

Electrophoresis was carried out in a disc electrophoresis tank (Shandon Scientific Co. Ltd., London, England) at 5 mA/gel until 15 minutes after the marker cleared the bottom of the gel. Gels were removed from the tubes by rimming with water, stained in Amido black (0.1% in methanol:acetic acid:water; 5:1:5;V:V:V) for 10 minutes and destained with 7.5% acetic acid.

Purified apo-AI and apo-AII were run on separate gels to compare and locate the apoproteins.

2.11 Preparative Separation of Apo-HDL:

Polyacrylamide gel columns were prepared as described previously and cast in 1.0 x 12 cm tubes. 8.0 ml of separation gel and 1.5 ml of concentration gel were used in each tube. Electrophoresis was performed at 2 mA/gel overnight and run for one extra hour after the marker dye left the gel. Gels were immersed (127,147) in 0.01% acetic acid solution of 8-anilino-1-naphthalene-sulphonic acid magnesium salt (BDH Chemicals Ltd., Poole, England) for 5 minutes and then placed under a UV light where the stained apoproteins were visualised as fluorescent bands. The bands corresponding to apo-AI and apo-AII were excised and the proteins eluted (148) by electrophoresis into dialysis bags (Spectraphor, Spectrum Medical Industries Inc., Los Angeles, C.A.). The purities of eluted apo-AI and AII were checked against anti-apo-AI and anti-apo-AII by immunodiffusion. They were found to be free from cross contamination.

2.12 Electroimmunoassay of Apo-AI and apo-AII:

1) Apo-AI.

Apo-AI in whole plasma or HDL subfractions was quantitated by electroimmunoassay (149,150).

Samples were diluted with barbitone buffer (10.3 gm sodium barbitone + 1.83 gm barbituric acid, pH 8.6) to give approximately 1.0 mg/ml final concentration of apo-AI, and applied to plates of 1% agarose and 1.5% antiserum in 0.05M barbitone buffer, pH 8.6. These plates were prepared as follows: 8.4 ml of boiling 2% agarose (BIO-RAD Laboratories) solution was mixed with 8.4 ml barbitone buffer to which 0.26 ml of anti-apo-AI had previously been added. The mixture was spread immediately on a levelled clean glass plate (14 x 8 cm) and left to cool. Holes (2.5 mm in diameter and 0.8 mm apart) were made in the formed gel (1.5 mm thick) and the plate was placed in the electrophoresis tank (2117 Multiphor, LKB). Samples (5 µl) were applied to the holes and electrophoresis carried out at 250V for 18 hours at a constant temperature of 10°C. At the end of the run the plate was placed in a saline bath for 2 hours, to remove the unreacted antibody, covered with filter paper and dried in an oven at 30°C. Dried plates were stained with Coomassie Brilliant Blue R (1% solution in MeOH:AcOH:H_2O;50:10:40) for 10 minutes and destained with MeOH:AcOH:H_2O (50:10:40). Apo-AI standards, dissolved in the same buffer, were also applied to each plate and the height of sample "rockets" compared to those of the standards.

2) Apo-AII.

Apo-AII was determined by a method similar to that for apo-AI, but diluted samples of apo-AII were heated at 52°C for 3 hours to achieve maximum immunoreactivity of apo-AII (151). Standards of apo-AII in the same buffer were also applied to each plate.

2.13 Beta-Quantification:

Principle

Plasma cholesterol concentrations in the lipoprotein density classes VLDL, LDL and HDL, were determined using standard methodology (152). Plasma was centrifuged to separate VLDL (and chylomicrons if present) as a floating fraction. The infranatant was then treated with heparin/ Mn^{2+} (at final concentrations of 1.3 mg/ml heparin and 0.092M Mn^{2+}) to precipitate LDL and leave HDL in solution. The cholesterol content of whole plasma, of the top (VLDL) and bottom (LDL and HDL) fractions and of the Mn^{++}/heparin supernatant (HDL) was measured using an enzymatic method (Boehringer Mannheim GmbH, Mannheim, W. Germany, Kit No. 236 691) designed for use in the Auto Analyser II (Technicon Instruments Corporation, Tarrytown, N.Y. 10591). The cholesterol content of LDL fraction was calculated as follows:

LDL-cholesterol = cholesterol in the bottom fraction
- HDL-cholesterol

Plasma triglycerides were measured by an enzymatic method (Boehringer, Kit No. 166 448) on the same double channel AAII autoanalyser.

1) Ultracentrifugation: 5.0 ml plasma were placed in a Beckman Ultra-Clear centrifuge tube (13 x 64 mm) (Spinco Division, 1117 California Avenue, Palo Alto, C.A.) and overlayered with 0.9% saline (d 1.006 kg/l).

The tube was capped and centrifuged overnight at 39,000 rpm and 4°C, then sliced approximately 20 mm from the top and the contents of the bottom fraction transferred to a 5.0 ml volumetric flask. The top of the tube was washed with saline and the wash added to the flask and the volume adjusted to 5.0 ml with saline.

2) Precipitation of LDL (68): 1.0 ml of the bottom fraction was placed in a Beckman centrifuge tube and 50 ul of precipitating reagent (10.39 gm $MnCl_2.4H_2O$ + 1.05 gm (≡ 5 x 10^5 units) heparin Na-salt (Sigma London Chemical Co., Fancy Road, Poole, England) in 25 ml saline were added and mixed. The mixture was kept at 4°C for 15 minutes and then centrifuged at 10,000 rpm for 30 minutes; the supernatant was separated immediately for subsequent assay of cholesterol.

2.14 Chemical Methods:

1) Protein was determined by the method of Lowry et al. (153).

2) Cholesterol, free and esterified, were determined by an enzymatic method using the Boehringer Kit. For the measurement of free cholesterol, cholesterol esterase in solution 3 was replaced by an equivalent volume of saline in the final reagent mix. Subtraction of this free cholesterol from total assayed cholesterol gives an estimate of the number of cholesterol 3'OH

groups esterified with fatty acid. In order to account for the unmeasured fatty acid component of cholesteryl ester (considered to be linoleate), the measured ester cholesterol concentration was multiplied by a correction factor of 1.68.

3) Phospholipids were assayed as inorganic phosphorus by the method of Bartlett (154). In this method a value of 25 is used to express the PO_4 detected as phospholipid. This is the mass ratio of lecithin/PO_4.

2.15 Molecular Weight Determination:

Molecular weights of HDL subfractions were determined by the meniscus depletion sedimentation equilibrium method according to Yphantis (155) in a Beckman Model L8 ultracentrifuge equipped with an ultraviolet scanning attachment using an An-F rotor with double sector centrepiece cells. Ultracentrifugation was performed at a sample concentration of 0.2-0.3 mg/ml in 0.15M NaCl/0.01% Na_2EDTA, pH 7.0, and the rotor was rotated at 19,000 rpm and 20°C for a minimum of 48 hours, during which time equilibrium was attained. This was checked by obtaining three identical scans at 3 hour intervals. The last scan was used for calculation of the molecular weight. The apparent weight-average molecular weight was calculated from the equation:

$$Mr_{app.} = \frac{2RT}{(1-\bar{\nu}\rho)\omega 2} \quad \frac{d\ln c}{d(r^2)}$$

where R: the gas constant

T: the absolute temperature

c: the concentration in absorbance units

$\bar{v}$: the partial specific volume which was taken as 0.913 cm^3g^{-1} for (AI)HDL_2 and HDL_2, and 0.874 cm^3g^{-1} for HDL_3 (156).

ρ: density of the solution (1.006 gm/ml)

ω: the angular velocity which was calculated from the relationship

$$\omega = \frac{2\pi rpm}{60} = 0.10472\ rpm$$

r: the distance to the centre of rotation which was calculated from the scan chart from the relation:

$$r = \frac{x}{f} + 5.7\ cm$$

where x is the distance from the inner reference edge of the cell on the scan chart.

f: the magnification factor which equals the distance between the inner and outer reference edges of the cell on the scan chart divided by the actual distance between the two reference edges of the cell (1.62 cm). 5.7 cm is the distance between the inner reference edge of the cell and the centre of rotation.

The value of $d\ln c/d(r^2)$ was obtained from the slope of the $\ln c$ versus r^2 plot.

2.16 Determination of Flotation Rates

Flotation rates of HDL subfractions were determined by the boundary flotation rate method (157) in the Beckman Model L8 ultracentrifuge equipped as above.

Samples were examined at a concentration of 0.3-0.4 mg/ml and a density of 1.2 kg/l (158). The rotor was centrifuged at 45,000 rpm and 26°C and scans were taken every 15-20 minutes.

The observed flotation rate was calculated from the relation:

$$F_{obs} = \frac{1}{\omega^2 r}\frac{dr}{dt} = \frac{2.303}{60\omega^2}\frac{(d \log x)}{dt'}$$

where r: the radial distance to the centre of rotation.

x: the distance between the boundary position and cell outer reference edge.

ω: the angular velocity.

t and t': time in seconds and minutes, respectively.

The distance x and t' were measured from each scan and the values log x were plotted versus t'. The slope of the resulting best fit straight line was taken as equal to d log x/dt'.

2.17 Precipitation of radiolabelled HDL with antibodies:

Radioiodinated HDL, HDL_2, or HDL_3 were incubated with anti-apo-AI and anti-apo-AII, separately, to precipitate immunochemically distinct HDL particles as follows. Three 10 µl aliquots of HDL or one of its subfractions (1 mg/ml protein) were treated with 300 µl of anti-apo-AI, anti-apo-AII, or non-immune rabbit serum and incubated at 4°C for 48 hours.

The radioactivity of each mixture was measured in a twin channel gamma spectrometer (Packard Instruments, Downers Grove, IL.), then the mixture was centrifuged and an aliquot of the supernatant counted. In addition a 10 µl aliquot of the supernatant from each incubation mixture was treated with 125 µl saline and 200 µl donkey-anti-rabbit serum then incubated at 4°C for a further 48 hours. The mixture was then counted and centrifuged to pellet any further precipitate. All counts were corrected for volume changes and results were expressed as percentage of the sample precipitated. Non-immune rabbit serum (NIRS) was used alongside the antibodies to correct for non-specific precipitation.

2.18 Assay of (AI)HDL and (AI/AII)HDL Particles

The apo-AI content of (AI)HDL and (AI/AII)HDL particles in whole plasma, total HDL, HDL_2 and HDL_3 was quantitated using two methods:

A) Immunoprecipitation of (AI/AII)HDL Particles

Samples were diluted with saline to give a concentration of apo-AI of about 1.0 mg/ml. 10 µl of the diluted sample were mixed with 250 µl of anti-apo-AII, or its gamma globulin fraction, and incubated at 4°C for 48 hours, during which (AI/AII)HDL was virtually quantitatively precipitated. The completeness of precipitation was confirmed using radioiodinated HDL. Further addition of anti-apo-AII or of donkey anti-rabbit serum to the supernatant of the initial incubation mixture did not precipitate any more label. To determine the apo-AI

present in (AI)HDL particles the incubation mixture was centrifuged and the supernatant diluted 1:1 with barbitone buffer, pH 8.6, then applied to a gel plate prepared, as described previously (Section 2.12), with anti-apo-AI. In addition, an appropriate dilution of the original sample was applied to the same plate and subjected to electrophoresis. After the plate was dried and stained, the concentrations of the total apo-AI content of the sample as well as the apo-AI content of (AI)HDL were determined by comparing their rocket heights with concurrently run standards. The difference between the two values represents the apo-AI content of (AI/AII)HDL in the sample.

B) Measurement of (AI)HDL Particles by Two-Phase Gel

A gel plate was prepared containing two antibody phases: the lower one contained 2.5% anti-apo-AII and the upper one contained 1.5% anti-apo-AI. The two gel phases were separated by a non-immune gel zone of 0.5 cm width to prevent cross diffusion of the antibodies. Samples were diluted with barbitone buffer, pH 8.6, and applied to the lower gel and electrophoresis performed as described earlier. Samples migrate through the lower gel phase which precipitates all the (AI/AII)HDL particles leaving the (AI)HDL particles to pass unreacted to the upper gel phase where they react with the anti-apo-AI antiserum forming rockets. Standards of apo-AI were run

on each plate. Each sample gave two peaks, one in the lower gel and the other in the upper which represents the amount of (AI)HDL in the sample.

2.19 Exchange of Apoproteins Between HDL Subfractions

In each of the following experiments the HDL subfractions used were from the same volunteer.

A) Exchange between (AI)HDL_2 and HDL subfractions: ^{125}I(AI)HDL_2 was prepared from ^{125}I HDL_2, as described in Section 2.8, and incubated with excess (15 fold) HDL_2 or HDL_3 for 24 hours at room temperature. An aliquot of the incubation mixture was counted and then applied to an anti-apo-AII iummunoaffinity column. Unretained lipoproteins were eluted with tris buffer, pH 8.0, as described before, and the radioactivity in the eluate determined.

B) Exchange between HDL_2 and HDL_3:

Equal specific activities of ^{125}I HDL_2 and ^{131}I HDL_3 (5 mg each) were incubated at room temperature for 30 minutes, then subjected to rate zonal ultracentrifugation to re-isolate the subfractions. Radioactivity in each fraction of the zonal profile was determined on a gamma counter and results were corrected for salt quenching of ^{125}I radioactivity (159). This was done by adding a known amount of radioactivity to each fraction of a zonal profile then the radioactivity was measured. The difference between the added and measured radioactivity is the amount quenched by the salt present in that fraction.

Peak fractions of HDL_2 and HDL_3 were pooled, dialysed against 0.15M NaCl/0.01% Na_2EDTA and concentrated. Aliquots of each subfraction were applied to an anti-apo-AII immunoaffinity column and elution carried out with tris buffer, pH 8.0. The radioactivity present in the aliquots before application and in the eluted material was measured.

C) Exchange Between (AI/AII)HDL and HDL Subfractions:

Anti-apo-AII was conjugated to Sepharose gel as described earlier. Then 1.0 ml of the conjugated gel was treated with excess ^{125}I HDL_3 (2.0 mg protein) in Tris buffer, pH 8.0, by incubating at room temperature for 24 hours. The mixture was centrifuged and the supernatant discarded. The gel was washed five times with saline prior to study of its exchange properties. Aliquots of 0.2 ml of the washed gel were incubated, separately, with excess (1.5 mg protein in 1.0 ml solution) (AI)HDL_2, HDL_2 and HDL_3 at room temperature for 24 hours. The radioactivity of the whole suspension was measured and then gel bound lipoproteins were separated by centrifugation. Supernatant lipoproteins were aspirated and the precipitate washed once with saline and the wash added to the supernatant. Radioactivities present in the supernatant and in the gel were determined.

2.20 Turnover Studies

The metabolism of the major HDL apoproteins, apo-AI and apo-AII, was examined in a group of subjects according to the following protocol. In some instances the kinetic properties of (AI)HDL particles were also determined.

Subjects were given intravenous injections of sterilised autologous ^{125}I labelled HDL (50 uCi) or a mixture of 25 μCi ^{131}I HDL and 25 μCi ^{125}I (AI)HDL_2 (approximately 1.0 mg protein per injection). The HDL for injection was separated from 10 ml plasma of the fasting donors by preparative ultracentrifugation and labelled with ^{131}I or ^{125}I as described previously. HDL_2 was separated from 30 ml of the fasting donor's plasma by rate zonal ultracentrifugation and labelled with ^{125}I, and then passed through an anti-apo-AII immunoaffinity column to prepare ^{125}I (AI)HDL_2. HDL_2 was used to prepare the (AI)HDL particle because it contains a higher concentration of (AI)HDL than total HDL or HDL_3.

Subjects were given 900 mg potassium iodide daily in three doses for three days prior to and throughout the turnover to prevent thyroid uptake of released radioiodide. After injection plasma samples were withdrawn at 10 minutes and thereafter daily, after a 14 hour fast, for 14 days. 24 hour urine samples were also collected throughout the study period. The radioactivity present in a 2.0 ml aliquot of plasma was measured for each time

point in a twin channel gamma spectrometer (Packard Instruments) and the results used to construct the plasma decay curves for each isotope.

HDL (1.063-1.21 kg/l) was re-isolated from 10 ml plasma at each time point by preparative ultracentrifugation and delipidated by addition of an equal volume of TMU.

The solubilised HDL apoproteins were separated on preparative urea/PAGE gels (1 x 8 cm) to isolate apo-AI and apo-AII, as described previously. The bands corresponding to apo-AI and apo-AII were eluted from the gel by electrophoresis and more than 90% of the radioactivity in each band was recovered. The specific activity of the eluted apoproteins was determined by measuring the protein content (153) and radioactivity of each fraction. The resulting specific activity curves were used to calculate the kinetic parameters of the individual apoproteins. These were derived from the plasma decay curves and urine data by three mathematical procedures. First by the curve peeling technique of Matthews (160), second by the integral excretion method of Nosslin (161) and finally by a computer-based, statistical method using multicompartmental analysis (162).

HDL_2 and HDL_3 concentrations in plasma were determined by rate zonal ultracentrifugation for each subject, and plasma total apo-AI and apo-AII were assayed by

electroimmunoassay. In some cases the plasma apo-AI content of (AI)HDL was also determined as described in Section 2.18.

2.21 Mathematical Analysis of Tracer Decay Data

Three main approaches were used to analyse the experimental data and obtain kinetic parameters.

2.21.1 Curve peeling method (Matthews) (160):

In this analysis the biological system is described as a central compartment, representing the intravascular pool, and outer compartments connected to the central either reversibly, representing the extravascular pools, or irreversibly, that is the urine excretion pool (Fig. 12). It was assumed that a) the system is in a dynamic equilibrium, b) all newly synthesised protein enters the intravascular compartment, c) protein catabolism takes place in the intravascular compartment only, and d) extravascular compartments are not connected to each other.

The differential equations describing these relationships (eqn.1)

$$\frac{dX}{dt} = \sum_{j=1}^{n} K_{ji}X_j - \sum_{j=1}^{n} K_{ij} X_i \quad (i = 1,2....n) \qquad \text{eqn. 1}$$

Kji is the fraction at time t of protein in compartment j passing into compartment i per day. X_i is the fraction of injected dose in the ith compartment.

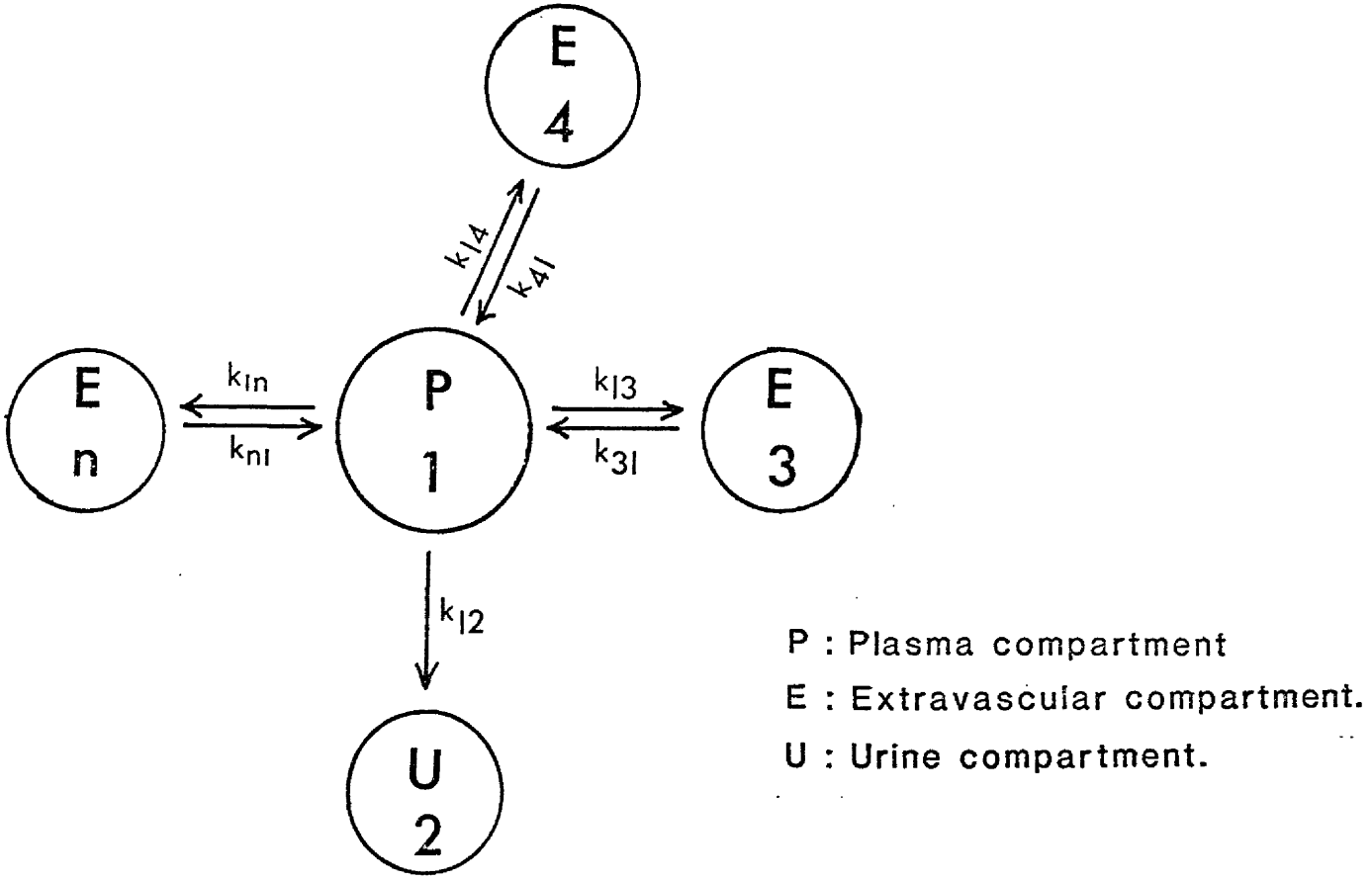


Figure 12. Mamillary model of n compartments.

are solved by means of Laplace transforms (Rescigno, 163) where the amount of label in each compartment (X_i) is represented by a number of exponential functions

$$X_i = C_1e^{-b_1t} + C_2e^{-b_2t} + C_3e^{-b_3t} + \ldots.$$

equivalent to n + 1 extravascular compartments. The number of exponential functions is determined from the radioactivity decay curve by the peeling method. That is the terminal single exponential component of the decay curve (of slope b_1) is extrapolated to intercept the ordinate at the point C_1. The extrapolated line is then subtracted from the original curve at suitable time intervals to give either a straight line, the slope of which equals b_2 and the intercept on the ordinate, C_2, or another curve which becomes linear at its end. If a curve is obtained, the process of extrapolation and subtraction is repeated until a straight line is finally achieved. The number of intercepts equals the number of exponential functions, and the kinetic parameters can be calculated from the slopes and intercepts of the straight lines obtained.

As an example where n = 3 (Fig. 13)

$$C_1 + C_2 = 1$$

$$C_1 = \frac{k_{3,1} - b_1}{b_2 - b_1}$$

$$b_1b_2 = k_{1,2}\, k_{3,1}$$

$$b_1 + b_2 = k_{1,2} + k_{3,1} + k_{1,3}$$

$k_{1,2}$ is the excretion rate or the fractional catabolic rate (FCR) as a fraction of the intravascular protein pool per day.

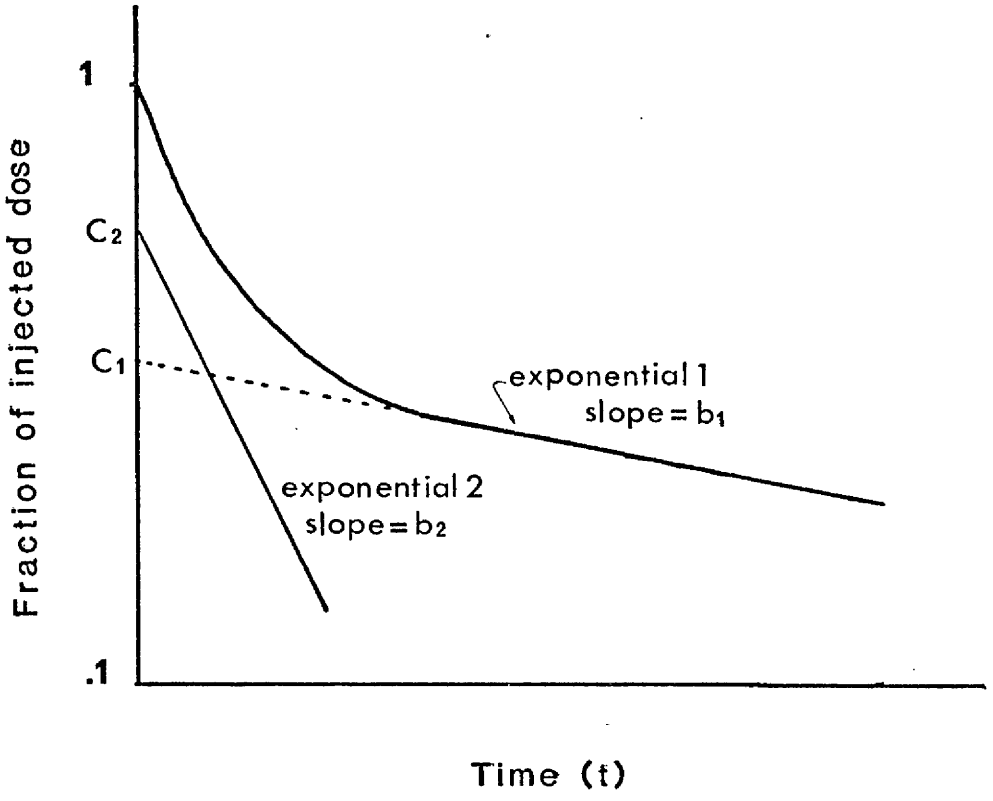


Figure 13. Resolution of a plasma decay curve into two exponential functions.

$k_{1,3}$ is the fractional capillary transfer or permeability rate which describes the fraction of the label passing from pool 1 (I.V.) to pool 3 (Ex.V.) daily.

$k_{3,1}$ is the reverse rate to $k_{1,3}$

Also $t_{1/2} = \frac{\ln 2}{b_1} = \frac{0.693}{b_1}$

$t_{1/2}$ is the half life of the protein during the terminal log-linear decay.

2.21.2 Computer assisted multicompartmental analysis (162,164,165).

This is a general statistical method for the analysis of biological systems described by linear or non-linear first order differential equations. In this method, a compartmental model is formulated which can be interpreted as a series of differential equations (see previous section). These can either be solved algebraically using a derived integrated rate equation

$$X_i(t) = \sum_{j=1}^{n} A_{ij}\, e^{-\alpha jt}$$

or by numerical integration using a 4th order Runge-Kutta method. The solutions yield calculated values at each time point for a given set of rate constants. The latter can then be adjusted in an iterative manner to give the best fit of observed to calculated data by a procedure which minimises the function

$$F = \sum_{1}^{n} (\text{obs} - \text{calc})^2 \qquad \text{over n points.}$$

2.21.3 Graphical Integration Method (Nosslin) (161).

In this technique the biological system is represented by a model comprising two exchanging pools, ie, an intravascular pool (P), and extravascular pool (E), and an excretion pool (U), Fig. 14. No assumptions are made about equal distribution of specific activity in all the extravascular spaces as well as the site of catabolism, thus an allowance is made for both intravascular as well as extravascular catabolism. To use this method both plasma and urine radioactivity should be measured.

The differential equations describing the system

$$\frac{dp}{dt} = -(k_1 + k_3)\ P + k_2E \qquad ...(a)$$

$$\frac{dE}{dt} = k_1P - (k_2 + k_4)\ E \qquad ...(b)$$

are rearranged and integrated from zero to time t.

Substituting for E in equation (a) and integrating from 0 to t, the following equation is obtained:

$$P - 1 = -\left(k_3 + \frac{k_1\ k_4}{k2 + k_4}\right)\int_o^t Pdt - \frac{k_2}{k_2 + k_4}\ E \qquad ...(c)$$

which on rearrangement gives:

$$\frac{1 - P}{E} = \frac{k_2}{k_2 + k_4} + \left(k3 + \frac{k_1\ k_4}{k_2 + k_4}\right)\frac{\int_o^t Pdt}{E} \qquad ...(d)$$

Rearranging the integrated form of equation (b) gives:

$$\frac{\int_o^t Edt}{E} = -\frac{1}{k_2 + k_4} + \left(\frac{k_1}{k_2 + k_4}\right)\frac{\int_o^t Pdt}{E} \qquad ...(e)$$

Equations (d) and (e) are in the form of straight line equations, thus the plot of the variables $\frac{1-P}{E}$

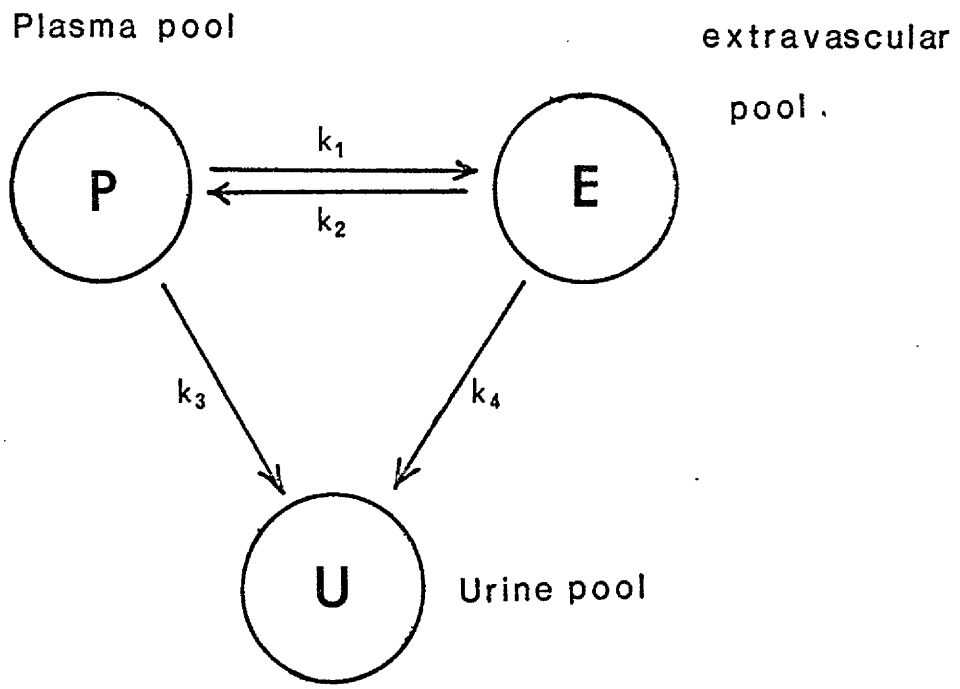


Figure 14. A system of three compartments in which catabolism occurs in both the intravascular and the extravascular pools.

versus $\frac{\int_0^t Pdt}{E}$ is a straight line of slope $k_3 + \frac{k_1 k_4}{k_2 + k_4}$ and intercept $\frac{k_2}{k_2 + k_4}$. Moreover, the plot of the variables $\frac{\int_0^t Edt}{E}$ versus $\frac{\int_0^t Pdt}{E}$ is another straight line of slope $\frac{k_1}{k_2 + k_4}$ and intercept $\frac{-1}{k_2 + k_4}$ (Fig. 15). From these slopes and intercepts the k values are calculated and the kinetic parameters calculated from the relations:

Total FCR = $k_3 + \frac{k_1 k_4}{k_2 + k_4}$ as a fraction of label in the intravascular pool per day.

Intravascular FCR = K_3 I.V. pools/day

Extravascular FCR = $\frac{k_1 k_4}{k_2 + k_4}$ I.V. pools/day

= k_4 as a fraction of label in the extravascular pool per day.

Also E/P ratio = $\frac{k_1}{k_2 + k_4}$

where E/P is the ratio of the mass concentrations in the extravascular and intravascular pools.

The values of the variables $\int_0^t Pdt$ and $\int_0^t Edt$, which correspond to the areas under the P and E curves, respectively, are calculated by cumulative summing of the graphed values of the curves at different time points.

If catabolism in the system takes place in both intravascular as well as extravascular compartments then the intercept of the plot from equation (d) must have the

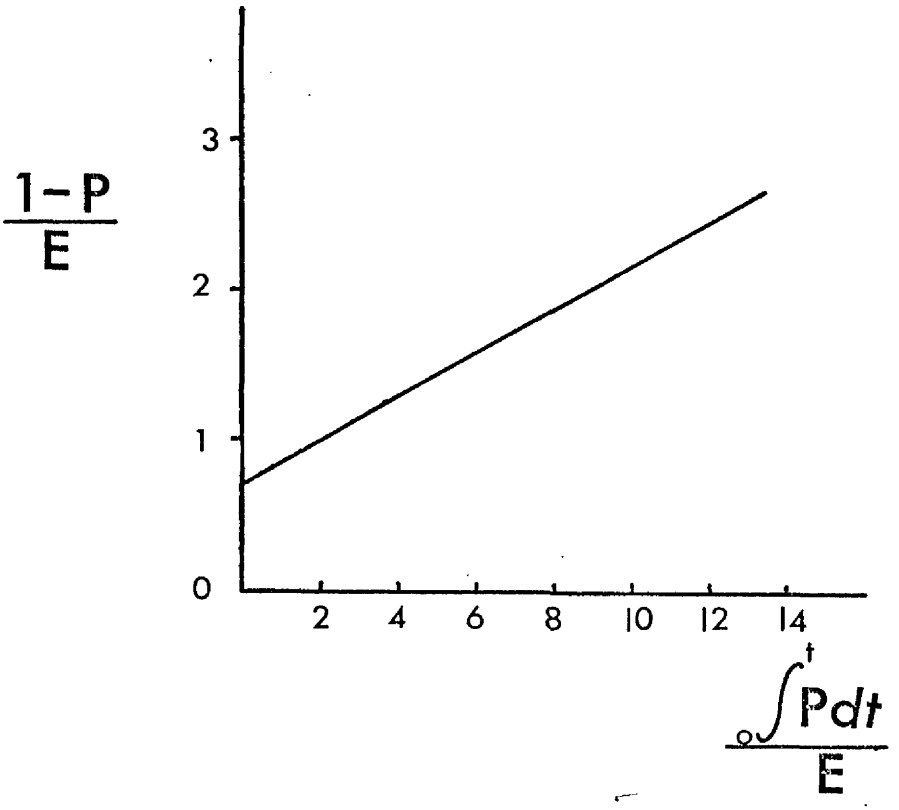


Figure 15a. Plot of equation d (page 59).

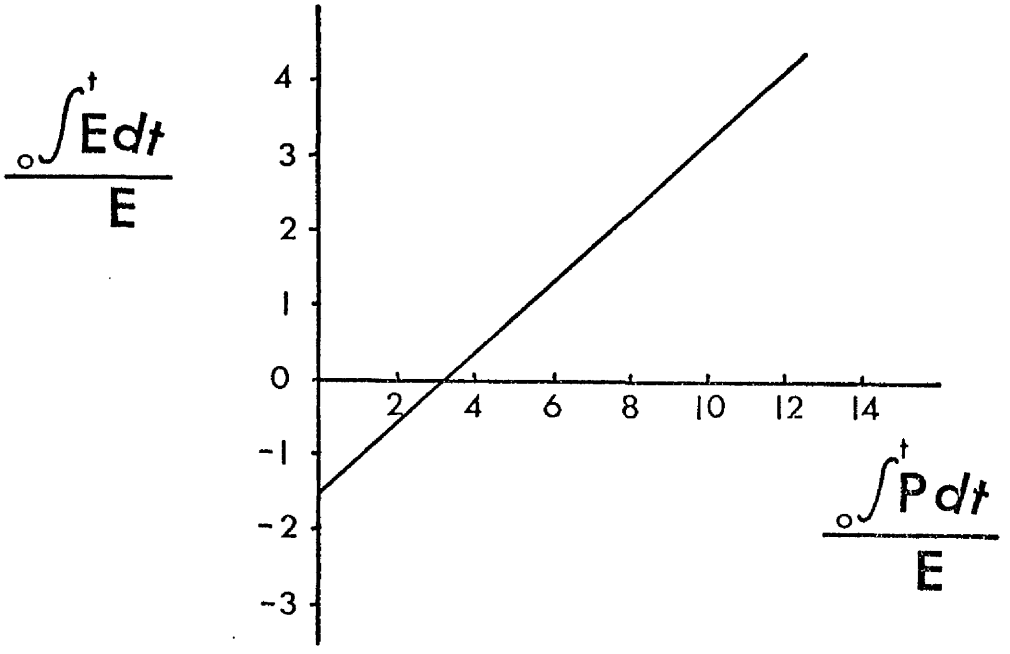


Figure 15b. Plot of equation e (page 59).

value of more than zero and less than unity. However, if the intercept is unity then catabolism takes place entirely in the intravascular compartment.

SECTION 3: RESULTS

3.1 STUDY I: Separation, properties and metabolism of (AI)HDL particle.

The following experiments describe an investigation of high density lipoproteins in which they were separated immunologically into populations with and without apo-AII.

3.1.1 Detection and separation of (AI)HDL

1. HDL_2 and HDL_3 were isolated from the plasma of normolipidaemic females and incubated with five different monospecific antisera raised against anti-apo-AI and anti-apo-AII as described in Section 2.17. The results are presented in Table 5. Little radioactivity was precipitated with the non-immune rabbit serum (NIRS) (less than 2%). The anti-apo-AI antisera precipitated most of the radioactivity present in HDL_2 and HDL_3, ie, 91.3 and 92.8%, respectively. In contrast, anti-apo-AII did not precipitate all the radioactivity, particularly in HDL_2. In fact, only 38.1 and 84.2% of the radioactivity in HDL_2 and HDL_3, respectively, were precipitated. This indicated the presence of two immunologically distinct types of particles in both HDL subfractions.

2. In order to obtain the non-AII containing lipoproteins in a usable form, immunoaffinity columns of sepharose-anti-apo-AII were utilised. When a known amount of radioiodinated HDL_2 was applied to the immunoaffinity column and eluted with tris buffer, pH 8.0 (as described in Section 2.7), 54% of the radioactivity was eluted from the column, leaving the remaining portion

Table 5: Precipitation of labelled HDL_2 and HDL_3 with antibodies.

Experiment Number	% of label precipitated with anti-apo-AI		% of label precipitated with anti-apo-AII		% of label precipitated with NIRS	
	HDL_2	HDL_3	HDL_2	HDL_3	HDL_2	HDL_3
					0.9	1.5
1	91.8	94.0	41.6	85.0	0.7	0.5
2	92.1	93.8	39.5	86.6		
3	90.8	92.0	33.0	78.1		
4	91.3	92.9	40.8	88.4		
5	90.3	91.5	35.4	83.0		
Mean	91.3	92.8	38.1	84.2		
$\pm$ 1 S D	0.7	1.1	3.7	4.0		

NIRS: Non-immune rabbit serum.

(46%) bound to the anti-apo-AII gel. This procedure removed all AII particles from the preparation since when this eluate was re-applied to fresh immunoaffinity column and eluted with the same buffer, 95% of the applied radioactivity was eluted from the second column.

The eluted material was dialysed and its density raised to 1.21 kg/l by adding solid KBr. On ultracentrifugation at 45,000 rpm for 24 hours, more than 70% of the radioactivity was collected from the top millilitre of the tube.

3. This eluate from the immunoaffinity column was further tested to determine its composition. On an immunodiffusion plate it reacted with anti-apo-AI but not with anti-apo-AII (Fig. 16) and when it was incubated with anti-apo-AI, 92% of the radioactivity was precipitated.

The lipoproteins retained during the separation of ^{125}I-HDL on columns of anti-apo-AII antibody conjugated to sepharose were washed with saline and then dissociated with 3M KCNS. 52% of the radioactivity was solubilised from the antibody-lipoprotein conjugate after 60 minutes incubation at room temperature. In a control tube where saline was used instead of KCNS, only 5% of the radioactivity was recovered from the complex. The dissociated fraction was found to have the flotation properties of a lipoprotein when centrifuged at d 1.21 kg/l. Moreover,

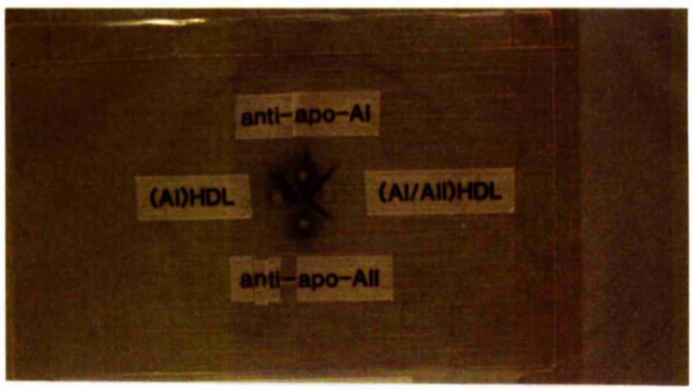


Figure 16. Immunodiffusion plate showing presence of apo-AI and absence of apo-AII in (AI)HDL particle isolated by immunoaffinity chromatography.

it reacted with both anti-apo-AI and anti-apo-AII antisera when tested in an immunodiffusion plate (Fig. 16).

4. A third method was used to show the presence of discrete AI and AI/AII particles in HDL. Fresh plasma, HDL, HDL_2 and HDL_3 were applied to a two-phase immuno-electrophoresis plate comprising anti-apo-AI as the upper phase and anti-apo-AII as the lower (as described in Section 2.18B), each of these samples showed a peak in each phase (Fig. 17). Plasma gave peaks of similar heights in both phases, while HDL_2 gave a small peak in the anti-apo-AII phase and much larger one in the anti-apo-AI phase. In contrast, HDL_3 (applied at the same total protein concentration as HDL_2) showed a larger peak in the first phase and a shorter one in the anti-apo-AI phase. When (AI)HDL_2, eluted from the immuno-affinity column, was run on the same plate, a peak was observed in the anti-apo-AI phase only (Fig. 17). In order to show that trapping of the HDL(AII) containing particles was complete, radioiodinated HDL_2 at five different dilutions was run on a similar plate. The peaks were cut from the wet gel and counted. Similar ratios were obtained in the two phases at all dilutions ie 61.3 and 38.7% in the anti-apo-AI and anti-apo-AII, respectively (Table 6).

The polypeptide composition of labelled (AI)HDL_2 was analysed by polyacrylamide gel electrophoresis. An aliquot of the material from an immunoaffinity column eluate was subjected to PAGE separation as described in

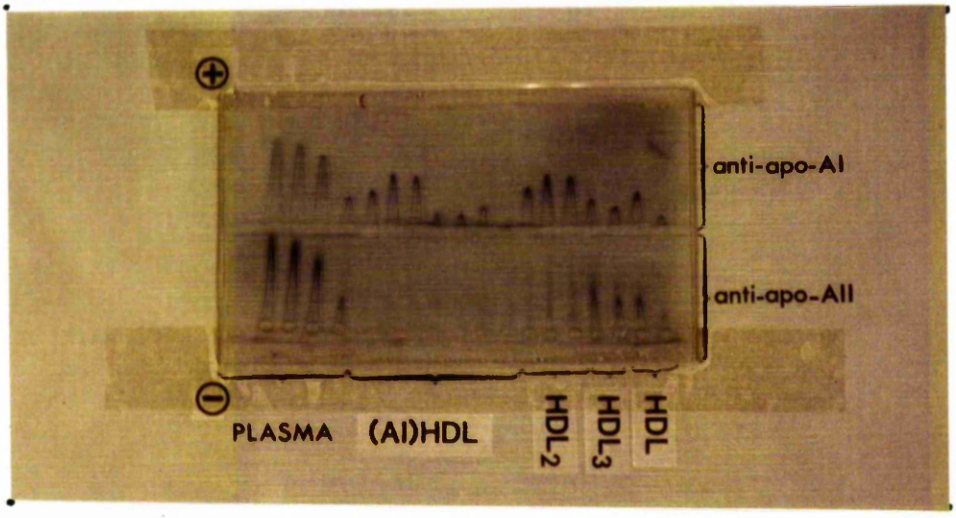


Figure 17. Two phase electroimmunoassay of the (AI)HDL particle.

Table 6: The measurement of (AI)HDL_2 particles in two phase electroimmunoassay: Effects of antigen dilution.

Dilution	% of radioactivity in	
	anti-apo-AI phase	anti-apo-AII phase
5/25	60.7	39.3
4/25	61.6	38.4
3/25	60.6	39.4
2/25	60.1	39.9
1/25	63.4	36.6
Mean	61.3	38.7
$\pm$ 1 S D	1.3	1.3

Section 2.10. The stained gels were sliced into 1mm slices and counted. 14.1% of the label was found associated with apo-C and the rest with apo-AI.

3.1.2 Exchange of apoproteins between (AI)HDL and (AI/AII)HDL particles

a. In vitro

Five separate experiments were performed in vitro to determine whether A apoproteins were exchanged between (AI) and (AI/AII) containing HDL particles.

1) Mixtures of ^{125}I(AI)HDL$_2$ with a 15 fold excess of either HDL$_2$ or HDL$_3$ were incubated, separately, at room temperature for 24 hours and then applied to immuno-affinity columns containing sepharose-anti-apo-AII. The majority, ie, 90 and 89% of the radioactivity present in these HDL$_2$ and HDL$_3$ mixtures, respectively, passed through the column.

2) Following an incubation (30 minutes) of ^{125}I-HDL$_2$ with ^{131}I-HDL$_3$ and re-isolation by rate zonal ultra-centrifugation (Fig. 18), as detailed in Section 2.19B, 71.1% of the radioactivity initially associated with HDL$_2$ remained in HDL$_2$. Of the radioactivity initially associated with HDL$_3$ 10.8% was found in HDL$_2$ subfraction (Table 7a) following incubation. The HDL$_3$ subfraction was found to contain 28.9 and 89.2% of the radioactivities initially associated with HDL$_2$ and HDL$_3$, respectively.

3) A mixture of ^{125}I(AI)HDL$_2$ and ^{131}I HDL (15 fold excess) was incubated at room temperature for 20 minutes then subjected to rate zonal ultracentrifugation. The

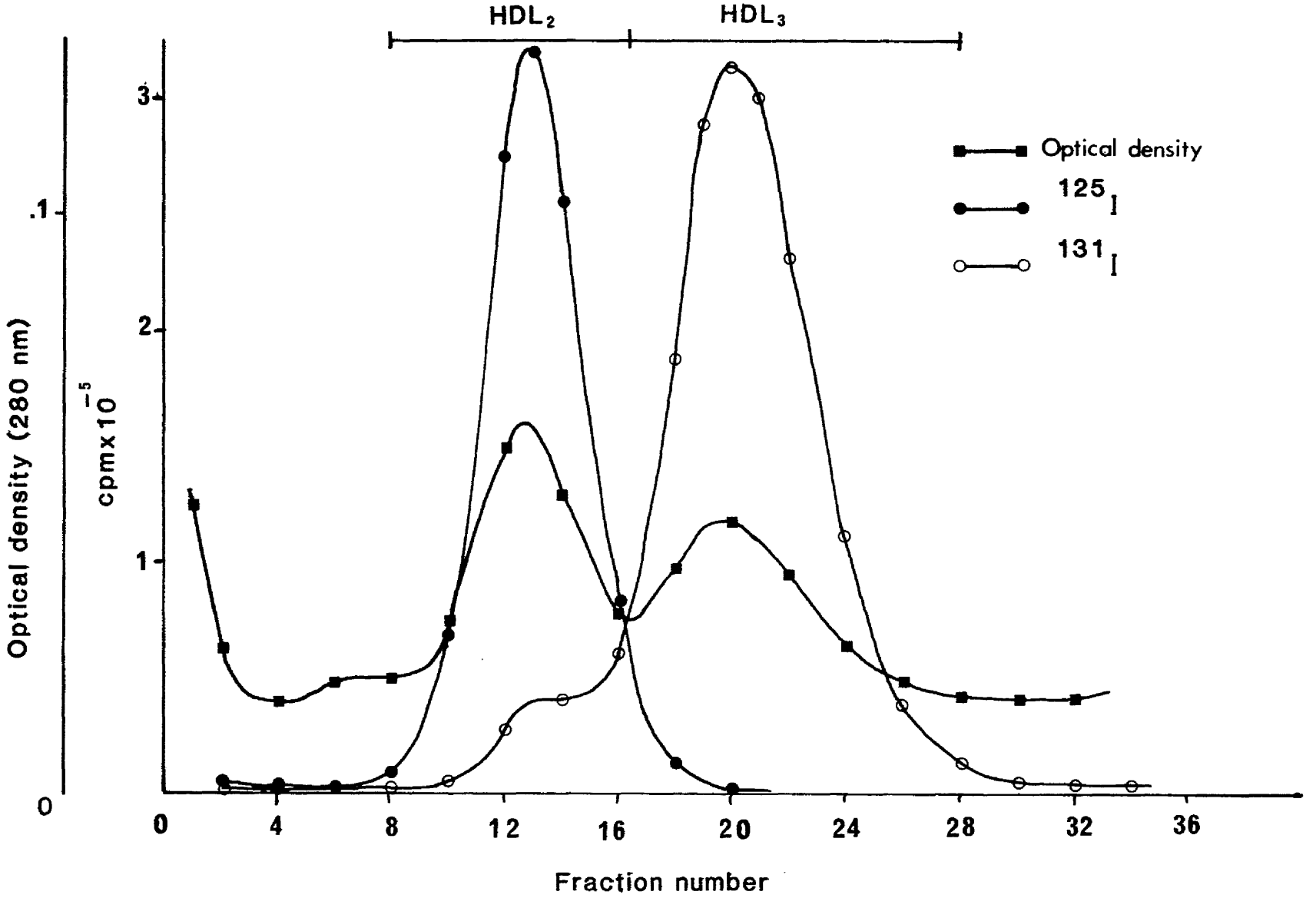


Figure 18. Rate zonal profile of the mixture ^{125}IHDL$_2$/^{131}IHDL$_3$ after incubation at room temperature for 30 minutes.

Table 7a: Distribution of radioactivity after incubation of ^{125}I-HDL_2 with ^{131}I-HDL_3.

Fraction Separated	of radioactivity initially associated with	
	HDL_2 (^{125}I)	HDL_3 (^{131}I)
HDL_2	71.1	10.8
HDL_3	28.9	89.2

Table 7b: Distribution of radioactivity after incubation of ^{125}I-(AI)HDL_2 with ^{131}I-HDL.

Fraction Separated	of radioactivity initially associated with	
	(AI)HDL_2 (^{125}I)	HDL (^{131}I)
HDL_2	78.5	15.5
HDL_3	21.5	84.5

distribution of radioactivity among the HDL subfractions is shown in Table 7b. The isolated HDL_2 subfraction was found to comprise 78.5 and 15.5% of the radioactivities initially associated with (AI)HDL_2 and HDL, respectively. On the other hand HDL_3 comprised 21.5 and 84.5% of these radioactivities.

4) When ^{125}I(AI)HDL_2 was incubated at room temperature for 20 minutes with a 15 fold molar excess of unlabelled HDL_3 and the mixture subjected to rate zonal ultracentrifugation, 18% of the radioactivity had transferred to the HDL_3 density range, and following prolonged incubation (72 hours), this rose to 34.3%.

5) In a further experiment labelled (AI/AII)HDL bound to anti-apo-AII-sepharose was incubated separately with equal protein concentrations of (AI)HDL_2, HDL_2 and HDL_3. Recovery of soluble radioactivity showed that 17.9% of the antibody bound label had transferred to the (AI)HDL_2 particle which may possibly be attributed to the exchange of C-apoprotein, since apo-C comprises approximately 15% of the (AI)HDL_2 apoproteins. On the other hand 37 and 51% of the label was transferred to HDL_2 and HDL_3, respectively. This increased exchange of radioactivity from the gel bound lipoprotein must be associated with the loss of radioactive apo-A into these fractions.

b. <u>In vivo</u>

A mixture of autologous ^{125}I(AI)HDL_2 and ^{131}I HDL of similar specific activities was injected into the bloodstream of two normolipidaemic young females. Thereafter, blood samples were withdrawn on three

different occasions, and the plasma subjected to rate zonal ultracentrifugation to determine the distribution of radioactivity among the HDL_2 and HDL_3 fractions. The results are shown in Table 8 and Figure 19. During the first day after the injection, most of the radioactivity initially associated with (AI)HDL_2 was found in the HDL_2 fraction. However, between the first and third days this decreased and reached a constant value of about 40% of total ^{125}I in the sample. In contrast, the radioactivity injected in association with total HDL was found to have a constant distribution throughout the study period both in HDL_2 and HDL_3.

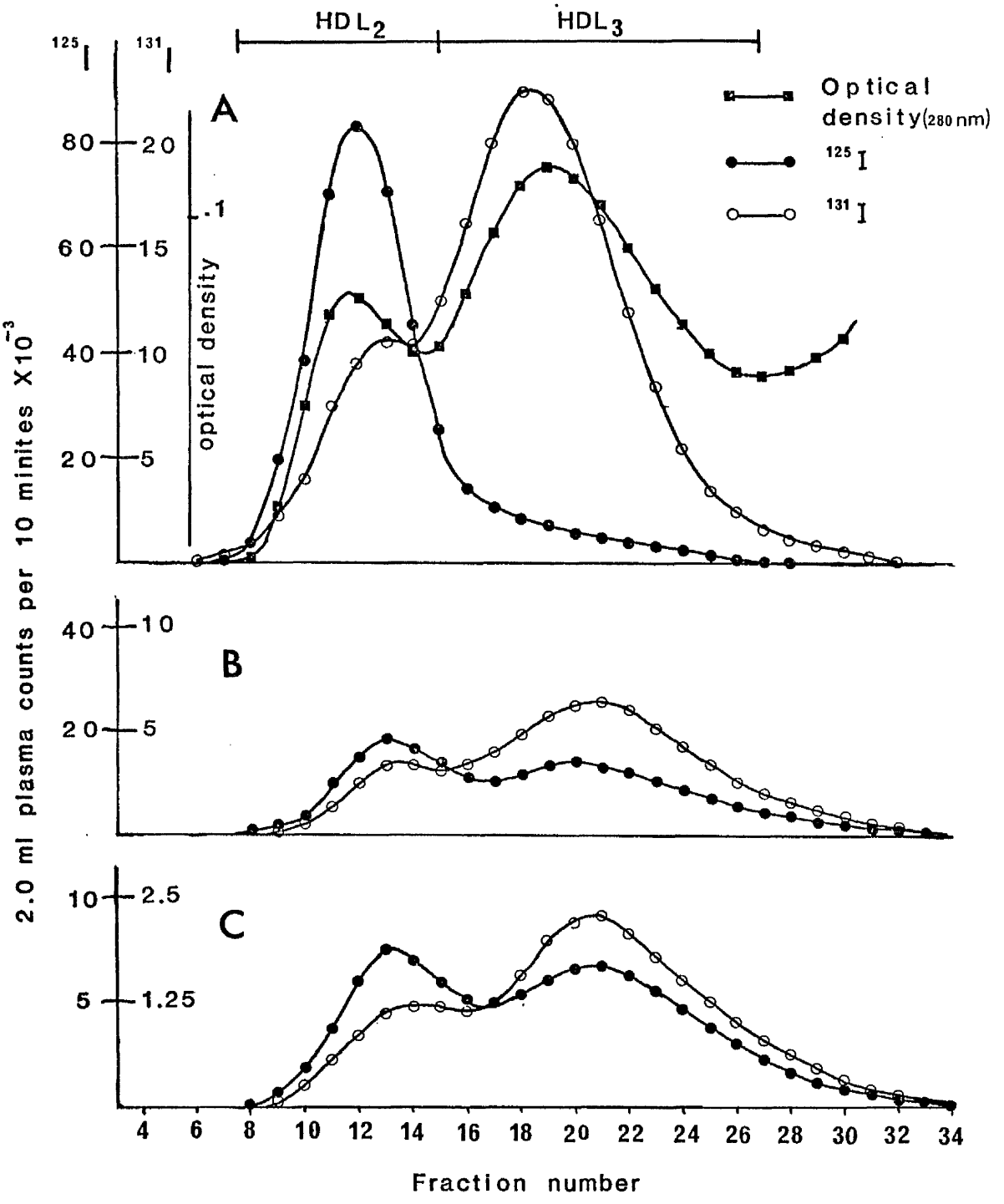
As is clear from Table 8 and Figure 19, the distribution of radioactivity initially associated with (AI)HDL_2, unlike that associated with total HDL, was not in accordance with the mass distribution of HDL_2 and HDL_3; but, rather, it was higher in HDL_2 than would be expected. This possibly indicated that minimal exchange had taken place between the (AI)HDL and (AI/AII)HDL in both density fractions. And this view was supported by the in vitro results where most of the radioactivity was recovered associated with (AI) HDL when the labelled (AI)HDL_2 was incubated with either HDL_2 or HDL_3; moreover, the results of other incubation experiments support this concept (Tables 7a and 7b).

3.1.3 Turnover of (AI)HDL and HDL

This study was designed to compare the kinetics of apo-AI in total HDL and in (AI)HDL particles. This was performed in two young normolipidaemic females, who were

Table 8: Distribution of radioactivity among HDL subfractions after the injection of a mixture of $^{125}I(AI)HDL_2$ and ^{131}I-HDL into normal human subjects (MP and JS).

Subject	Time of sample after the injection	% of radioactivity initially associated with (AI)HDL_2 (^{125}I) in:		% of radioactivity initially associated with total HDL (^{131}I) in:		HDL_2/HDL_3 mass ratio
		HDL_2	HDL_3	HDL_2	HDL_3	
Case 1 (MP)	10 min	78.1	21.9	21.5	78.5	
	3 days	42.9	57.1	23.4	76.6	0.21
	7 days	42.1	57.9	27.6	72.4	
Case 2 (JS)	1 day	89.4	10.6	19.1	80.9	
	5 days	43.3	56.7	17.5	82.5	0.28
	10 days	44.5	55.5	21.9	78.1	



A : 10 minutes sample after injection.

B : 3 days sample after injection.

C : 7 days sample after injection.

Figure 19. Rate zonal profiles after injection of ^{125}I(AI)HDL$_2$ and ^{131}IHDL mixture into a normal female (MP).

each given an injection of a mixture of autologous ^{131}I HDL and $^{125}I(AI)HDL_2$. Blood samples were withdrawn and urine was collected as described in Methods (Section 2.20). Their plasma lipoprotein concentrations are documented in Table 9.

The resulting plasma decay curves (Fig. 20) were analysed by the three mathematical methods detailed earlier, and the results are presented in Table 10. When these results were analysed by the Matthews (160) method, similar total FCR values were obtained for both HDL particles. However, analysing these results by the other two methods, ie, the multicompartmental (Berman) (162) and the graphical integration method (Nosslin) (161), showed that an appreciable proportion of both particles was catabolised in the extravascular compartment. The intravascular FCR for both particles was higher than their extravascular FCR. However, although the total FCRs of the two particles were similar, small differences in the intravascular as well as the extravascular FCRs were observed.

The specific activity decay curves of apo-AI and apo-AII (Fig. 21), isolated from HDL of the two subjects, were analysed by the method of Matthews (160) and the results are shown in Table 11. In one subject (MP) apo-AI in (AI)HDL and total HDL particles decayed at similar rates as did apo-AII in total HDL. On the other hand, data from the other subject (JS) showed that the three apoproteins decayed in different rates. Apo-AI in (AI)HDL had a slower fractional catabolic rate than that

Table 9: Plasma lipoprotein concentrations in MP and JS.

Subject	Body Weight kg	plasma volume ml	Triglycerides mmol/l	Total Chol. mmol/l	VLDL-Chol. mmol/l	LDL-Chol. mmol/l	HDL-Chol. mmol/l	Total apo-AI mg%	Free apo-AI mg%	apo-AII mg%	HDL_2 mg%	HDL_3 mg%	Total HDL mg%
M.P.	57.3	2264	0.70	4.7	0.15	2.7	1.85	165	76	47	60	282	342
J.S.	51.8	2220	0.50	5.2	0.10	3.4	1.70	154	70	45	53	190	243

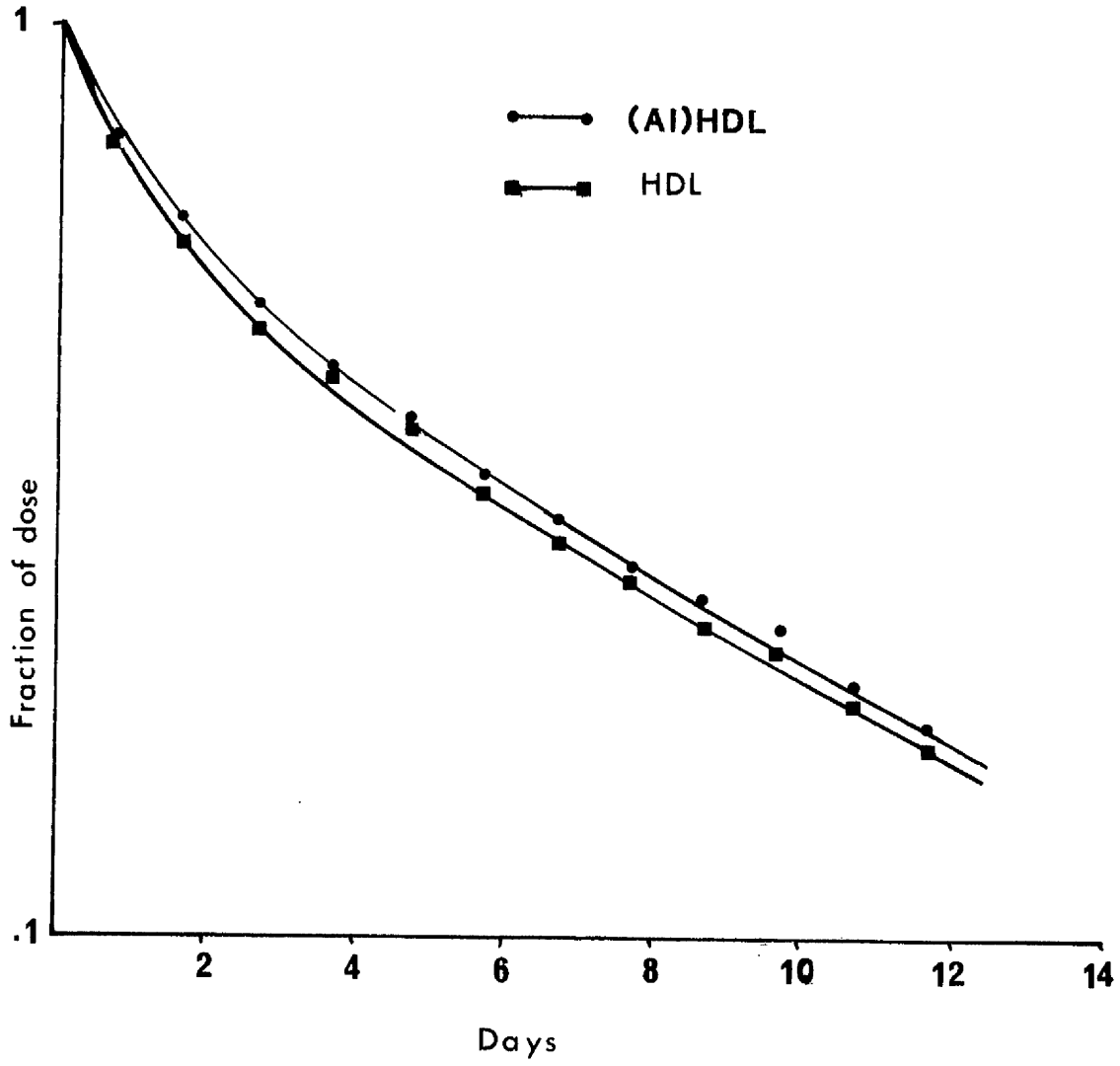


Figure 20. Plasma decay curves of (AI)HDL and HDL (JS).

Table 10: Kinetic parameters of HDL and (AI)HDL in two normal females (MP and JS).

Subject/ Particle	Method of Analysis	Total FCR[a]	I.V. FCR[a]	Ex.V. FCR[a]	k_1	k_2	k_3	k_4		$t_{½}$ days
M.P. HDL	Mathews	.185	-	-	.089	.431	.185	-	%I.V. = 83	4.91
	Nosslin	.157	.107	.050	.146	.114	.107	.059	E/P = .85	
	Berman	.165	.104	.061	.143	.088	.104	.065	E/P = .93	
(AI)HDL	Matthews	.180	-	-	.088	.456	.180	-	%I.V. = 84	4.91
	Nosslin	.165	.094	.071	.147	.100	.094	.092	E/P = .77	
	Berman	.167	.099	.066	.145	.100	.099	.084	E/P = .79	
J.S. HDL	Matthews	.174	-	-	.224	.499	.174	-	%I.V. = 68	6.38
	Nosslin	.160	.116	.044	.241	.250	.116	.056	E/P = .80	
	Berman	.153	.113	.040	.262	.274	.113	.049	E/P = .81	
(AI)HDL	Matthews	.167	-	-	.175	.503	.167	-	%I.V. = 72	6.17
	Noslin	.160	.097	.063	.236	.249	.097	.090	E/P = .70	
	Berman	.153	.092	.061	.261	.278	.092	.084	E/P = .72	

a. Plasma pools/day

FCR: Fractional catabolic rate

I.V.: Intravascular

Ex.V.: Extravascular

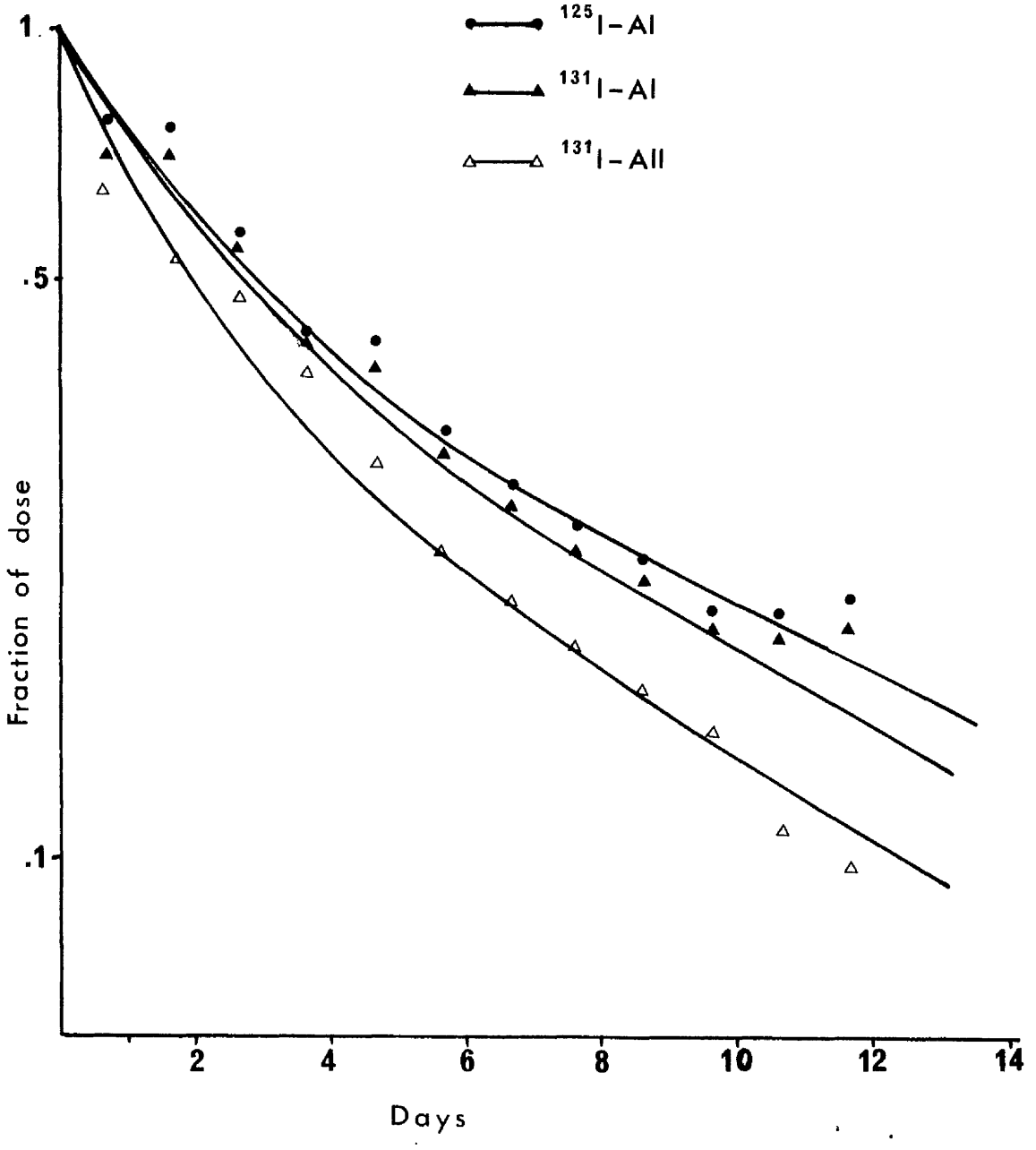
k_1: The fractional capillary transfer rate which describes the fraction of the label passing from the I.V. to the Ex.V. pool per day.

k_2: The reverse rate of k_1.

k_3: The I.V. excretion rate or FCR as a fraction of the I.V. pool per day.

k_4: The Ex.V. excretion rate or FCR as a fraction of the Ex.V. pool per day.

Total, I.V. and Ex.V. FCRs are expressed as fractions of the intravascular pool per day.



Figre 21. Specific activity decay curves of apo-AI and apo-AII after the injection of ^{125}I(AI)HDL and ^{131}IHDL mixture (JS).

Each curve represents the specific activity decay of apo-AI or apo-AII protein isolated from HDL by PAGE electrophoresis.

Table 11: Apo-AI and apo-AII kinetics after the injection of $^{125}I(AI)HDL_2$ and ^{131}I-HDL mixture.

Subject	apoprotein	FCR pools/day	%I.V.	$t_{1/2}$ days
M.P.	^{125}I AI	0.200	70.1	5.97
	^{131}I AI	0.206	69.4	5.68
	^{131}I AII	0.200	82.1	4.57
J.S.	^{125}I AI	0.158	69.9	7.02
	^{131}I AI	0.173	73.4	6.13
	^{131}I AII	0.226	63.6	5.46

in the total HDL, 0.158 and 0.173 pools/day, respectively. But, apo-AII in total HDL had a faster FCR (0.226 pools/day) than either of the AI apoprotein preparations.

The small differences in the FCRs observed between (AI)HDL and total HDL as well as those between their apoproteins are not readily explained, but they might have been more pronounced had the (AI/AII)HDL particle been used instead of the total HDL. The former had not been used due to the difficulties encountered in its isolation.

3.1.4 Physical and chemical properties of (AI)HDL_2

Fasting blood samples from three normolipidaemic young females were used to isolate HDL_2 from the plasma prior to preparing the (AI)HDL_2 particle. The molecular weights, flotation rates and chemical compositions of both lipoproteins were determined. The results are documented in Table 12 and Figure 22a. These results show that (AI)HDL_2 does not differ markedly from its parent HDL_2, which is in accord with the fact that the former accounts for 67% of the total HDL_2. Both lipoproteins have similar composition, molecular weight and flotation rate.

When the flotation pattern of (AI)HDL, isolated from total HDL, is compared with that of the total HDL, the former showed four maxima in the range $F_{1.2}$ 1-8 (Fig. 22b). The first two maxima correspond to the HDL_3 and the other two occur in the HDL_2 density range.

Table 12: Physical and chemical properties of (AI)HDL_2 compared with HDL_2.

	$M_{app.}$ X 10^{-5}	$F_{1.20}$	Composition gm/100gm				% in HDL_2
			Free Cholesterol	Cholesteryl ester	Phospholipid	protein	
HDL_2	3.28 ± 0.26[a]	6.7 ± .08	6.8 ± .8	20.9 ± 1.7	31.0 ± 2.4	41.3 ± 1.1	100
(AI)HDL_2	3.12 ± 0.18	5.95 ± .19	6.1 ± .8	18.7 ± 1.2	31.2 ± 1.6	44.0 ± 3.5	67 ± 13.1

a. Mean ± 1 S.D. (n = 3)

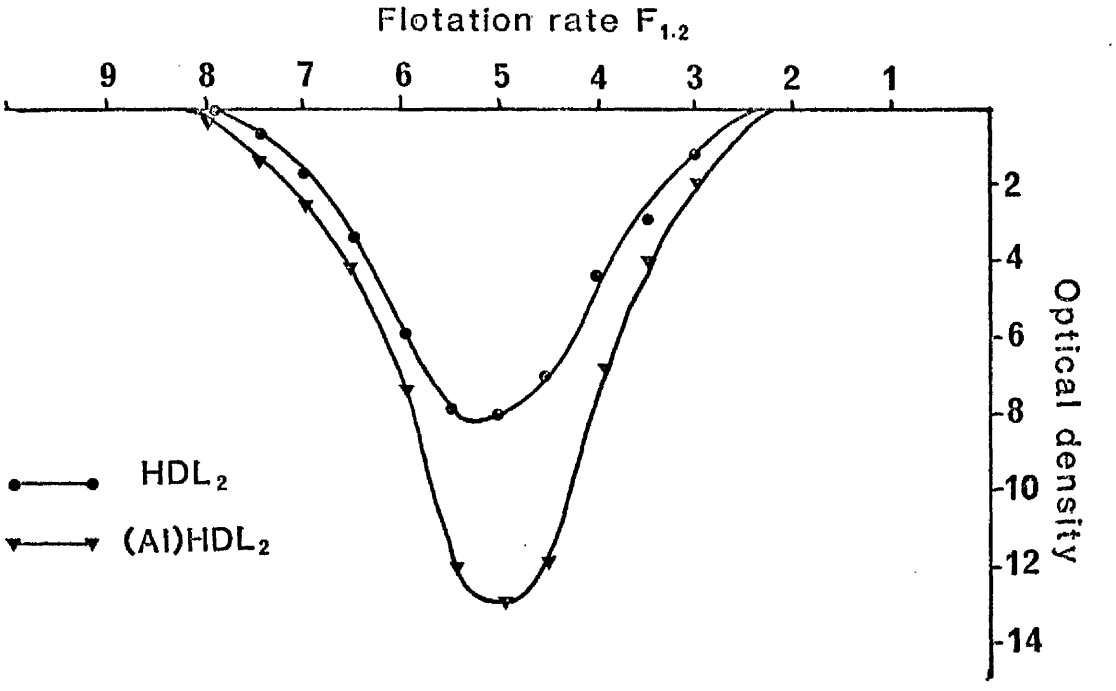


Figure 22a. Flotation patterns of HDL$_2$ and (AI)HDL$_2$ of a normolipidaemic young female (BD).

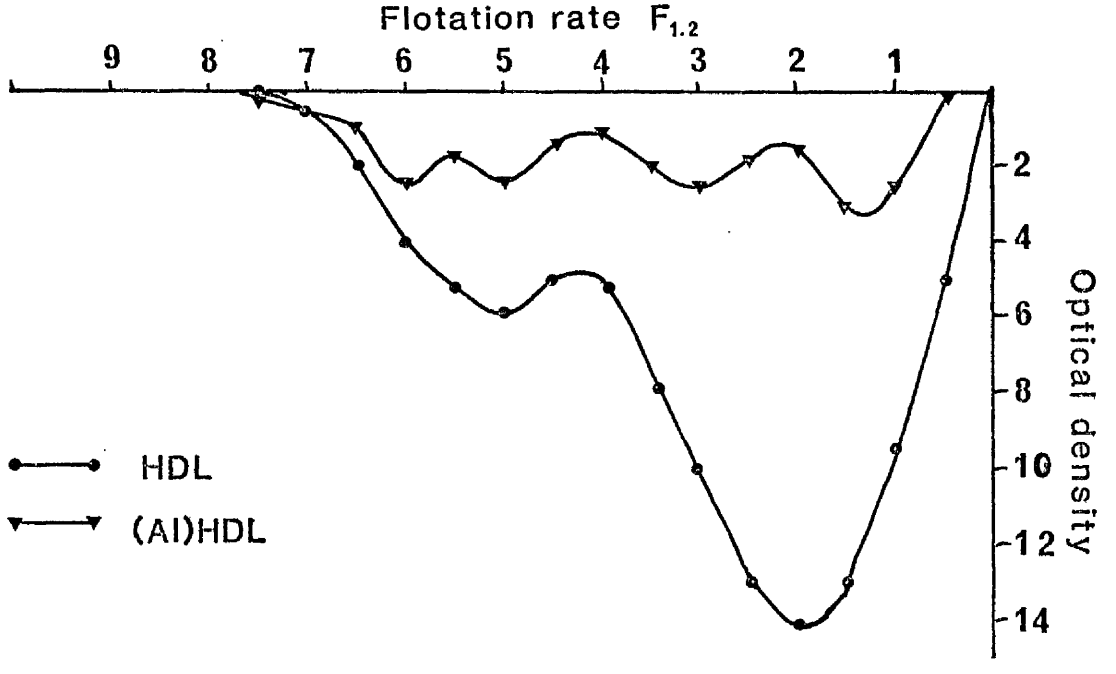


Figure 22b. Flotation patterns of total HDL and (AI)HDL (BD).

3.1.5 Distribution of (AI)HDL in plasma

Plasma samples from a group of eleven normolipidaemic young volunteers were analysed for their HDL subfractions and apoprotein A content, and electroimmunoassay measurements were made of the distribution of the latter between the HDL_2 and HDL_3 fractions (see Sections 2.12 and 2.18). The results are presented in Tables 13 and 14. They demonstrate that apo-AI associated with the (AI)HDL fraction of plasma, with HDL_2 and with HDL_3 accounts for 42, 72 and 44%, respectively, of their total apo-AI content. Moreover, the molar ratio of apo-AI/apo-AII in the (AI/AII)HDL fraction of these materials is similar, ie 1.2, 1.1 and 1.1, respectively.

3.1.6 Effect of nicotinic acid and probucol on the distribution of (AI)HDL particle in plasma and HDL subfractions

Six healthy pre-menopausal non-pregnant females were examined on three occasions, none having taken any drugs (except where specified) during the period of the study. On the first study period, plasma concentrations of HDL and its subfractions were measured and the apo-A content of these fractions determined. These analyses were repeated following two weeks of nicotinic acid therapy (2 gm/day, 5 subjects) and then after one month of probucol (1 gm/day). The results are shown in Tables 15-19.

i) Nicotinic acid: This drug lowered the plasma levels of LDL-cholesterol by 47% ($p<0.01$), but it had no consistent effect on HDL-cholesterol. In spite of this,

Table 13: (AI)HDL distribution in plasma

Subject	Plasma Cholesterol mmol/l	HDL-Chol mmol/l	HDL_2 mg/dl	HDL_3 mg/dl	total apo-AI mg/dl	total apo-AII mg/dl	apo-AI associated with (AI)HDL mg/dl	apo-AI associated with (AI/AII)HDL mg/dl	apo-AI/AII ratio in (AI/AII)HDL mol:mol
H.T.	5.1	1.95	80	201	152	46	76	76	1.02
B.D.	4.4	2.45	113	310	160	46	76	84	1.12
E.M.	6.65	1.85	35	244	149	43	62	87	1.24
D.L.	5.80	1.40	27	224	135	40	52	83	1.27
D.B.	5.25	2.40	74	189	176	48	81	95	1.21
E.B.	5.4	1.80	69	279	149	41	60	89	1.30
M.P.	4.7	1.85	60	282	165	47	76	89	1.16
J.S.	5.2	1.70	53	190	154	45	70	84	1.14
R.A.	4.8	1.85	56	245	134	42	49	85	1.24
V.P.	4.5	n.d.	n.d.	n.d.	158	53	44	114	1.32
Mean	5.20	1.92	63	240	153.2	45.1	64.6	88.6	1.20
± 1 S D	0.67	0.33	25.5	43.4	12.8	3.8	13.0	10.2	0.09

Table 14: Apoprotein A distribution between HDL_2 and HDL_3.

Subject	HDL_2				HDL_3			
	total apo-AII gm/100 gm A protein	apo-AI associated with		apo-AI/AII ratio in $(AI/AII)HDL_2$ mol:mol	total apo-AII gm/100 gm A protein	apo-AI associated with		apo-AI/AII ratio in $(AI/AII)HDL_3$ mol:mol
		$(AI)HDL_2$ gm/100 gm A protein	$(AI/AII)HDL_2$ gm/100 gm A protein			$(AI)HDL_3$ gm/100 gm A protein	$(AI/AII)HDL_3$ gm/100 gm A protein	
H.T.	12.9	63.4	23.7	1.1	26.1	36.2	37.7	0.89
B.D.	12.6	60.6	26.8	1.3	20.0	43.8	37.2	1.20
E.M.	14.7	55.7	29.6	1.2	18.3	43.3	38.4	1.29
D.L.	14.3	59.5	26.2	1.1	22.2	33.0	44.8	1.24
D.B.	13.2	62.5	24.3	1.1	25.6	34.4	40.0	0.96
E.B.	14.6	57.6	27.8	1.18	22.1	30.1	47.8	1.32
J.S.	15.4	57.2	27.4	1.08	n.d.	n.d.	n.d.	n.d.
R.A.	11.2	65.2	23.6	1.30	20.1	40.8	39.1	1.19
M.A.	12.6	66.4	21.0	1.02	32.2	23.6	44.1	0.84
F.M.	12.8	68.1	19.1	0.92	30.0	21.2	48.8	1.00
M.C.	13.1	68.5	18.4	0.87	24.8	29.1	46.1	1.14
Mean	13.4	62.2	24.4	1.11	24.2	33.6	42.4	1.11
± 1 S D	1.6	4.5	3.7	0.137	4.5	7.8	4.4	0.17

Table 15: Effect of nicotinic acid on (AI)HDL particle distribution in plasma

Subject	LDL-cholesterol mmol/l		HDL-cholesterol mmol/l		Plasma HDL_2 mg/dl		Plasma HDL_3 mg/dl		total apo-AI mg/dl		apo-AI associated with (AI)HDL mg/dl		total apo-AII mg/dl		apo-AI associated with (AI/AII)HDL mg/dl		apo-AI/AII ratio in (AI/AII)HDL mol:mol		HDL_2/HDL_3 ratio	
	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N
B.D.	1.85	0.95	2.45	2.65	113	158	310	222	160	173	76	96	46	41	84	77	1.12	1.15	.36	.71
E.M.	4.65	2.35	1.85	1.55	35	56	244	167	149	141	62	73	43	34	87	68	1.24	1.22	.14	.34
D.L.	3.55	2.31	1.40	1.44	27	64	244	168	135	152	52	73	40	34	83	79	1.27	1.42	.12	.38
D.M.	2.35	1.25	2.40	2.00	74	97	189	155	176	156	81	89	48	34	95	67	1.21	1.20	.39	.63
E.B.	3.30	1.45	1.80	1.25	69	82	279	187	149	120	60	63	41	27	89	57	1.30	1.29	.25	.44
Mean	3.14	1.66	1.98	1.78	63.4	91.3	249	179.8	153.8	148.4	66.4	78.8	43.6	34.0	87.6	69.6	1.23	1.25	.25	.50
± 1 S D	1.09	0.64	0.44	0.56	34.4	40.6	46.9	26.4	15.3	19.6	12.0	13.4	3.4	5.0	4.8	8.8	0.07	0.10	.12	.16
p<	.01		NS		.01		.01		NS		.05		.01		.05		NS		.001	

C: Control

N: Nicotinic Acid

Table 16: Effect of nicotinic acid on (AI)HDL distribution in HDL_2 and HDL_3

Subject	HDL_2								HDL_3							
	apo-AI associated with (AI)HDL_2 gm/100 gm A protein		total apo-AII gm/100 gm A protein		apo-AI associated with (AI/AII)HDL_2 gm/100 gm A protein		apo-AI/AII ratio in (AI/AII)HDL_2 mol:mol		apo-AI associated with (AI)HDL_3 gm/100 gm A protein		total apo-AII gm/100 gm A protein		apo-AI associated with (AI/AII)HDL_3 gm/100 gm A protein		apo-AI/AII ratio in (AI/AII)HDL_3 mol:mol	
	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N
B.D.	60.6	74.2	12.6	8.5	26.8	17.3	1.3	1.2	43.8	30.8	19.0	23.3	37.2	45.9	1.20	1.20
E.M.	55.7	75.0	14.7	9.1	29.6	15.9	1.2	1.07	43.3	40.0	18.3	19.2	38.4	40.8	1.29	1.30
D.L.	59.5	63.8	14.3	13.0	26.2	23.2	1.1	1.10	33.0	41.1	22.2	21.0	44.8	37.9	1.24	1.10
D.B.	62.5	70.6	13.2	10.8	24.3	18.6	1.1	1.06	34.4	37.5	25.6	21.4	40.0	41.1	0.96	1.18
E.B.	57.6	64.5	14.6	13.5	27.8	22.0	1.8	1.00	30.1	27.1	22.1	27.6	47.8	45.3	1.32	1.00
Mean	59.2	69.6	13.9	11.0	26.9	19.4	1.18	1.09	36.9	35.3	21.4	22.5	41.6	42.2	1.20	1.16
± 1 S D	4.4	5.2	.9	2.2	2.0	3.1	.09	.07	6.3	6.1	2.9	3.2	4.5	3.4	.14	.11
p <	.01		.05		.02		NS		NS		NS		NS		NS	

C: Control

N: Nicotinic Acid

Table 17a: Changes in (AI)HDL_2 and HDL_3 composition during nicotinic acid therapy.

	(AI)HDL_2		HDL_2	
	C	N	C	N
Protein	44.1[a,b]	43.2	40.7	42.6
Phospholipid	31.4	31.9	32.2	31.1
F C	5.9	6.2	7.2	7.0
E C	18.6	18.9	20.0	19.1

a. Mean

b. gm/100gm

F C : Free Cholesterol

E C : Esterified Cholesterol

Table 17b: Effect of nicotinic acid on (AI)HDL_2 concentration.

Subject	% of (AI)HDL_2 in HDL_2		Plasma concentration mg/dl			
			(AI)HDL_2		(AI/AII)HDL_2	
	C	N	C	N	C	N
B.D.	59	83.7	66	132	46	26
E.M.	59.8	74.4	18	41	12	14
Mean ± 1 S D	59.4	79.1	42	86.5	29	20

C: Control

N: Nicotinic Acid

Table 18: Effect of probucol on (AI)HDL particle distribution in plasma

Subject	LDL-cholesterol mmol/l		HDL-cholesterol mmol/l		Plasma HDL_2 mg/dl		Plasma HDL_3 mg/dl		total apo-AI mg/dl		apo-AI associated with (AI)HDL mg/dl		total apo-AII mg/dl		apo-AI associated with (AI/AII)HDL mg/dl		apo-AI/AII ratio in (AI/AII)HDL mol:mol		HDL_2/HDL_3 ratio	
	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
H.T.	2.70	1.95	1.95	1.55	80	23	201	193	152	142	76	60	46	40	76	82	1.02	1.26	.4	.12
B.D.	1.85	1.75	2.45	1.35	113	26	310	197	160	156	76	57	46	42	84	99	1.12	1.44	.36	.13
E.M.	4.65	3.35	1.85	0.80	35	32	244	203	149	137	62	49	43	38	87	88	1.24	1.42	.14	.16
D.L.	3.55	2.65	1.40	1.25	27	25	224	261	135	125	52	46	40	38	83	79	1.27	1.28	.12	.10
D.B.	2.35	2.25	2.40	1.65	74	37	189	408	176	150	81	55	48	44	95	95	1.21	1.32	.39	.09
E.B.	3.30	2.80	1.80	1.55	69	26	279	273	149	146	60	49	41	40	89	97	1.30	1.40	.25	.10
Mean	3.07	2.46	1.98	1.36	66.2	28.1	241.1	256	153.5	142.7	67.8	52.7	44.0	40.3	85.7	90.0	1.19	1.34	.28	.12
± 1 S D	0.99	0.59	0.40	0.31	31.5	5.3	46.4	82	13.7	10.8	11.4	5.5	3.2	2.3	6.4	8.4	0.1	0.1	.13	.10
p <	.05		.02		.05		NS		.02		.01		.01		NS		NS		.05	

C: Control

P: Probucol

Table 19: Effect of probucol on (AI)HDL distribution in HDL_2 and HDL_3

Subject	HDL_2								HDL_3							
	apo-AI associated with (AI)HDL_2 gm/100 gm A protein		total apo-AII gm/100 gm A protein		apo-AI associated with (AI/AII)HDL_2 gm/100 gm A protein		apo-AI/AII ratio in (AI/AII)HDL_2 mol:mol		apo-AI associated with (AI)HDL_3 gm/100 gm A protein		total apo-AII gm/100 gm A protein		apo-AI associated with (AI/AII)HDL_3 gm/100 gm A protein		apo-AI/AII ratio in (AI/AII)HDL_3 mol:mol	
	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
H.T.	63.4	62.4	12.9	14.4	23.7	23.2	1.1	0.99	36.2	33.1	26.1	20.4	37.7	46.6	0.89	1.40
B.D.	60.6	57.6	12.6	12.9	26.8	29.5	1.3	1.40	43.8	36.3	19.0	21.1	37.2	41.9	1.20	1.20
E.M.	55.7	48.4	14.7	16.1	29.6	35.5	1.2	1.35	43.3	37.3	18.3	25.9	38.4	36.8	1.29	.87
D.L.	59.5	39.5	14.3	21.0	26.2	39.5	1.1	1.14	33.0	34.0	22.2	23.7	44.8	42.3	1.24	1.10
D.B.	62.5	56.8	13.2	15.7	24.3	27.5	1.1	1.07	34.4	33.3	25.6	23.7	40.0	43.0	0.96	1.10
E.B.	57.6	45.0	14.6	18.3	27.8	36.7	1.18	1.20	30.1	30.1	22.1	25.1	47.8	44.8	1.32	1.10
Mean	59.9	51.6	13.7	16.4	26.4	32.0	1.18	1.19	36.8	34.0	22.2	23.3	41.0	42.6	1.15	1.13
± 1 S D	2.9	8.7	0.9	2.9	2.2	6.2	.09	.16	5.6	2.6	3.2	2.2	4.3	3.3	.18	.17
p<	.05		.05		.05		NS		NS		NS		NS		NS	

C: Control

P: Probucol

it had a dramatic influence on the distribution of HDL_2 and HDL_3 in plasma (Fig. 23). It raised the HDL_2 level by 44% ($p<0.01$) and lowered that of HDL_3 by 28% ($p<0.01$). Consequently, the plasma HDL_2/HDL_3 ratio was doubled ($p<0.001$) (Table 15). Similarly, although it had no consistent effect on the plasma total apo-AI concentration, it increased the plasma concentration of apo-AI associated with the (AI)HDL by 19% ($p<0.05$) and decreased that associated with the (AI/AII)HDL by 21% ($p<0.05$). In addition to that the plasma level of apo-AII was lowered by 22% ($p<0.01$). The drug did not change the apo-AI/apo-AII ratio in (AI/AII)HDL fraction of plasma, HDL_2 or HDL_3 (Tables 15 and 16). In spite of the reduction in the plasma HDL_3 level, its apoprotein composition did not change significantly. However, the rise in the plasma HDL_2 concentration is attributable to the increase in its (AI)HDL_2 content since the proportion of apo-AI associated with the latter was increased by 18% ($p<0.01$). Moreover, the apo-AI and apo-AII in (AI/AII)HDL_2 decreased by 27% ($p<0.02$) and 21% ($p<0.05$), respectively (Table 16).

HDL_2 and (AI)HDL_2 were isolated from the plasma of two subjects before and during the therapy. Their chemical composition and the (AI)HDL_2 content of HDL_2 were measured. No change was observed in their composition (Table 17a). However, the percentage of (AI)HDL_2 in HDL_2 and its concentration in plasma were increased while those of (AI/AII)HDL_2 were decreased (Table 17b).

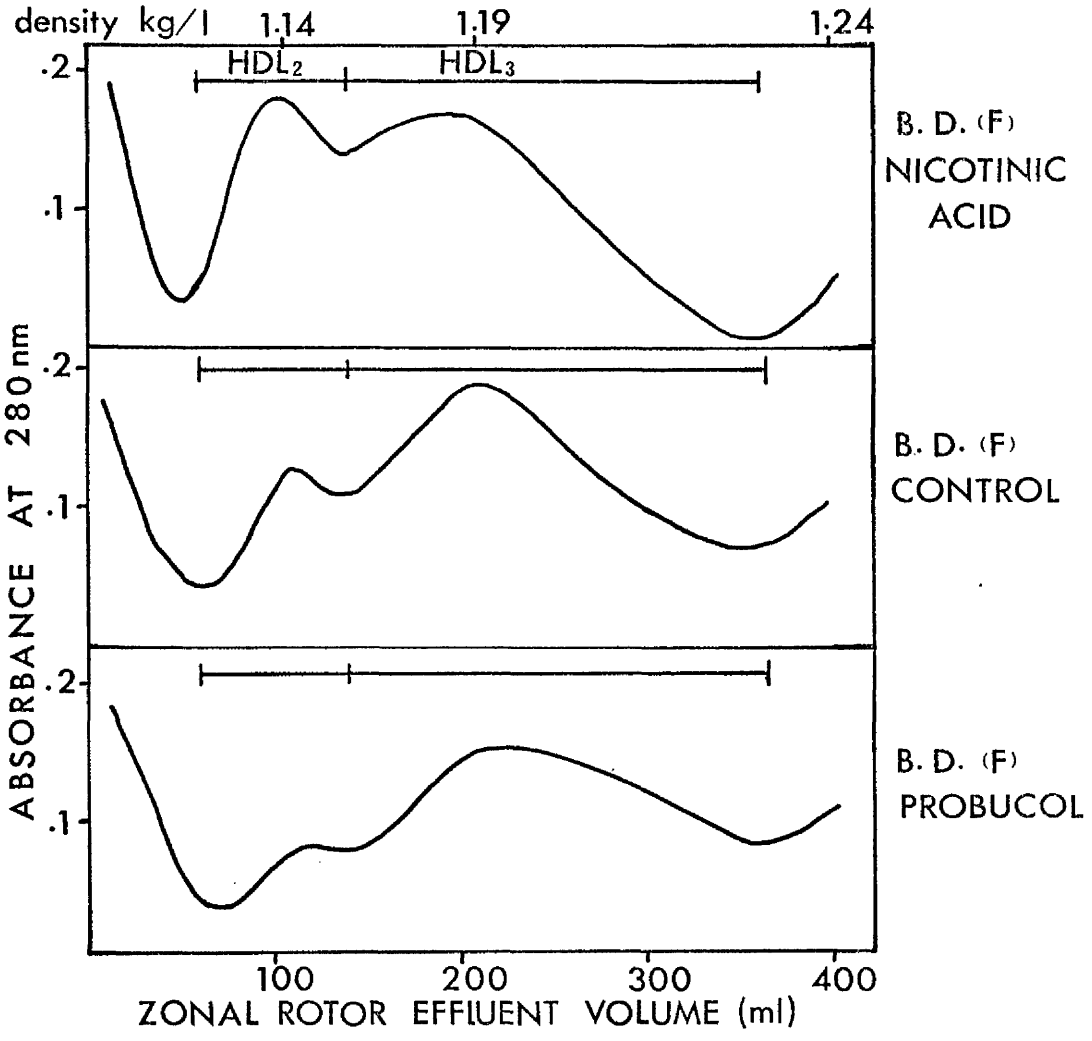


Figure 23. Effect of drugs on the rate zonal profile of normal subjects.

ii) Probucol: In contrast to nicotinic acid, this drug caused a dramatic drop in the plasma level of HDL_2 (Fig. 23 and Table 18); moreover, the HDL_2/HDL_3 ratio in the plasma was decreased by 57% ($p<0.05$). The drug lowered both HDL- and LDL-cholesterol concentrations in plasma by 31% ($p<0.02$) and 20% ($p<0.05$), respectively. In addition to that, the plasma levels of total apo-AI, apo-AII and apo-AI associated with the (AI)HDL fell significantly (by 7, 22 and 8%, respectively (Table 18)). It seems that the drop in the (AI)HDL_2 accounts for the reduction in the plasma HDL_2 level, since the concentration of the apo-AI associated with the former lipoprotein fell by 14% ($p<0.05$). In contrast, the concentration of the apo-AI and apo-AII associated with (AI/AII)HDL_2 increased by 20% ($p<0.05$) and 21% ($p<0.05$), respectively. On the other hand, HDL_3 apoproteins did not show any significant change on treatment (Table 19). Similar to nicotinic acid, probucol did not affect the apo-AI/apo-AII ratio in the (AI/AII)HDL fraction of plasma, of HDL_2 or of HDL_3 (Tables 18 and 19).

3.1.7 Discussion

These results demonstrate that HDL contains two immunologically distinct types of particles: one type has apo-AI as its major apoprotein ((AI)HDL) and the other ((AI/AII)HDL) contains both apo-AI and apo-AII. Both particles behave as lipoproteins in that they float at a solution density of 1.21 kg/l. Moreover, they appear to be physiologically relevant entities since they were detected in fresh untreated plasma by the two-phase gel

technique. Their concentrations can be measured in whole plasma or in isolated HDL subfractions by more than one method. The (AI)HDL particles were quantitated directly by three procedures:

a) the immunoaffinity chromatography using anti-apo-AII;
b) the two-phase agarose gel immuno-electrophoresis; and
c) immunoprecipitation with anti-apo-AII antibody and measuring the apo-AI content of the supernatant. The last two techniques are applicable to plasma samples. In the three methods the amount of the (AI/ AII)HDL can be calculated from the difference between the amount of total HDL in the starting material and that of the (AI)HDL. However, the first method was used for preparation of the (AI)HDL particles.

In spite of the fact that both particles contain apo-AI, it appears that there is little exchange of apo-AI between them either in vitro or in vivo. The results of the exchange experiments demonstrated that the transfer of AI radioactivity was minimal in both density ranges, HDL_2 and HDL_3 (Tables 7 and 8). In contrast, exchange between similar particles apparently took place in both density ranges. Bearing in mind that the exchange between (AI)HDL and (AI/AII)HDL was minimal, it could be concluded that both particles are biologically distinct although they may have similar fractional catabolic rates. This concept was further supported by the drug perturbation study which established that nicotinic acid treatment markedly increased the plasma concentration of (AI)HDL with the concomitant decrease in

that of (AI/AII)HDL (Table 15). Moreover, probucol therapy decreased the plasma levels of both lipoproteins but not to the same extent (Table 18). However, neither drug influenced the ratio of apo-AI/apo-AII in (AI/AII)HDL particles; in addition, their major influence was on the HDL_2 fraction. It was increased on nicotinic acid and decreased on probucol. Consequently plasma HDL_2/HDL_3 ratio was doubled by the former and halved by the latter (Tables 15 and 18).

3.2 STUDY II: Effect of bezafibrate and probucol on HDL metabolism in hypertriglyceridaemic patients.

This study aimed to examine the effects of two lipid lowering drugs, ie, bezafibrate and probucol, on the plasma concentration, composition and metabolism of high density lipoprotein in six hyperlipidaemic subjects; one of the participants (RB) did not undertake the probucol trial for personal reasons. Subjects were studied in a control phase in which they did not receive any drug therapy, after which they received bezafibrate therapy at a dose of 200 mg thrice daily for a minimum of four weeks. In a third phase of the study they were given probucol therapy in a dose of 1 gm/day for a minimum period of three months. In each of the three phases, body weight was recorded daily and plasma concentrations of triglycerides, total, VLDL-, LDL- and HDL-cholesterol as well as apo-AI and apo-AII were determined on several occasions. HDL_2 and HDL_3 concentrations in plasma were quantitated from rate zonal profiles. Moreover, HDL

apo-AI and apo-AII metabolism was followed by separation of the apoproteins from HDL and determination of their specific activities.

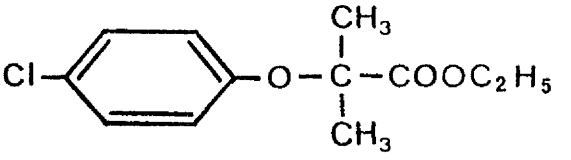
3.2.1 Results

A. Bezafibrate (2-{4-[2-(4-chlorobenzamido)ethyl]-phenoxy}-2-methylpropionic acid)

This is a lipid lowering drug of the clofibrate family (Fig. 24). It had a significant effect on the plasma levels of triglycerides and VLDL-cholesterol. They were lowered by 49% ($p<0.01$) and 46% ($p<0.001$), respectively (Table 20). Although plasma total cholesterol was lowered by 9%, this drop was not significant. Furthermore, no consistent change was observed in the plasma levels of LDL- and HDL-cholesterol, nor did apo-AI and apo-AII levels in plasma or their fractional catabolic and synthetic rates change significantly (Tables 21 and 22). The drug induced a moderate but not significant increase in the plasma levels of HDL, HDL_2 and HDL_3 by 17, 70 and 10%, respectively. Treatment did not alter the chemical composition of HDL subfractions (Table 23).

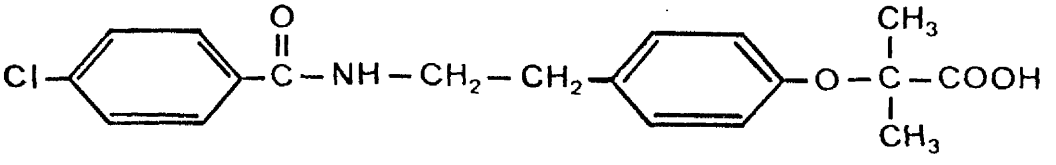
B. Probucol (4,4'-[isopropylidenedithio]bis[2,6-di-t-butylphenol])

Probucol is a bis-phenol which has no apparent structural similarity to other lipid lowering agents (Fig. 24). It showed no effect on plasma concentrations of triglycerides, total cholesterol, VLDL-, and LDL-cholesterol; plasma HDL_2, which was initially low, was



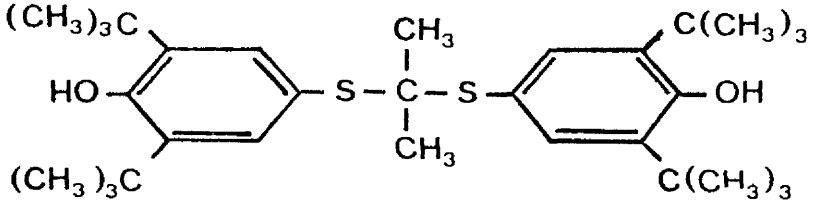
Ethyl ∝-(4-chlorophenoxy)-isobutyrate

(CLOFIBRATE)



2-{4-[2-(4-chlorobenzamido)ethyl]phenoxy}-2-methylpropionic acid

(BEZAFIBRATE)



4,4`-(isopropylidenedithio)bis(2,6-di-t-butylphenol)

(PROBUCOL)

Figure 24. Chemical structure of clofibrate, bezafibrate, and probucol.

Table 20: Effects of bezafibrate on plasma lipids and lipoproteins

Subject	Hyperlipo-proteinaemic Phenotype	Age (yr)	Body Weight (kg)		Plasma Triglyceride (mg/dl)		Plasma Cholesterol (mg/dl)		Cholesterol (mg/dl) in VLDL		LDL		HDL	
			Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug
					n = 5		n = 5				n = 5			
D.C.	IIb	49	82.3[a] ± 0.4	81.3 ± 0.2	235.4 ± 39.8	135.4 ± 14.7	323.1 ± 15.1	245.0 ± 7.5	50.3 ± 36.8	19.4 ± 4.3	218.7 ± 33.7	166.0 ± 11.2	54.2 ± 2.7	59.6 ± 1.9
J.McD.	IV	58	61.2 ± 0.1	65.3 ± 0.3	290.3 ± 121.2	140.7 ± 24.3	236.1 ± 23.2	224.5 ± 8.1	38.7 ± 17.8	23.2 ± 9.7	135.5 ± 5.8	143.2 ± 22.1	61.9 ± 3.5	54.2 ± 9.3
A.K.	IV	61	78.8 ± 0.1	81.9 ± 0.2	577.1 ± 117.7	349.6 ± 63.7	224.5 ± 10.8	236.0 ± 14.7	96.8 ± 24.4	65.8 ± 10.8	85.1 ± 22.4	139.3 ± 8.5	45.3 ± 18.5	31.0 ± 7.0
M.C.	IV	52	65.3 ± 0.4	67.0 ± 0.4	462.9 ± 108.0	194.7 ± 34.5	263.2 ± 15.9	278.6 ± 14.3	81.3 ± 16.6	38.7 ± 7.0	143.2 ± 19.4	189.6 ± 14.7	42.6 ± 4.3	52.6 ± 3.9
J.F.	IV	57	84.7 ± 0.6	85.0 ± 0.7	294.7 ± 17.7	174.3 ± 33.6	260.5 ± 13.5	205.1 ± 12.4	107.2 ± 7.0	69.7 ± 12.0	110.3 ± 1.4	81.3 ± 2.3	43.7 ± 1.2	54.2 ± 2.9
R.B.	IV	29	80.8 ± 0.5	79.2 ± 0.7	380.6 ± 193.8	153.1 ± 32.7	249.6 ± 17.0	203.6 ± 3.3	59.2 ± 26.3	19.4 ± 5.8	142.0 ± 9.3	129.3 ± 18.2	48.4 ± 5.8	55.3 ± 14.3
paired t-test vs. control			NS		p<0.01		NS		p<0.001		NS		NS	

a. mean ± 1 S D

Table 21 Effects of bezafibrate on HDL apoprotein and subfraction concentrations

Subject	Plasma Apo AI (mg/dl)		Plasma Apo AII (mg/dl)		Plasma HDL_2 (mg/dl)		Plasma HDL_3 (mg/dl)	
	Control	Drug	Control	Drug	Control	Drug	Control	Drug
D.C.	129 ± 14	125 ± 13	33 ± 5	30 ± 4	13.4	44.8	348	387
J.McD.	127 ± 11	135 ± 11	45 ± 2	48 ± 3	14.5	15.3	416	396
A.K.	132 ± 4	142 ± 11	34 ± 3	35 ± 5	15.0	26.9	352	355
M.C.	141 ± 4	137 ± 10	49 ± 3	36 ± 3	8.0	19.5	309	355
J.F.	132 ± 14	131 ± 8	40 ± 4	39 ± 3	16.9	20.1	259	383
R.B.	149 ± 10	141 ± 9	39 ± 4	36 ± 2	14.7	20.2	277	367
Mean ± 1 S.D.	135 ± 8	135 ± 6	40 ± 6	37 ± 6	13.8 ± 3.0	24.5 ± 10.6	327 ± 57	357 ± 47
Paired t-test vs. control		NS		NS		NS		NS

Table 22: Effects of bezafibrate on high density lipoprotein metabolism

Subject	Apolipoprotein AI Kinetics				Apolipoprotein AII Kinetics			
	Fractional Clearance Rate (pools/d)		Absolute Clearance Rate (mg/kg/d)		Fractional Clearance Rate (pools/d)		Absolute Clearance Rate (mg/kg/d)	
	Control	Drug	Control	Drug	Control	Drug	Control	Drug
D.C.	0.156	0.185	5.6	8.2	0.395	0.175	3.63	1.87
J.McD.	0.255	0.209	10.9	11.5	0.221	0.243	3.35	4.68
A.K.	0.280	0.264	11.1	11.2	0.284	0.247	2.90	2.58
M.C.	0.264	0.308	15.9	15.9	0.150	0.269	3.14	3.65
J.F.	0.268	0.242	10.9	10.3	0.155	0.229	1.91	2.91
R.B.	0.375	0.424	17.8	19.6	0.291	0.200	3.62	2.38
Mean ± 1 S D	0.264	0.272	12.04	12.79	0.249	0.227	3.09	3.01
	+ 0.075	+ 0.086	+ 4.34	+ 4.20	+ 0.094	+ 0.034	+ 0.64	+ 1.01
Paired t-test vs. control		NS		NS		NS		NS

Table 23: Effects of bezafibrate on lipoprotein compositions

Lipoprotein	Composition (gm/100 gm)											
	Free Cholesterol		Esterified Cholesterol		Triglyceride		Phospholipid		Protein		Cholesterol/Protein ratio	
	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug
HDL_2	4.9[a]	3.6	16.4	15.8	6.2	5.1	28.7	29.0	43.7	45.3	0.34	0.32
(n = 6)	± 2.8	± 0.8	± 5.5	± 6.0	± 2.6	± 3.1	± 5.6	± 5.1	± 4.8	± 3.4	± 0.18	± 0.08
HDL_3	1.8	2.1	12.1	12.7	2.9	3.6	29.4	26.0	53.7	54.7	0.17	0.18
(n = 6)	± 0.4	± 0.7	± 2.4	± 3.1	± 2.2	± 3.4	± 4.4	± 3.0	± 3.6	± 3.4	± 0.04	± 0.04

a. Mean ± 1 S D

not consistently affected (Tables 24 and 25). The main effect of this drug on the HDL fraction whose concentration decreased by 35% ($p<0.05$) was accounted for by the decrease in HDL_3 level (37%, $p<0.05$) (Table 25).

Both apo-AI and apo-AII concentrations in plasma were lowered by 21 and 13%, respectively, but the lowering of apo-AI was not statistically significant while it was significant in the case of apo-AII ($p<0.05$) (Table 25). This lowering of HDL apoprotein levels was reflected in their kinetics (Table 26). Probucol induced a 39% decrease in apo-AI synthesis ($p<0.01$) and in four out of the five subjects, also reduced its fractional catabolic rate. The fifth (M.C., Tables 25 and 26) showed the greatest drop in apo-AI level due to a combination of reduced synthesis and increased catabolism, and interestingly his plasma triglyceride level (Table 24) doubled during treatment. The changes which the drug effected on apo-AII metabolism were quantitatively similar to those described for apo-AI to the extent that subject M.C. again showed an atypical response. Overall, treatment lowered plasma apo-AII by 13% ($p<0.05$) due to a reduction in its synthesis. The fractional catabolic rate of the protein fell substantially in all subjects except M.C., but this drop was not statistically significant. The reduction of total plasma apo-AI was less than that of HDL-cholesterol suggesting a change in HDL composition and this was confirmed by the direct measurement of the composition of HDL_2 and HDL_3. Their protein content rose (19.1%, $p<0.02$

Table 24: Effects of probucol on plasma lipids and lipoproteins

Subject	Hyperlipo-proteinaemic Phenotype	Age (yr)	Body Weight (kg)		Plasma Triglyceride (mg/dl)		Plasma Cholesterol (mg/dl)		Cholesterol (mg/dl) in VLDL		LDL		HDL	
			Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug
					n = 5		n = 5				n = 5			
D.C.	IIb	49	82.3[a] ± 0.4	81.3 ± 0.2	235.4 ± 39.8	230.0 ± 38.9	323.1 ± 15.1	240.3 ± 10.1	50.3 ± 36.8	23.2 ± 15.5	218.7 ± 33.7	175.3 ± 10.1	54.2 ± 2.7	42.6 ± 2.3
J.McD.	IV	58	61.2 ± 0.1	65.3 ± 0.3	290.3 ± 121.2	289.4 ± 40.7	236.1 ± 23.2	253.5 ± 8.5	38.7 ± 17.8	53.4 36.4	135.5 ± 5.8	152.9 ± 1.5	61.9 ± 3.5	47.2 ± 2.0
A.K.	ICV	61	78.8 ± 0.1	81.9 ± 0.2	577.1 ± 117.7	332.7 ± 50.4	224.5 ± 10.8	195.4 ± 22.4	96.8 ± 24.4	44.9 ± 12.0	85.1 ± 22.4	108.7 ± 16.3	45.3 ± 18.6	41.8 ± 2.3
M.C.	IV	52	65.3 ± 0.4	67.0 ± 0.4	462.9 ± 108.0	874.4 ± 102.7	263.2 ± 15.9	256.6 ± 23.2	81.3 ± 16.6	137.4 ± 19.4	143.2 ± 19.4	102.1 ± 6.9	42.6 ± 4.3	17.0 ± 3.5
J.F.	IV	57	84.7 ± 0.6	85.0 ±0.7	294.7 ± 17.7	270.8 ± 72.6	260.5 ± 13.5	233.7 ± 15.9	107.2 ± 7.0	113.8 ±24.8	110.3 ± 1.4	84.8 ± 5.4	43.7 ± 1.2	35.2 ± 3.9
paired t-test vs. control				NS		NS		NS		NS		NS		p<0.05

a. mean ± 1 S D

Table 25: Effects of probucol on HDL apoprotein and subfraction concentrations.

Subject	Plasma Apo AI (mg/dl)		Plasma Apo AII (mg/dl)		Plasma HDL_2 (mg/dl)		Plasma HDL_3 (mg/dl)	
	Control	Drug	Control	Drug	Control	Drug	Control	Drug
D.C.	129 ± 14	105 ± 9	33 ± 5	28 ± 5	13.4	10.6	348	190
J.McD.	127 ± 11	114 ± 11	45 ± 2	40 ± 3	14.5	12.5	416	257
A.K.	132 ± 4	114 ± 10	34 ± 3	33 ± 2	15.0	21.6	352	268
M.C.	141 ± 4	69 ± 9	49 ± 3	39 ± 3	8.0	16.0	309	99
J.F.	132 ± 14	117 ± 12	40 ± 4	37 ± 4	16.9	10.0	259	247
Mean ± 1 S D	132 ± 5	104 ± 20	40 ± 7	35 ± 5	13.6 ± 3.4	14.2 ± 4.8	337 ± 58	212 ± 70
Paired t-test vs. control	NS		$p<0.05$		NS		$p<0.05$	

Table 26: Effects of probucol on high density lipoprotein metabolism

Subject	Apolipoprotein AI Kinetics				Apolipoprotein AII Kinetics			
	Fractional Clearance Rate (pools/day)		Absolute Clearance Rate (mg/kg/d)		Fractional Clearance Rate (pools/day)		Absolute Clearance Rate (mg/kg/d)	
	Control	Drug	Control	Drug	Control	Drug	Control	Drug
D.C.	0.156	0.095	5.6	3.2	0.395	0.145	3.63	1.30
J.McD.	0.255	0.205	10.9	7.3	0.221	0.138	3.35	1.72
A.K.	0.280	0.240	11.1	8.6	0.284	0.251	2.90	2.60
M.C.	0.264	0.306	15.9	9.0	0.150	0.149	3.14	2.48
J.F.	0.268	0.142	10.9	5.4	0.155	0.114	1.91	1.37
Mean	0.236	0.198	10.9	6.7	0.241	0.159	2.99	1.89
± 1 S D	± 0.049	± 0.082	± 3.6	± 2.4	± 0.102	± 0.052	± 0.66	± 0.61
Paired t-test vs. control	NS		p<0.01		NS		p<0.05	

and 12.1%, $p<0.05$, respectively) and there was a significant decrease in the esterified cholesterol content of HDL_2 ($p<0.02$) as well as in the cholesterol/protein ratio in HDL_3 ($p<0.05$) (Table 27).

3.2.2 Discussion

A. Probucol has been reported to lower plasma cholesterol levels, but the response was variable among patients (166-168). The results of this study reflect this situation where plasma total cholesterol changed by a mean of 10% with a range of 26% to 3%. Although both LDL and HDL contributed to the fall in cholesterol, its variability was attributable to the former since the drop in HDL in response to the drug was consistent in all subjects. Treatment lowered both HDL-cholesterol and apo-AI and apo-AII levels, the latter as a result of suppressed synthesis. These changes were accompanied by a fall in the level of HDL_3, the major apo-A containing particle in the plasma (48,169). Since the percentage fall in plasma HDL-cholesterol (26%) was higher than that of total apo-A (19%) it could be inferred that probucol treatment lowered the cholesterol/protein ratio in the HDL_3 fraction. This in fact was confirmed by direct compositional analysis (Table 27). Thus, a major effect of probucol is to lower plasma HDL and reduce its cholesterol saturation. Despite the fact that HDL is regarded as a negative risk factor for IHD the multiplicity of effects of probucol on this parameter makes it difficult to assess how the drug will influence the apparent antiatherogenic function of the lipoprotein.

Table 27: Effects of probucol on lipoprotein compositions

Lipoprotein	Composition (gm/100 gm)											
	Free Cholesterol		Esterified Cholesterol		Triglyceride		Phospholipid		Protein		Cholesterol/Protein ratio	
	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug
HDL_2 (n = 5)	5.3 ± 2.9[a]	4.0 ± 3.0	17.8 ± 4.8	13.3 ± 2.5[c]	6.9 ± 2.3	4.3 ± 1.0	27.6 ± 5.6	28.1 ± 3.2	42.3 ± 3.6	50.4 ± 1.8[c]	0.37 ± 0.18	0.24 ± 0.05
HDL_3 (n = 5)	1.9 ± 0.3	1.6 ± 1.0	12.6 ± 2.3	9.5 ± 2.5	3.2 ± 2.2	3.2 ± 1.9	29.2 ± 4.9	26.4 ± 3.3	53.0 ± 3.5	59.4 ± 3.7[b]	0.18 ± 0.03	0.12 ± 0.02[b]

a. Mean ± 1 S D

Paired t-test vs. control b. $p<0.05$

c. $p<0.02$

Indeed, preliminary evidence (170) suggests that such therapy may reduce cardiovascular risk. Nevertheless, since the efficacy of the drug is variable from patient to patient, it is prudent to match probucol treatment with those subjects whose response in terms of LDL lowering is good.

B. Bezafibrate has been shown to lower plasma concentrations of VLDL, triglyceride and total cholesterol in hyperlipoproteinaemia; on the other hand, plasma HDL-cholesterol levels were not influenced by treatment. Its influence on LDL-cholesterol was variable depending on the type of hyperlipoproteinaemia treated. The level decreased in type II but did not change in type IV patients (171). The results of the present study showed a decrease in the plasma levels of VLDL-cholesterol and triglyceride (46 and 49%, respectively) which is in good agreement with the above mentioned report; however, total cholesterol concentrations were not consistently affected.

The reduction in plasma triglyceride was attributed to the decrease in the VLDL-triglyceride (171). Moreover, since the drug had no influence on the apo-AI and apo-AII levels in plasma nor on their catabolic and synthetic rates (Tables 21 and 22), it can be concluded that this drug affects mainly VLDL metabolism which is similar to the effect of clofibrate (172,173).

3.3 STUDY III: Mathematical analysis of tracer data

The aim of this study was to compare the kinetic parameters derived from HDL turnover data by three techniques i.e., curve peeling (Matthews) (160), multi-compartmental simulation (Berman) (164,165) and graphical integration (Nosslin) (161). The utility of the Matthews approach for analysis of tracer data is limited due to the fact that extravascular catabolism is not taken into consideration. This gives a true physiological picture only in the case where the protein under study is catabolised exclusively in the intravascular compartment. Recent studies *in vitro* (119,122,123) and *in vivo* (174) have suggested that HDL is catabolised in the liver as well as in other tissues, and so is subject to extra-vascular degradation. Moreover, previous *in vitro* studies (128) have shown that the tracer data of HDL can best be fitted to a model which allows for extravascular catabolism.

3.3.1 Results

Plasma and urine data from twenty subjects obtained after injection of radioiodinated HDL were analysed by the three methods and the results are presented in Tables 28 and 29. The shapes of the decay curves from the three compartments i.e., the plasma (P) extravascular (E) and urine (U), are shown in Figure 25. The total FCRs calculated by the three methods were not significantly different. Moreover, strong correlations existed between the methods when compared by linear regression. However, the closest correlation was between the values calculated

Table 28: HDL decay data analysed by three different methods.

Case No	Total FCR			I.V. FCR		Ex.V. FCR*		E/P ratio			Total Flux mg/kg/day			Plasma Flux mg/kg/day		Non Plasma Flux mg/kg/day	
	M	N	B	N	B	N	B	M	N	B	M	N	B	N	B	N	B
1.	.174	.160	.153	.116	.113	.044	.040	.45	.80	.81	9.4	9.4	8.8	6.3	6.1	3.1	2.7
2.	.167	.160	.153	.097	.092	.063	.061	.35	.70	.72	4.1	3.9	3.8	2.4	2.3	1.5	1.5
3.	.185	.157	.165	.107	.104	.050	.061	.21	.85	.93	13.2	11.2	11.7	7.6	7.4	3.6	4.3
4.	.180	.165	.167	.094	.099	.071	.066	.19	.77	.79	5.9	5.4	5.4	3.1	3.2	2.3	2.2
5.	.259	.263	.262	.194	.197	.069	.065	.26	.74	1.80	21.4	21.6	21.5	16.0	16.1	5.6	5.4
6.	.228	.204	.211	.163	.168	.041	.043	.31	.86	2.16	20.2	18.0	18.7	14.4	14.9	3.6	3.8
7.	.268	.262	.270	.200	.210	.063	.060	.40	1.38	2.13	11.9	11.6	12.0	8.9	9.3	2.7	2.7
8.	.324	.328	.342	.260	.279	.068	.063	.44	.40	2.16	18.8	19.1	19.9	15.1	16.2	4.0	3.7
9.	.325	.337	.336	.284	.288	.053	.049	.42	.51	2.31	19.5	20.3	19.2	17.1	17.3	3.2	2.9
10.	.311	.324	.324	.270	.275	.054	.049	.35	.55	2.12	15.0	15.6	15.7	13.0	13.3	2.6	2.4
11.	.239	.239	.240	.198	.200	.041	.040	.40	.77	1.75	17.9	18.0	18.0	14.9	15.0	3.1	3.0
12.	.446	.413	.427	.349	.356	.064	.071	.38	1.69	3.56	13.3	12.3	12.6	10.4	10.6	1.9	2.1
13.	.250	.227	.231	.169	.171	.058	.060	.45	1.30	1.99	11.3	10.3	10.4	7.7	7.7	2.6	2.7
14.	.233	.241	.241	.185	.187	.055	.054	.53	.75	1.79	16.1	16.6	16.6	12.8	12.9	3.8	3.7
15.	.286	.239	.235	.132	.139	.100	.096	.41	1.54	1.85	9.4	8.8	8.9	4.3	4.5	4.5	4.4
16.	.231	.229	.226	.167	.169	.062	.057	.42	.87	1.78	13.6	13.4	13.3	9.8	9.9	3.6	3.4
17.	.246	.210	.208	.102	.104	.108	.104	.36	2.12	1.62	19.9	17.0	16.8	8.3	8.4	8.7	8.4
18.	.240	.256	.256	.210	.214	.046	.042	.43	.52	1.34	8.7	9.3	9.2	7.6	7.7	1.7	1.5
19.	.280	.293	.300	.189	.196	.105	.104	.41	1.26	1.86	13.9	14.6	12.4	9.4	9.7	5.2	5.1
20.	.274	.325	.337	.301	.307	.024	.030	.44	1.55	2.31	16.4	19.5	20.2	18.1	18.4	1.4	1.8
Mean	.257	.252	.254	.189	.193	.062	.061	.38	1.0	1.79	14.0	13.7	13.8	10.4	10.5	3.3	3.3
+ 1SD	.06	.07	.07	.07	.08	.02	.02	.08	.46	.66	4.9	5.1	5.2	4.6	4.8	1.7	1.8

Nosslin	r: 0.996	0.997	0.969
Berman	P: N.S.	N.S.	N.S.

Matthews	r: 0.953
Nosslin	P: N.S.
Matthews	r: 0.955
Berman	P: N.S.

M: Matthews
N: Nosslin
B: Berman

r: correlation coefficient
k_1-k_4: as mentioned in Table 10.

* as a fraction of the I.V. pool

Table 29: Rate constants of HDL turnover

k_1			k_2			k_3			k_4	
M	N	B	M	N	B	M	N	B	N	B
.224	.241	.262	.499	.250	.274	.174	.116	.113	.056	.049
.175	.236	.261	.503	.249	.278	.167	.097	.092	.090	.084
.089	.146	.143	.431	.114	.088	.185	.107	.104	.059	.065
.088	.147	.145	.456	.100	.100	.180	.094	.099	.092	.084
.152	.143	.131	.484	.099	.037	.259	.194	.197	.092	.036
.156	.192	.138	.391	.177	.044	.228	.163	.168	.047	.020
.248	.213	.220	.562	.109	.075	.268	.200	.210	.045	.028
.236	.129	.121	.561	.155	.027	.324	.260	.279	.171	.029
.191	.116	.104	.549	.123	.024	.325	.284	.288	.104	.021
.361	.139	.123	.899	.156	.035	.311	.270	.275	.098	.023
.195	.135	.128	.509	.122	.050	.239	.198	.200	.053	.023
.262	.255	.249	.584	.113	.050	.446	.349	.356	.038	.020
.421	.172	.167	.798	.087	.054	.250	.169	.171	.044	.030
.193	.133	.125	.468	.104	.040	.233	.185	.187	.074	.030
.422	.198	.189	1.00	.064	.050	.286	.132	.139	.064	.052
.246	.147	.135	.674	.098	.044	.231	.167	.169	.071	.032
.555	.236	.224	1.31	.060	.074	.246	.102	.104	.051	.064
.374	.165	.145	.904	.232	.077	.240	.210	.214	.089	.031
.400	.230	.221	1.03	.099	.063	.280	.189	.196	.083	.056
.372	.319	.314	.844	.190	.123	.274	.301	.307	.015	.013
Nosslin r Berman	0.97		0.66			0.997			0.212	
Matthews r Nosslin	0.413		-0.169			0.849				
Matthews r Berman	0.351		-0.149			0.859				

Symbols as in Table 28.

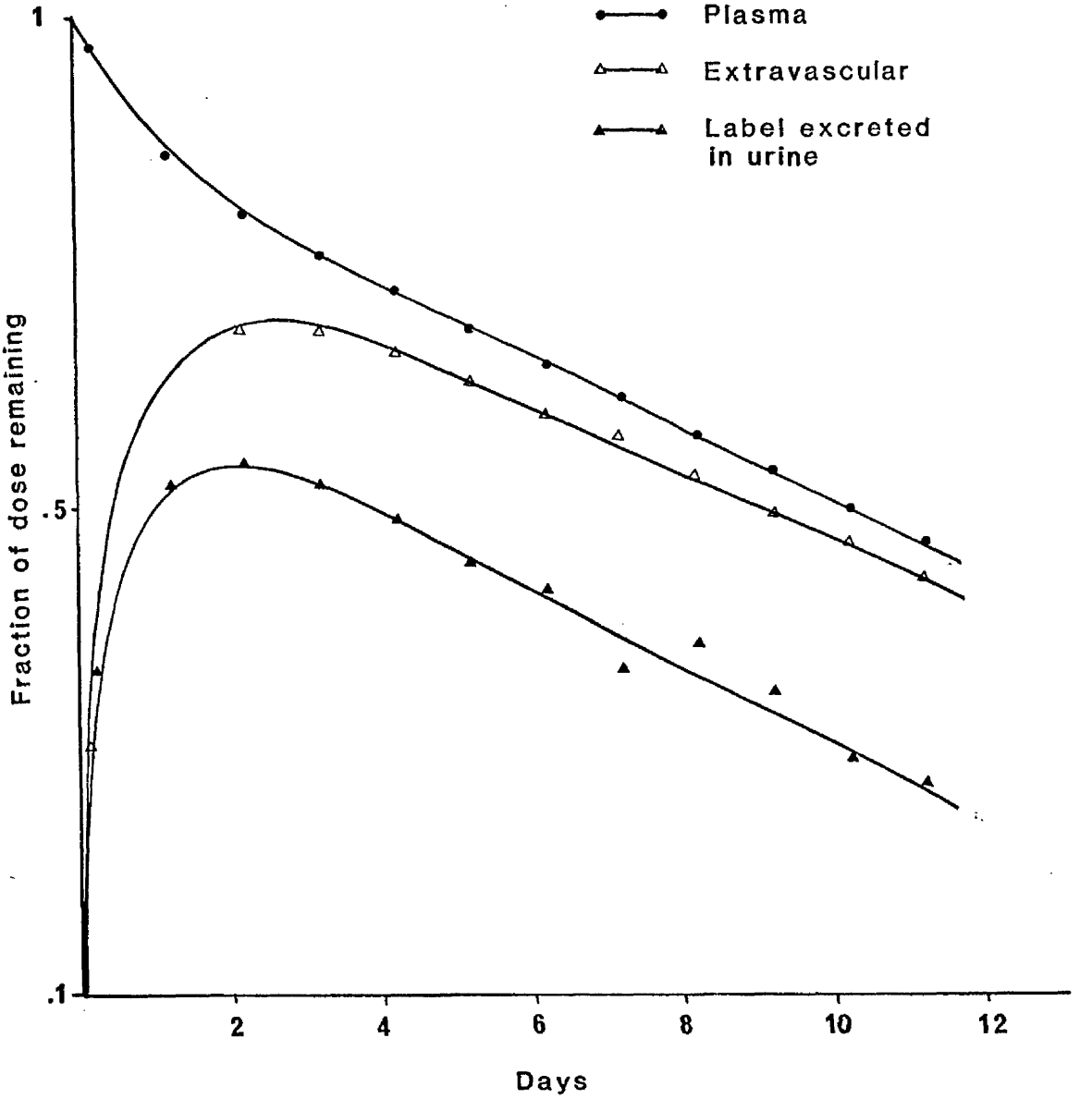


Figure 25. HDL radioactivity decay curves(JM).

The extravascular clearance of radioactivity was calculated from the observed plasma and urinary excretion data.

by the graphical integration and Berman's methods, r = 0.996. Intravascular and extravascular FCRs were in excellent agreement, (for intravascular FCR, r = 0.997; for extravascular FCR, r = 0.969). Interestingly, these two approaches are in agreement in demonstrating the extravascular catabolism of HDL which has a fractional rate of 36% of the total FCR in the normal subjects. This is in accord with the work of Blum et al. (128) who indicated that HDL was catabolised both in the intravascular and extravascular compartments. In his study the extravascular fractional catabolic rate was 52% of the total. Moreover, the average total flux calculated by the three methods was 14.0 (Matthews), 13.7 (Nosslin) and 13.8 (Berman) mg/kg/day. Similarly, the last two methods produced the same values for the HDL average flux into plasma and non-plasma pools (10.4 and 3.3 mg/kg/day (Nosslin) and 10.5 and 3.3 mg/kg/day (Berman), respectively).

3.3.2 Discussion

These results show that regardless of the method used, the same total FCR is obtained from plasma and urine data. However, the method of Matthews does not allow for extravascular catabolism, in addition it gives prime importance to the slope of the terminal exponential of the plasma decay curve which is taken as the starting point in calculating the other exponentials. This is often the time when measurements are least precise being 7-14 days after injection of the label when only 10% remains. On the other hand, the other two techniques,

i.e., the multicompartmental and graphical integration, allows for extravascular catabolism, and calculations start from the beginning of the decay curve where equal weight is given to all the points on the curve. Moreover, in Nosslin's method inspection of the plot $\frac{1-P}{E}$ versus $\frac{\int pdt}{E}$ (Fig. 15a) gives a good visual impression of the fit between experimental data and theory. This inspection may be of value in selecting the proper time intervals where the early values (which are sensitive to the presence of free iodine and denatured protein in the preparation) may be left out. This means that a kind of biological self-screening of the protein is made possible. Interestingly, Nosslin's method fits well with Berman's despite the difference in their mathematical approaches. This gives credence to the former since it is easy to perform and does not need computer facilities.

Studies of the HDL turnover should consider extravascular catabolism. Since changes in the ratio of the amount catabolised intravascularly versus that in the extravascular compartment may have physiological meaning when interpreting the effects of diet, drugs etc on HDL metabolism. In this respect it is informative to compare the results obtained from HDL with LDL and 1,2-cyclohexanedione treated LDL (CHD-LDL). This treatment selectively modifies the arginyl residues of the apoprotein and almost totally abolish the binding of the LDL to the high affinity cell surface receptors (124). By using Nosslin's procedure, LDL was found to undergo

catabolism in both the intravascular and extravascular compartments and the FCR in the latter was 20% of the total. In contrast, CHD-LDL was catabolised entirely in the intravascular compartment. Thus if HDL is catabolised in the liver alone it would have similar ratio of its extravascular FCR to that of LDL since it has been suggested that the liver is the major site of extravascular LDL catabolism (175). But since it was found that the extravascular catabolic rate accounted for 36% of the total FCR from the present results and 52% from those of Blum et al. (128), it could be inferred that HDL should be catabolised, in part, in an extrahepatic tissue. However, HDL contains more than one apoprotein, i.e., apo-AI, AII and C which may individually influence the total HDL turnover rate. Thus to gain a better understanding of HDL metabolism and function, the turnover of its major apoproteins, i.e., apo-AI and AII must be studied and their intravascular and extravascular kinetic parameters calculated. This cannot be done using the present data since each apoprotein contributes individually to the total urinary radioactivity pool. This emphasises the need for an approach which overcomes this obstacle and is able to predict such urinary data for each apoprotein.

SECTION 4: GENERAL DISCUSSION

4.1 The existence and function of Lp-AI in human HDL

Plasma lipoproteins can be separated by density gradient centrifugation or column chromatography into fractions clearly different in their chemical and physical properties. However it is recognised that these isolated fractions may not accurately represent the functional units of the human lipid-transporting system. In an alternative approach to the classification of plasma lipoproteins, Alaupovic and co-workers introduced the concept of lipoprotein families (176), in which the physiologically relevant entities were considered to be lipid-protein complexes containing a single apolipoprotein type e.g. Lp-B containing apo-B, Lp-A with AI and AII etc. For LDL the major fraction of density 1.019-1.050 kg/l which contains virtually exclusively Lp-B, both classifications agree this is a homogeneous species which from metabolic evidence would appear to have a well defined function in delivering cholesterol to cells. In the less dense triglyceride-rich lipoproteins (VLDL) the particles possess a number of apolipoproteins on their surface as evidenced by, for example, fractionation of VLDL on heparin-sepharose chromatography (177). Here the gel binds only particles containing apoE and it can be seen that apo-B and apo-C are also retained. Thus these lipoproteins are best characterised by their size and density. On this basis they are separated into particles which deliver triglyceride to tissues via the agency of

lipoprotein lipase. The situation in the high density lipoproteins is different. These, on account of their limited size are not able to contain any more than a small number of polypeptides on their surface. Furthermore, from the measured abundance of apo-E in HDL it is clear that there is not enough E protein present on each HDL particle and the same argument follows for the C peptides. Thus lipoproteins with the physico-chemical properties of HDL but rich in apo-E or in apo-C can be separated from the bulk of apolipoprotein A containing material. Most investigators assumed that the AI and AII proteins were present uniformly on the majority of high density lipoproteins. The presence of lipoprotein family characterised by the presence of only apo-AI (Lp-AI) in human plasma was suggested earlier (76), but it was detected as only a minor component (less than 10%) of HDL_2 and HDL_3 subfractions by immunoprecipitation (178) and hydroxyapatite chromatography (76). However, the structure and function of these particles have not been examined in detail nor has their quantitative importance been established in different subjects under a variety of conditions. In a recent report Cheung and Albers (179) have fractionated HDL into seven fractions by means of equilibrium CsCl gradient ultracentrifugation. On subjecting these fractions to immunoprecipitation with anti-apo-AI and anti-apo-AII antibodies, they have found that the former precipitated almost all of the apo-AI and apo-AII present in these fractions. On the other hand, anti-apo-AII precipitated all the apo-AII but only a part

of the apo-AI content of these fractions. Moreover, the amount of non-precipitable apo-AI varied from fraction to fraction, being greater in those of lower density, i.e., d 1.063-1.093 kg/l, and less in those of the higher density, i.e., d 1.093-1.149 kg/l. However, although they estimated that 25% of the apo-AI in HDL was not associated with apo-AII, they did not isolate such particles nor demonstrate that they were not artifacts formed during the several days of ultracentrifugation.

The present study provided a simple and reproducible method for the isolation of the apo-AI containing particles from HDL without lengthy manipulations of the sample. Furthermore, three procedures for the quantification of these particles and their distribution among HDL subfractions were presented. The results for HDL_3 were in good agreement with those of Albers and Aladjem (178) who reported the precipitation of 84-90% of the radioactivity in this fraction upon addition of an anti-apo-AII antibody. In contrast to their findings for HDL_2 isolated from males (they did not examine females), less than half of the HDL_2 protein radioactivity prepared from our female subjects was precipitated by the antibody, although virtually all reacted with anti-apo-AI.

The results of this study support the concept of the lipoprotein families of differing functionality and properties, since two distinct types of particles, i.e. Lp-AI and Lp-A, were identified in the HDL density range. L-pA containing apo-AI and apo-AII in a molar ratio of 1:1 was the major fraction present. This ratio is close

to that reported by Norfeldt et al. (180) (1.3:1), who separated the Lp-A particles by hydroxyapatite chromatography. His HDL particles possessed 2 AI and 2 AII polypeptides with a total particle protein content of 90,000 daltons or 50% of a HDL mass of 175,000. Moreover, the present findings raised the possibility that the two lipoprotein families were metabolically distinct since their major protein apo-AI did not exchange between them. The functional heterogeneity in the apo-AI of HDL is in agreement with the report of Shepherd et al. (181) who postulated the presence of two pools of apo-AI within the HDL class, one which was readily exchangeable with lipid-free apo-AI in the solution while the other was non-exchangeable, in spite of the presence of a large molar excess of the free apoprotein. Further evidence for the inhomogeneity of apo-AI and AII contained in HDL was obtained by Grow and Fried (182) who demonstrated that while apoproteins exchanged between HDL_2 and HDL_3 subfractions, equilibration of the proteins was not observed when labelled HDL_2 was incubated over a long period with unlabelled HDL_3 and vice versa. This is in accord with the present results of exchange experiments both <u>in vitro</u> and <u>in vivo</u> where no equilibrium was attained upon incubation of labelled Lp-AI with HDL_2 or HDL_3.

The distribution of these lipoprotein families extended throughout the entire density spectrum of HDL. However, Lp-AI predominates at the lower end of the density range whereas the Lp-A is the major particle at

the higher densities (Fig. 22b). This distribution accords well with that found by Cheung and Albers (179) and is evident from the finding that HDL_2 is rich in Lp-AI and HDL_3 comprises a higher proportion of Lp-A (Table 14). The co-presence of the two populations in both density ranges, i.e., HDL_2 and HDL_3, may explain the apparent discrepancy from laboratory to laboratory in the reported ratio of apo-AI/apo-AII in these density subfractions (91). It also offers new insight into the metabolic and structural properties of HDL. It supplies an explanation for the repeated, although not universal finding that apo-AI and AII are removed from the plasma at different rates (125-127). Furthermore, divergent metabolic fates of these apoproteins have been reported in non-human primates (183,184). Apo-AI and AII also decay disproportionately in hypertriglyceridaemic (126, 127) and Tangier disease (37,185) patients. Interestingly, apo-AI in Tangier disease is catabolised faster than apo-AII even when normal HDL is injected into homozygotes with this disease (185). Moreover, disparate decay of the apoproteins was demonstrated when human HDL was injected into rats (121). These data taken together support the view that there are divergent metabolic pathways for apo-AI and AII which are more pronounced in the dyslipoproteinaemic states. The inconsistent difference in the decay of apo-AI and apo-AII from the plasma of normal subjects is reflected in the removal rates of these apoproteins in the two cases presented in Table 11 where a mixture of ^{125}I-Lp-AI and ^{131}I-HDL was injected.

In one case (MP) ^{125}I-apo-AI, ^{131}I-apo-AI and ^{131}I-apo-AII were catabolised at similar rates, while in the other subject (JS) the three apoproteins were catabolised differently with ^{131}I-apo-AII decaying faster than ^{131}-apo-AI, and the latter faster than ^{125}I-apo-AI. The results in JS suggest that two kinetically distinct apo-AI pools exist in plasma, one representing Lp-AI and the other Lp-A.

The divergent metabolic behaviour of apoprotein AI and AII is further seen in the different response of the lipoproteins Lp-AI and Lp-A during nicotinic acid treatment. Quantitative immunoprecipitation demonstrated that the drug increased the plasma levels of the former and decreased those of the latter. In addition, the apo-AI/AII ratio was increased in plasma and HDL_2 with a slight decrease in this ratio in HDL_3 (Tables 15 and 16). The latter changes are in good agreement with those reported by Shepherd et al. (186) and Blum et al. (128) who demonstrated an increase in the ratio of apo-AI/AII in HDL coupled with a rise in the plasma HDL_2/ HDL_3 ratio during nicotinic acid administration. Moreover, the latter workers and Gonen et al. (187) demonstrated the reverse effect following administration of an 80% carbohydrate diet; furthermore,they reported an increase in the lower density fractions of HDL on nicotinic acid (128) and an increase in the higher density fractions on the carbohydrate treatment (128,187). In this regard it is notable that in the present study nicotinic acid therapy caused a shift of the HDL spectrum to a lower

density (Fig. 23). Accompanying these changes in subfraction distribution Blum et al. (128) reported an alteration in the HDL intravascular and extravascular fractional catabolic rates on both treatments. Nicotinic acid decreased the former and increased the latter. Interestingly a high carbohydrate diet was able to produce reciprocal effects to nicotinic acid on both the HDL subfraction distribution and its intravascular and extravascular catabolism. From these experiments it may be postulated that the 80% carbohydrate diet decreased the plasma level of Lp-AI and increased that of Lp-A.

The effect of probucol on both lipoprotein types was similar to carbohydrate feeding in that it lowered their concentration in plasma (Table 18). This resulted from a decrease in the synthesis of both apo-AI and AII (Table 26).

It is possible that other dietary manipulations affect the plasma concentrations of Lp-AI and Lp-A. Shepherd et al. (188) reported a decrease in plasma apo-AI concentration and HDL_2/HDL_3 ratio (suggesting a decreased plasma Lp-AI) in normolipidaemic humans when their diet was changed from that containing saturated fat, to polyunsaturated fat. A similar decrease in apo-AI concentration was also reported in monkeys (183). On the other hand, diets high in saturated fat and cholesterol were shown to increase plasma apo-AI levels in humans (189).

4.2 Potential sources of Lp-AI:

Recent quantitative studies of the changes in HDL during alimentary lipaemia have shown that following ingestion of a fatty meal, plasma levels of HDL_2 and HDL_3 are increased largely due to a rise in the phospholipid, cholesteryl ester and apo-AI content of the former, and apo-AI, AII, phospholipid, free cholesterol and cholesteryl ester in the latter. Moreover, six hours after the ingestion of a corn oil meal the plasma levels of apo-AI and AII are increased from 118 and 40 to 138 and 51 mg/dl, respectively (190). This suggests that the two lipoprotein species, i.e. Lp-AI and Lp-A, are secreted by the intestine during alimentary lipaemia. Conversely, Zannis et al. have demonstrated that cultures of human foetal intestine (191) secrete apo-AI but hardly any apo-AII unlike hepatoma (192) or human foetal liver cells (191) which elaborate both apoproteins. From these studies it can be postulated that apo-AI and AII are secreted, possibly in the form of Lp-A, by the liver at all times and by the intestine in chylomicrons following a fatty meal. Lp-AI on the other hand is apparently secreted mainly by the intestine in the fasting state.

Support for this suggestion comes from the examination of intestinal lymph HDL which is generally larger than its plasma counterpart (105,193) and contains a much higher ratio of apo-AI to AII possibly indicating the presence of a high Lp-AI concentration.

Lp-A may also be formed in the plasma as apo-AII released from chylomicrons associates avidly with Lp-AI and displaces apo-AI from the HDL particle. These interactions are discussed in more detail below.

4.3 The role of Lp-AI in body cholesterol transport:

As mentioned in Section 1, there is now a body of evidence that supports the concept that HDL enhances the removal of cellular cholesterol. It seems likely that Lp-AI plays a major role in the assimilation of cholesterol by HDL for the following reasons: 1) apo-AI is a potent activator of LCAT (131) which esterifies the free cholesterol lost from cells; 2) LCAT activity is reported to be directly proportional to the apo-AI content of vesicles containing different proportions of apo-AI and AII (133); 3) canine HDL, which is primarily (AI)HDL, was shown to have higher capacity to accept the polar lipid constituents (including free cholesterol) of VLDL, during its lipolysis in vitro, than apo-II hybrids of HDL (194); 4) Oram et al. (195) reported that apo-AI containing particles are effective acceptors for cellular cholesterol in vitro; and the greater the density of the particles the greater their ability to remove cholesterol from cells.

Assuming that it is the (AI)HDL particle that acts as a cholesterol "shuttle" between the peripheral cells and the liver, the following pathway can be proposed (Fig. 26) to explain this role. Knowing that free apo-AI exists in plasma at a low concentration (198), it could be postulated that there is an equilibrium between apo-AI

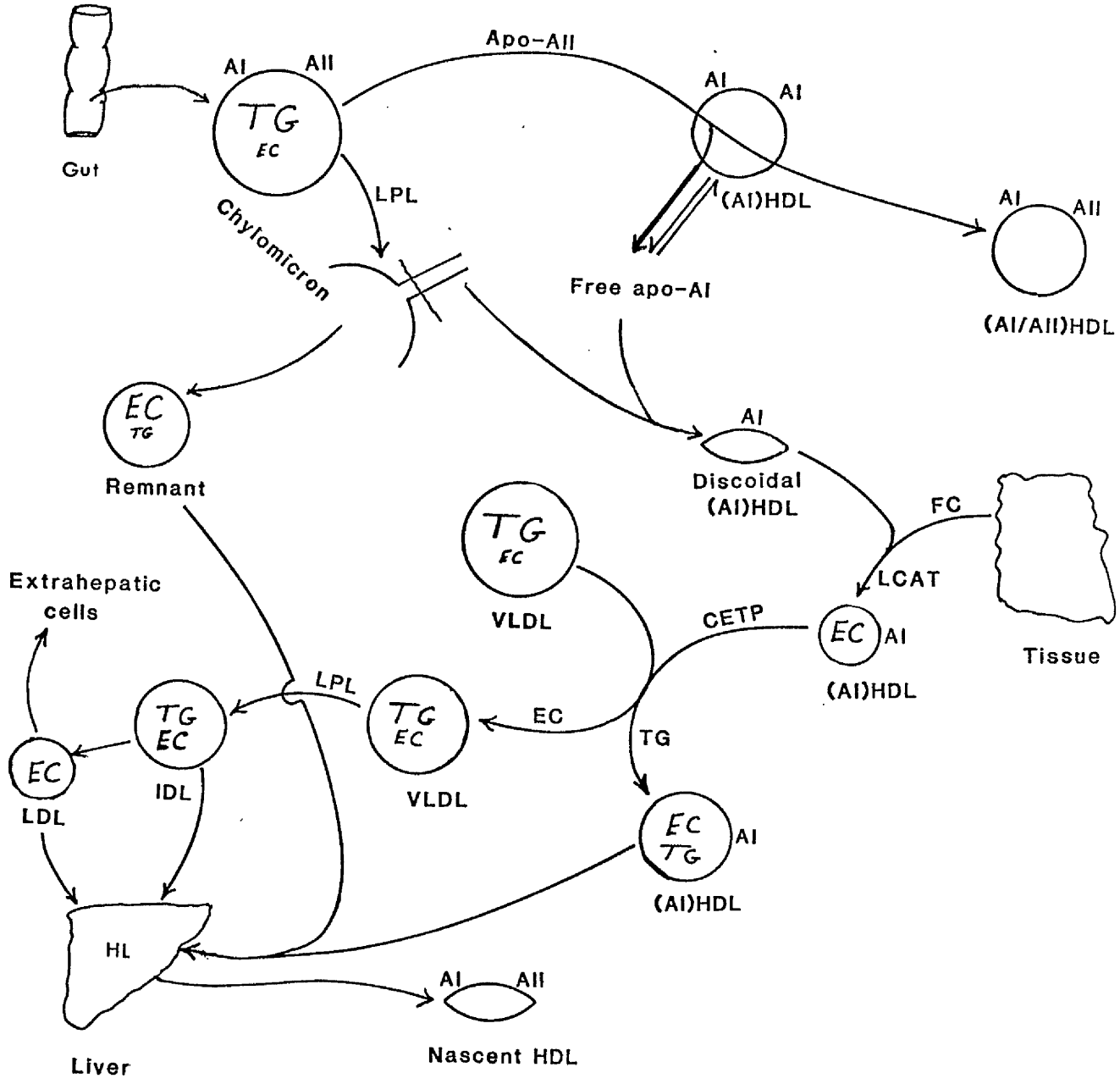


Figure 26. A hypothetical role for Lp-AI in cholesterol transport.

in the free form and that in HDL, since the enthalpy (ΔH) of the conformational change of apo-AI associated with its release from HDL (2.7 cal/gm) is only slightly higher than that for thermal unfolding of lipid-free apo-AI (2.4 cal/gm) (92). Moreover, this equilibrium can be shifted in the direction of apo-AI release from HDL in the presence of apo-AI acceptors like the phospholipid bilayers formed during chylomicron and VLDL lipolysis. This phenomenon is seen when the co-incubation of HDL with turbid suspensions of phospholipid results in the disappearance of the turbidity as a result of dissolution of the liposomes by the HDL-released apo-AI (93). The equilibrium shift, in turn, possibly enhances lipolytic processing of the chylomicrons and VLDL by facilitating the removal of excess surface components. In addition, when chylomicrons enter the circulation from lymph their apo-AII immediately (in less than 1 minute) transfers into HDL, whereas part of their apo-AI transfers after some delay (1-3 hours) (184). Once the chylomicrons are in the circulation they are acted upon by lipoprotein lipase which results in shrinkage of the particles with the protrusion from their surface of bilayer folds (90). These are subsequently detached from the particle and form bilayer sheets or vesicles which contain little apo-AI as discussed in Section 1.5.2. However, the released apo-AII may displace some of the apo-AI from (AI)HDL particles leading to the formation of (AI/AII) HDL. Subsequently, the displaced apo-AI combines with the formed bilayer sheets and vesicles to produce the

discoidal (AI)HDL (nascent) particles. These discs, which comprise mainly phospholipid with a little free cholesterol, are excellent substrates for the LCAT reaction where the free cholesterol is esterified and directed towards the core of the particle inducing the formation of a spherical lipoprotein. This, in consequence, renders the particle ready to accept more free cholesterol from cells and enriches its core with cholesteryl ester. The result is an increase in particle size. Further cycles of this cholesterol assimilating process can be induced when cholesteryl ester is exchanged for triglyceride from VLDL by means of the cholesteryl ester transfer protein (CETP). The (AI)HDL particle then becomes richer in triglycerides and poorer in cholesteryl ester while the VLDL particle becomes enriched with the latter. Subsequently, VLDL delivers its cholesteryl esters to LDL which is taken up via a high affinity receptor mechanism located in liver and peripheral cells (197). On the other hand, the modified (AI)HDL particle which is now large in size and contains a higher proportion of lipids may fuse with chylomicron remnants and hence be degraded by the liver. Alternatively liver lipase (129) may act directly on the phospholipid and triglyceride of (AI)HDL to reduce the particle size and cholesterol content. These processes lead to either an irreversible removal of the particle or to its conversion to smaller HDL.

The above scheme accounts for many of the known physical and chemical properties of HDL e.g. its ready ability to accept lipid and change its particle size. It also explains the observation that apo-AI and AII are removed slowly from the circulation whereas HDL cholesteryl esters have a much shorter turnover time (117); and it allows for the formation in the plasma of Lp-AI and Lp-A. Since the chylomicron is postulated to be a major source of the Lp-AI then the latter should be sensitive to diet as discussed above i.e. carbohydrate feeding which reduces chylomicron formation decreases Lp-AI. Similarly, polyunsaturated fat feeding leads to the synthesis of smaller chylomicrons with less A protein on their surface. Saturated fat feeding in this scheme conversely is able to augment the plasma content of both Lp-AI and Lp-A in line with the observed data.

SECTION 5: REFERENCES

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