The Role of the Reticuloendothelial System in LDL Metabolism

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To Anne.

DECLARATION

The work presented in this thesis was performed solely by the author with the exception of areas of acknowledged collaboration.

ABBREVIATIONS: The following abbreviations were used in this thesis.

Ac-BSA	:	acetylated bovine serum albumin
apo	:	apolipoprotein
d	:	density
FCR	:	fractional clearance rate
FH	:	familial hypercholesterolaemic
HDL	:	high density lipoprotein
IDL	:	intermediate density lipoprotein
IHD	:	ischaemic heart disease
K	:	phagocytic index
LDL	:	low density lipoprotein
MDGF	:	macrophage derived growth factor
MDP	:	muramyl dipeptide
Met	:	reductively methylated
MTP	:	muramyl tripeptide
PDGF	:	platelet derived growth factor
PE-LDL	:	LDL from ethyl oleate treated animals
RE	:	reticuloendothelial
SD	:	standard deviation
Sf	:	Svedberg flotation coefficient
VLDL	:	very low density lipoprotein

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INTRODUCTION

RESULTS

SUMMARY

Prospective epidemiological studies show that raised plasma cholesterol is a major risk factor for the development of ischaemic heart disease. Recent evidence indicates that reducing plasma cholesterol concentration reduces risk from the disease.

Cholesterol is transported in the plasma mainly by low density lipoproteins (LDL) and these particles also confer risk if present in high concentrations.

It is important to determine the mechanisms by which LDL is removed from the plasma and catabolised by the tissues of the body. The arterial wall is especially important in this respect as it can accumulate excessive quantities of cholesterol.

LDL catabolism is divided into two pathways:

1) LDL-receptor-dependent catabolism is now a well understood process by which a receptor recognises apolipoprotein B (the sole apolipoprotein moiety on LDL) and mediates the internalisation of the lipoprotein. It is then transported to the lysosome, degraded and the cholesterol released for cellular needs. The pathway is autoregulated by cellular requirements for the sterol. In most animals, including man, approximately one half of LDL catabolism occurs by this route.

2) LDL-receptor-independent catabolism is less well defined. Studies have indicated that the reticulo-endothelial system plays a role in this scavenger pathway.

The purpose of the present study is to increase understanding of the role played by the reticuloendothelial system (RE system) in LDL metabolism. However, because the RE system is a disseminated tissue it is not as easy to study as, for example, the liver which can even be removed from the animal intact.

One approach to this problem is functionally to block the cells of the system by injection of material which will be phagocytoced by these macrophages. This was achieved using three agents; a) ethyl oleate emulsion, b) muramyl tripeptide incorporated into triolein emulsion and c) muramyl dipeptide conjugated to acetylated bovine serum albumin (for which the cells of the RE system have a receptor). The end result was the same regardless of the agent employed.

The following summarises the essential conclusions of the thesis.

1) The RE system is important in lipoprotein catabolism. Blockage of the system causes an increase in plasma triglyceride and cholesterol levels. The raised total cholesterol is almost entirely accounted for by an increase in LDL cholesterol.

2) RE cells are important in the clearance of LDL from the plasma. Administration of RE cell activity inhibiting agents causes a decrease in the rate of the clearance of the lipoprotein. Kinetic analysis shows that this was due to a decrease in LDL-receptor-independent catabolism.

3) Lipoprotein catabolism by cultured macrophages is down regulated by RE system suppressants. Incubation of macrophages with these agents reduces the amount of LDL and ß-VLDL catabolised by the cells. This supports the in vivo findings.

4) LDL is altered in the plasma of RE blocked animals. It has an increased density, an increased electrophoretic mobility (negative charge), an increased cholesterol to protein ratio and is cleared from the plasma more rapidly than normal LDL. Control LDL injected into RE suppressed animals assumes the characteristics of this abnormal lipoprotein.

5) Blocking the RE system of cholesterol fed animals appears to promote the atheromatous infiltration of their aortae. Plasma cholesterol levels in the RE blocked animals were not significantly different from those in cholesterol fed animals with functional RE

systems but the overall negative charge of their LDL was greater.

6) It follows that the RE system is working to clear an abnormal form of LDL which may be formed by "ageing" in the circulation. Potentially this LDL is catabolised by atherosclerotic plaques leading to lesion advancement.

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INTRODUCTION

1.1:

Ischaemic heart disease (IHD) accounts for more deaths in the "western" industrialised world than any other single cause. For example, Levy reported in 1981 (1) that over a million people in the United States suffered a heart attack in 1978 with 650,000 mortalities. This compares to 400,000 deaths from all forms of cancer combined. IHD costs the United States 60 thousand million dollars per year in health care costs, lost wages and productivity (2).

The rest of North America and most of Europe and Australasia suffer similar human and monetary losses. Within the United Kingdom, Scotland has the highest mortality rate from IHD with over 18,300 deaths in 1983 (356 deaths per 100,000 population, male and female) compared with 14,200 from cancer (276 deaths per 100,000 population, male and female) (3), (Table 1).

Although the majority of people exhibiting symptoms of IHD (such as angina, myocardial infarction and sudden death) are over the age of 55 years (Table 2) it is understood that the underlying atherosclerosis develops, unobserved, at a much earlier stage. This presents difficulties in elucidating the aetiology of the disease.

TABLE 1

CAUSE OF DEATH	TOTAL DEATHS 1982	TOTAL DEATHS 1983
MALIGNANT NEOPLASMS	14077	14210
ISCHAEMIC HEART DISEASE	18633	18335

Deaths from IHD compared with those from all forms of cancer in 1982 and 1983. The death rates from IHD make Scotland the worst affected country in Britain.

TABLE 2:

AGE GROUP (YEARS)	1950	1960	1968	1976
35-44	74.3	87.8	134.9	90.3
45-54	254.2	297.0	419.4	326.0
55-64	554.1	723.7	998.1	794.4
65-74	943.1	1340.2	1920.2	1487.9

Ischaemic heart disease rates per 100,000 population in the United States (non-white males), showing increasing incidence of the disease with increasing age.

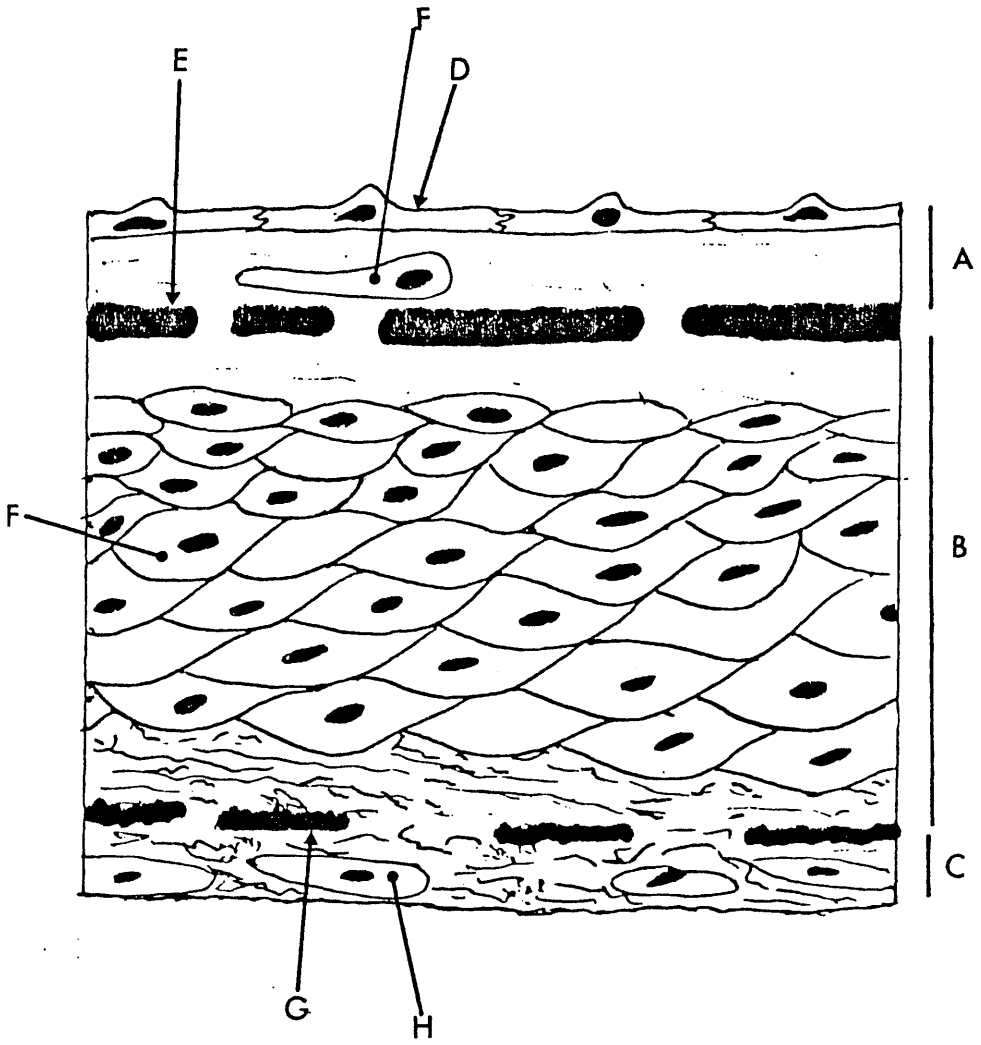
1.2: Atherosclerosis.

Before discussing those factors which are believed to be responsible for the initiation and advancement of the atherosclerotic lesion it would be advantageous to define the structure of arteries. The artery wall consists of three morphologically distinct layers, the intima or innermost layer, the media and the adventitia (Figure 1).

The intima is bounded on the lumen side by a monolayer of endothelial cells and separated from the media by a sheet of fenestrated elastic tissue known as the inner elastic lamina. The space between these boundaries is filled with connective tissue surrounding, in younger people, the occasional smooth muscle cell (SMC). The number of these SMC increases with age resulting in a thickening of the intima. It is here in the internal elastic lamina. The space between these formed. The current counsel is that damage to the vessel wall first occurs to the endothelial cells which afford protection from abnormal intrusion of blood components. Functioning normally, these cells allow metabolic exchange to occur between the blood and the vascular cells (4,5). Chemical, mechanical and immunological insults can disrupt this barrier and initiate the development of the lesion. The media may sustain secondary changes, especially in the more severe types of lesion.

There are three classically recognised types of lesion; 1) the fatty streak, 2) the fibrous plaque and 3) the complicated lesion.

FIGURE 1: Diagmatic representation of a section through the wall of the aorta.



A: Intima

B: Media

C: Adventitia

D: Endothelium

E: Internal Elastic Lamina

F: Smooth muscle cell

G: External Elastic Lamina

H: Fibroblast

The first of these is common in people over the age of 10 years (6). It does not generally interfere with blood flow because of its flattened topography. The yellow colour of the fatty streak is due to both intra- and extracellular lipid deposits. Intracellular deposits are mainly contained in macrophages and smooth muscle cells (7). These cells are thought to be the source of foam cells found in many lesions (8). The lipid deposits, mainly cholesterol and a cholesteryl ester, are derived from the circulating pool in the blood. Endothelial cells covering some of the lesions have a greater volume of cytoplasm than those over normal intima (9), though the significance of this finding is not understood.

Fatty streaks may be the precursors of the second type of lesion, the fibrous plaque for two main reasons; a) their lipid rich nature and b) the similarity in distribution of the two types. This connection has, however, been disputed because some workers have found fibrous plaques occurring in sites normally unaffected by fatty streaks (10).

Fibrous plaques are whitish in colour and are implicated, through their elevated structure, in interference with blood flow which may lead to tissue ischaemia. The media plays an important role in the formation of these plaques. Smooth muscle cells from the media accumulate and proliferate in the intima, contributing to the raised nature of the lesion. The

reasons for the elevation of the intima are, therefore, threefold; 1) migration and proliferation of smooth muscle cells from the media and macrophages from the blood born monocytes, 2) deposition of extracellular matrix components and 3) expansion and necrosis of the core of the plaque.

Smooth muscle cells and macrophages in the lesions are rich in lipid. As with the extracellular deposits the lipid present in these cells is mainly cholesterol and cholesteryl esters. In the early form of the fibrous plaque the intracellular ester is mostly cholesteryl oleate, presumably produced by the action of ACAT within the cell. This means that, at this stage, the smooth muscle cells hydrolyse cholesteryl ester obtained from the plasma and re-esterify the free cholesterol with oleic acid as do normal cells. In more advanced plaques, however, the metabolic efficiency of the cell is reduced and the ester which accumulates is predominantly linoleate, the most common ester found in plasma lipoproteins (11).

Free cholesterol also plays an important part in the progression of the plaque. It has been suggested by Jackson and Gotto (12) that initially, free cholesterol is incorporated into the cellular membranes decreasing their fluidity and further reducing the metabolic efficiency of the cell. Once the cellular assimilation of free cholesterol has reached the level of supersaturation of intracellular membranes, cholesterol can partition into

crystalline structures of cholesterol monohydrate (18). This contributes to the destruction of the cell and necrosis of the lesion.

The lipid laden cells promote the development of a fibrous capsule which eventually surrounds the lesion and envelopes the necrotic core of extracellular lipid and cell debris.

The third and most advanced type of lesion, the complicated lesion, is characterised by the presence of calcification. Indeed they are thought to develop by a combination of haemorrhage, cell necrosis, mural thrombosis and calcification.

1.3: Current Hypotheses of Lesion Initiation and Advancement.

1.3.1: The Endothelial Cell Injury Hypothesis.

An early form of the hypothesis was outlined by Virchow in 1856. Since then it has been modified and updated but the original concept remains the same. In essence the hypothesis states that the endothelial barrier protecting the intimal layer is breached allowing contact with factors from the blood such as platelets, monocytes and lipoproteins. This results in the proliferation of smooth muscle cells, formation of foam cells and other biochemical and cellular sequelae described in section 1.2.

Endothelial cells are known to be injured by various factors including mechanical forces (13,14),

chemical agents, such as lipoproteins (15) and immunological factors (16). The injury leads to adherence of platelets and monocytes to the unprotected connective tissue matrix of the mid-intima. According to Ross, monocytes are then activated to become macrophages and migrate into the intima releasing macrophage derived growth factor (MDGF) (17) which adds to the effect of platelet derived growth factor (PDGF) released from the adherent platelets (19). These growth factors stimulate smooth muscle cells to migrate through the inner elastic lamina and proliferate in the intimal layer, they also increase the number of lipoprotein receptors expressed on the surface of the cells. The smooth muscle cells synthesise and secrete connective tissue which is laid down in the intima. Rapid thickening of the intima and lipid deposition ensues.

In an attempt to repair the damage the endothelial cells bordering this area migrate over the intimal surface (20). If the migration is insufficient to cover the lesion, the endothelial cells divide. In cases where the extent of the injury and the tissue response to that injury are limited the endothelium will be successful and the lesion regresses. If, on the other hand, the damage is more severe and the endothelial layer is left incomplete, further cycles of smooth muscle cell proliferation and lipid deposition occur and the lesion recognisable as atheroma results.

What causes the failure of the endothelial cells to recover the intima? At sites where the injury is continuous, such as mechanical injury due to hypertension, the endothelial cells try repeatedly to cover the exposed mid-intima. They are capable of only a limited number of divisions, after which they can no longer respond in this way to aid in re-endothelialisation. Thus cell senescence may play an important role in a long term cycle of damage and repair.

1.3.2: The Lipid Hypothesis

The lipid hypothesis states that lipoproteins can cause atherosclerosis without the involvement of other risk factors. This does not mean that other risk factors are not present or indeed contributory to atherogenesis and lesion advancement. Lipoproteins, which will be more fully discussed in a later section, are responsible for the transport of lipid, including cholesterol, in the plasma. The lipoproteins which are most frequently documented as being atherogenic are low density lipoproteins (LDL) but other lipoproteins, such as beta migrating very low density lipoproteins (β VLDL) and the remnants of chylomicrons and VLDL may be equally as potent.

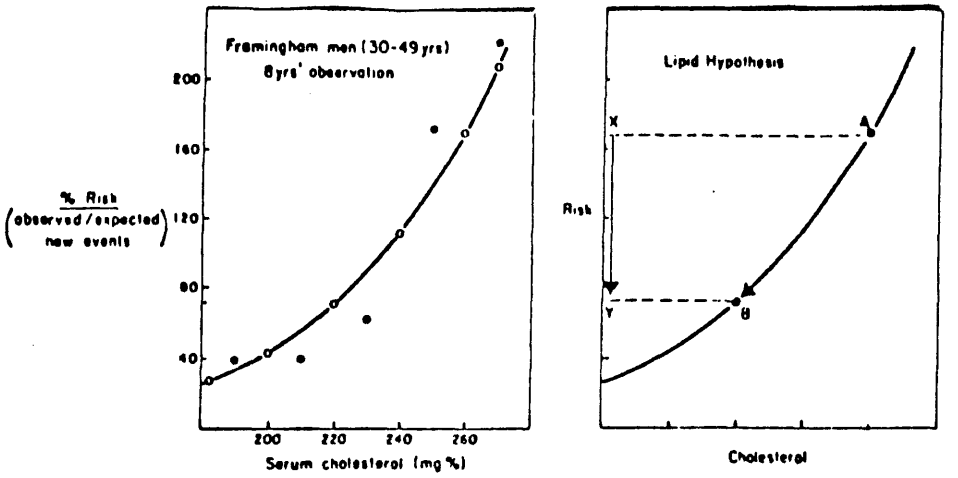
There is extensive literature to support the lipid hypothesis covering many years. Anitschkow and Chatlow showed in 1913 that feeding a cholesterol rich diet to rabbits accelerated the development of athero-

sclerosis and following this observation much work has been done in animals and humans to confirm and expand the finding. In animals, including non-human primates, the experimentally induced lesions, produced by feeding a diet which increases the plasma cholesterol level, closely resemble those found in humans (21,22). It has been shown that cholesterol in the atherosclerotic lesions of both animals (24) and man (25) is derived from the plasma. Using immunofluorescence techniques, Smith (23) demonstrated that LDL itself is present in these lesions and further investigation by Walton (26) revealed that most of it is extracellular and intact. An earlier investigation by Smith (27) showed that the concentration of LDL in the intima in atherosclerotic regions is proportional to the plasma LDL concentration and according to Katz (28), by the age of 40 years, 72% of the intimal lipid and even higher percentages of the lipid in the lesions is plasma derived.

In a person with hyperlipidaemia, damage to endothelial cells results in the formation of atherosclerotic plaques at a much more rapid rate than in those with normal plasma lipid levels (Fig. 2) (29,30) and, as has been stated, lipoproteins themselves can damage the endothelial monolayer (15).

Strong evidence for the lipid hypothesis comes from genetically determined abnormalities of the lipid transport system. Patients with familial hypercholesterolaemia (FH) have grossly elevated plasma LDL

FIGURE 2: The Lipid Hypothesis.



Left:- the relation between serum cholesterol levels in man (30-49 years) and their relative risk of coronary heart disease (●) and a curve of best fit to the data (o), from Framingham data (101).

Right:- the lipid hypothesis proposes that lowering serum cholesterol level from A to B reduces the relative risk from X to Y.

TABLE 3:

PERCENT REDUCTION IN	CHOLESTYRAMINE GROUP	PLACEBO GROUP
LDL CHOLESTEROL	20.3	7.7
TOTAL CHOLESTEROL	13.4	4.9

REDUCTION OVER CONTROL GROUP IN	PERCENT DROP IN CHOLESTYRAMINE GROUP OVER PLACEBO
RISK OF IHD OR SUDDEN DEATH	19
POSITIVE EXERCISE TEST	25
ANGINA	20
CORONARY BYPASS	21

Reduction in cholesterol level reduced the risk of heart disease.

levels, indeed those with the homozygous abnormal form of FH frequently suffer their first myocardial infarction before the age of fifteen years and rarely survive past their late twenties. Patients aged between 5 and 10 years who have IHD are unlikely to have been exposed to other risk factors such as hypertension and cigarette smoking (31).

Further evidence was obtained from epidemiological studies undertaken in Japan. Here hypertension and cigarette smoking are greater than in the West but Robertson showed that plasma cholesterol levels and the incidence of heart disease are lower. He speculated that other risk factors may not operate, or operate at much reduced levels, if plasma cholesterol is not raised (32).

Until recently clinical trials, undertaken to show a relationship between reducing cholesterol levels and lowering incidence of heart disease, have been inconclusive. It has now been shown that a moderately low cholesterol diet in tandem with an oral bile acid sequestrant reduced the cholesterol level in the plasma of asymptomatic men with hypercholesterolaemia by 8%. LDL cholesterol levels were reduced by 11% with a concomitant drop of 19.0% in the risk of contracting coronary heart disease (33) (Table 3).

In this section the lipid hypothesis has been examined independently but perhaps it should be viewed with other existing hypotheses to gain fuller insight into the causes and effects of atherosclerosis. Elevated

LDL levels can damage endothelial cells (15) and thus cause an increase in LDL infiltration into the vessel wall and in platelet adherence and aggregation. Indeed, Carvalho et al (34) has reported that hyperlipoproteinaemia may induce platelet aggregation and the release of PDGF. It is possible that this contributes to atherogenesis in patients with hyperlipoproteinaemia even if the extent of injury to the endothelium is modest.

Evidence is now available which shows that injury to the endothelial transport barrier, even if the cells are not stripped off, will cause an increase in the rate of infiltration, cellular uptake and degration of LDL at any given plasma lipoprotein level. Platelet derived growth factor causes an increase in the number of sites on smooth muscle cells by which LDL is actively taken up (36). Stimulation of smooth muscle cell growth, which can occur as a result of interaction with LDL as well as growth factors, causes the deposition of large amounts of extra cellular matrix components, most notably glycosaminoglycans, which can trap LDL in the extracellular spaces of the intima (38).

Thus, although there is good evidence for individual hypotheses, perhaps a compromise between them gives a more realistic, overall picture of atherosclerosis.

2: Plasma Lipid Transport: The Lipoproteins.

2.1: Nomenclature of the Lipoprotein Classes.

Various methods for isolating the classes of lipoproteins will be discussed in a later section but at this point it is necessary to mention only one of these; density gradient ultracentrifugation. Due to their lipid content, lipoproteins have a low buoyant density which allows them to be isolated by flotation through a salt gradient.

Five major classes of lipoprotein (39) have been identified in human plasma. These are i) chylomicrons; density range <0.95 kg/l: ii) very low density lipoproteins (VLDL); density range 0.95 - 1.006 kg/l: iii) intermediate density lipoproteins (IDL); density range 1.006 - 1.019 kg/l: iv) low density lipoproteins (LDL); density range 1.019-1.063 kg/l: and v) high density lipoproteins (HDL); density range 1.063 - 1.210 kg/l (Table 4).

Each class has a characteristic composition and metabolic fate although most of its components are not fixed but exist in a state of dynamic equilibrium, exchanging freely between fractions.

Lipoproteins with similar density ranges are found in other mammals but their relative amounts and compositions may vary (40).

TABLE 4:

LIPOPROTEIN CLASS	DENSITY (kg/l)	FLOTATION RANGE (Sf)	DIAMETER (n.m.)
Chylomicrons	< 0.95	< 400[a]	100 - 500
VLDL	0.95 - 1.006	60 - 400[a]	30 - 100
IDL	1.006 - 1.019	12 - 60[a]	25 - 30
LDL	1.019 - 1.063	0 - 12[a]	20 - 25
HDL	1.063 - 1.210	1 - 10[b]	10

Physical properties of the Lipoprotein Classes.

a - at d = 1.063 kg/l

b - at d = 1.210 kg/l

2.2: The Composition of Lipoproteins.

As the name suggests lipoproteins are composed of a mixture of lipids and proteins. The relative proportions and types of lipid and protein in each lipoprotein determines the fraction in which it will be found. Chylomicrons, for example, have a large triglyceride component and a low content of protein. The triglyceride content decreases and that of cholesterol and protein increases as the density of the particle increases (Table 5).

The protein component, apolipoproteins or simply apoproteins, are named alphabetically according to the classification proposed by Alaupovic (41). Different lipoprotein fractions have characteristic apoprotein contents (Table 5). It is likely that the apoproteins direct the metabolic fate of the lipoprotein. VLDL, for instance, is a substrate for lipoprotein lipase which removes triglyceride from the particle by hydrolysis. This reaction, however, only proceeds in the presence of one of the apolipoprotein C peptides, apoCII, which is an activating cofactor for the enzyme. Absence or dysfunction of apoCII results in an accumulation of VLDL and chylomicrons, the triglyceride of which is also hydrolysed by lipoprotein lipase, in the plasma (42).

2.3: The Structure of Lipoproteins.

Transport of hydrophobic lipids through the aqueous medium of the plasma poses a structural problem in the

TABLE 5:

CLASS	DENSITY (kg/l)	MAJOR LIPIDS	APO-PROTEINS
CHYLO-MICRONS	< 0.95	TRIGLYCERIDE 90%	A,B,C
VLDL	0.95 - 1.006	GLYDERIDE 60% PHOSPHOLIPID 15% CHOLESTEROL 20%	B,C,E
IDL	1.006 - 1.019	GLYCERIDE 35% CHOLESTEROL 45% PHOSPHOLIPID 15%	B,C,E
LDL	1.019 - 1.063	CHOLESTEROL 60% PHOSPHOLIPID 15%	B
HDL	1.063 - 1.210	PHOSPHOLIPID 40% CHOLESTEROL 40%	A,C,E

Composition of the lipoprotein classes.

organisation of lipoproteins. Non-polar lipids, such as triglycerides and cholesteryl esters, are completely insoluble in aqueous solution and cannot be carried in free form. Amphipathic lipids, such as phospholipids and free cholesterol, contain both polar and non-polar regions within their structure and can therefore interact with both hydrophobic and hydrophilic environments. These lipids form the outer shell of the lipoprotein, their hydrophobic regions interacting with the non-polar core of triglyceride and cholesteryl esters and their hydrophilic region with the plasma (Fig. 3). The apoproteins are found associated with the shell of phospholipids and free cholesterol.

Sequence analysis of certain of the apoproteins reveal an unusual alpha helical structure. These helices, known as amphipathic helices (43), have hydrophobic and hydrophilic faces. The hydrophobic face is believed to interact with the non-polar regions of the phospholipid shell.

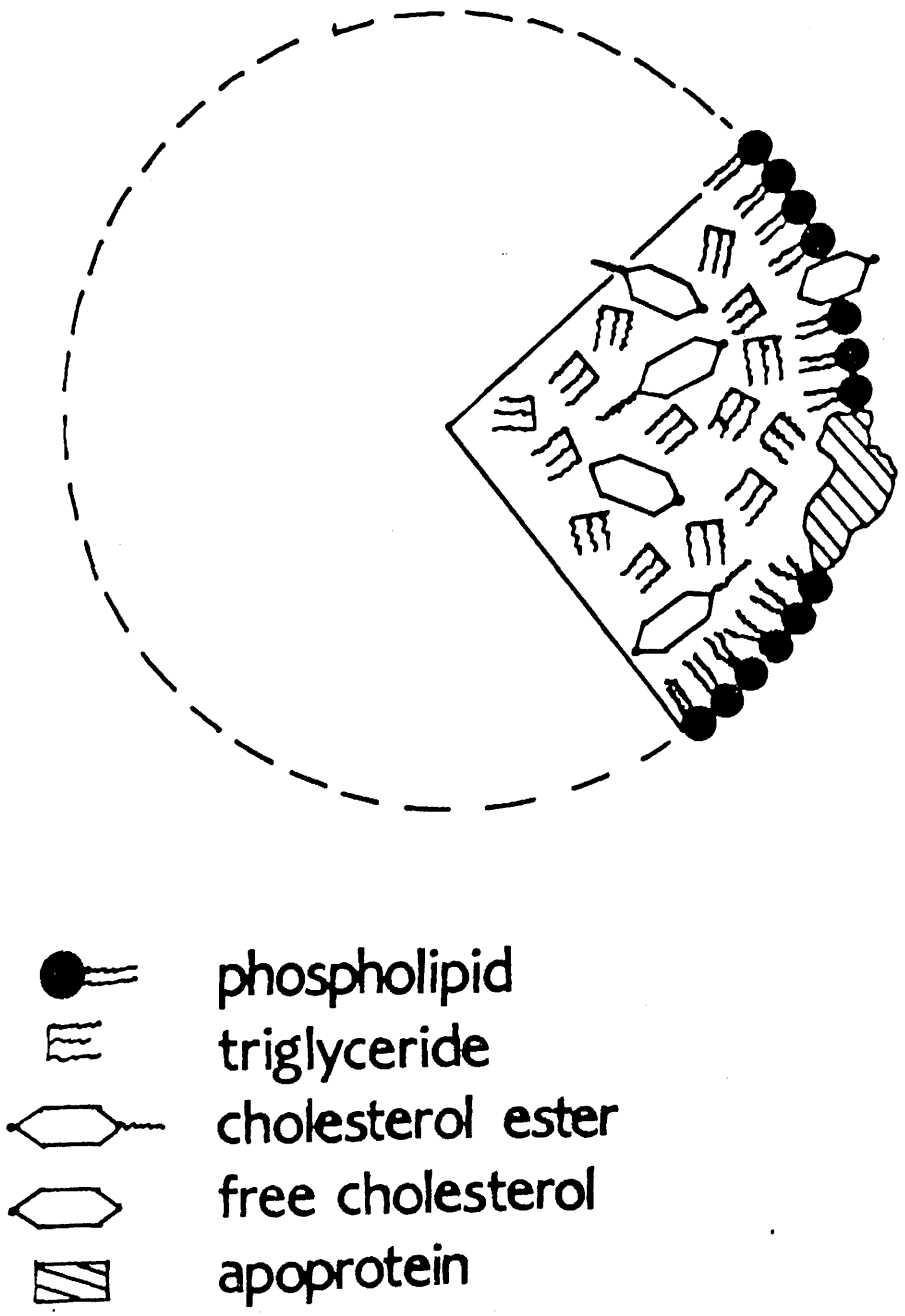
Apoproteins C and E appear to be only loosely attached to the lipoprotein and exchange freely between lipoproteins of the same and different classes. ApoB, however, seems to be an integral component of the lipoprotein and exhibits no such exchange.

2.4: The Apolipoproteins (Table 6)

i) ApoAI

ApoAI is a single polypeptide chain with 243 amino acids

Fig. 3: Diagramatic representation of generalised lipoprotein structure.



Apolipoprotein	Molecular Wt. x 10^{-3}	Site of Synthesis	Serum Concentration (mg/dl)	Function
A-I	28.3	Intestine Liver	100 - 150	Cofactor of lecithin:cholesterol acyltransferase; structural protein of HDL
A-II	17.0	Intestine Liver?	30 - 50	Phospholipid-binding properties; structural protein of HDL
A-IV	~ 46.0	Intestine	15	Unknown
B-100	549	Liver	80 - 100	Intracellular formation or transcellular transport of VLDL; receptor interaction (LDL) with "apolipoprotein B, E receptor cells"
B-48	~ 265	Intestine Liver ?	?	Intracellular formation or transcellular transport of chylomicrons and VLDL; receptor interaction with "apolipoprotein B, E receptor cells"?
C-I	6.5	Liver	< 10	Cofactor of lipoprotein lipase of adipose tissue (particularly saturated fatty acids)
C-II	8.8	Liver	3 - 8	Cofactor of lipoprotein lipase of adipose tissue
$C\text{-}III_{0,1,2}$	8.9	Liver	8 - 15	Unknown
D	20	?	10 -	Unknown; some question of a role in LCAT reaction and metabolism of cholesteryl esters
E	~ 39.0	Liver	3 - 5	Receptor interaction with "apo B, E receptor cells" and with hepatic apo E receptors; inhibitor of lipoprotein lipase of adipose tissue?

	Chylo-microns %	VLDL %	LDL %	HDL_2 %	HDL_3 %
A-I	33	trace	trace	65	62
A-II	trace	trace	trace	10	23
A-IV	14	-	-	?	trace
B	5	25	95	3	-
C	32	55	2	13	5
D	?	?	?	2	4
E	10	15	3	3	1
other	6	5	5	4	5

TABLE 6: The Apolipoproteins.

and a molecular weight of 28,500 daltons. The protein has no cystine or leucine and there are no carbohydrate residues linked to the peptide chain. It is rich in amphipathic helix and avidly binds phospholipid (44).

ii) ApoAII

ApoAII consists of two identical polypeptide chains linked by a single disulphide bridge at cystine-6. Each polypeptide has 77 amino acid residues giving a total molecular weight of 17,500 daltons. The molecule is devoid of histidine, arginine and tryptophan and, like apoAI, has no carbohydrate side chains. ApoAII has less amphipathic helix and more disordered structure than apoAI but still avidly forms phospholipid complexes (44).

iii) ApoAIV

Little is known about apoAIV except that it has a molecular weight of 46,000 daltons and that it is freely soluble in aqueous solution, indeed the majority may exist unassociated with plasma lipoproteins.

iv) ApoB

ApoB is extremely insoluble in aqueous solution after the removal of associated lipids. It can be solubilised with high concentrations of detergent, such as sodium dodecyl sulphate, though it is likely that aggregates form under these conditions. Estimates of molecular weight range from 10,000 to 550,000 daltons. Kane et al have isolated four species of apoB (45) which are named, after their electrophoretic mobility in sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS PAGE), by a centile system. ApoB 100 has an apparent molecular weight of 550,000 daltons and is predominant in LDL and VLDL. It appears that this form of apoB is synthesised mainly in the liver and, as the major form of apoB in LDL, shows that chylomicrons are unlikely to be the precursors of higher density lipoproteins (46). Two other forms of apoB are reported to be found on LDL, apoB74 and apoB26. These may be complimentary fragments or components of apoB100 but are as likely to be artifacts.

ApoB48, molecular weight 260,000 daltons, is the major species present on triglyceride rich particles produced by the gut. The synthesis of apoB48 is independent of that of apoB100 (46) and occurs in the gut. Unlike apoB74 and apoB26, no component can be added to apoB48 so that it has the same amino acid composition as apoB100. There are also differences in the absolute amounts of certain amino acids present (45). Krishnaiah et al (47) showed that this kind of heterogeneity also occurs in other mammals, including the rat.

Although apoB is a structural protein in chylomicrons, VLDL and IDL, upwards of 90% of apoB is found in LDL in normal individuals.

v) ApoCI

ApoCI activates the enzyme lecithin:cholesterol acyltransferase (LCAT) although the *in vivo* significance of this cofactor activity remains to be determined. Ten

percent of the total apoprotein content of VLDL and approximately two percent of that of HDL is apoCI. The amino acid sequence of apoCI is known and the protein has been synthesised in the laboratory (48). Like apoA, apoCI avidly binds phospholipid. The binding region in apoCI has been identified as a peptide of 26 amino acids occurring between residues 32 and 57 in the peptide chain.

vi) ApoCII

As stated in section 2.2 apoCII is a specific cofactor for lipoprotein lipase. The apoprotein comprises ten percent of VLDL protein and approximately 1.5 percent of HDL protein. The phospholipid binding region and the lipoprotein lipase cofactor region are separate domains. Like the other apoC proteins apoCII is transferred easily among the triglyceride rich lipoproteins and HDL.

vii) ApoCIII

ApoCIII can be resolved into three distinct bands on polyacrylamide gel electrophoresis. These bands are designated apoCIII$_0$, CIII$_1$ and CIII$_2$ and differ in their content of sialic acid. ApoCIII comprises about 50% of the total apoprotein content of VLDL in normal individuals but a higher percentage of apoCIII is found in those suffering from hypertriglyceridaemia. It has been found that apoCIII inhibits lipoprotein lipase under certain *in vitro* conditions but the relevance of this finding *in vivo* remains to be determined (49).

viii) ApoD

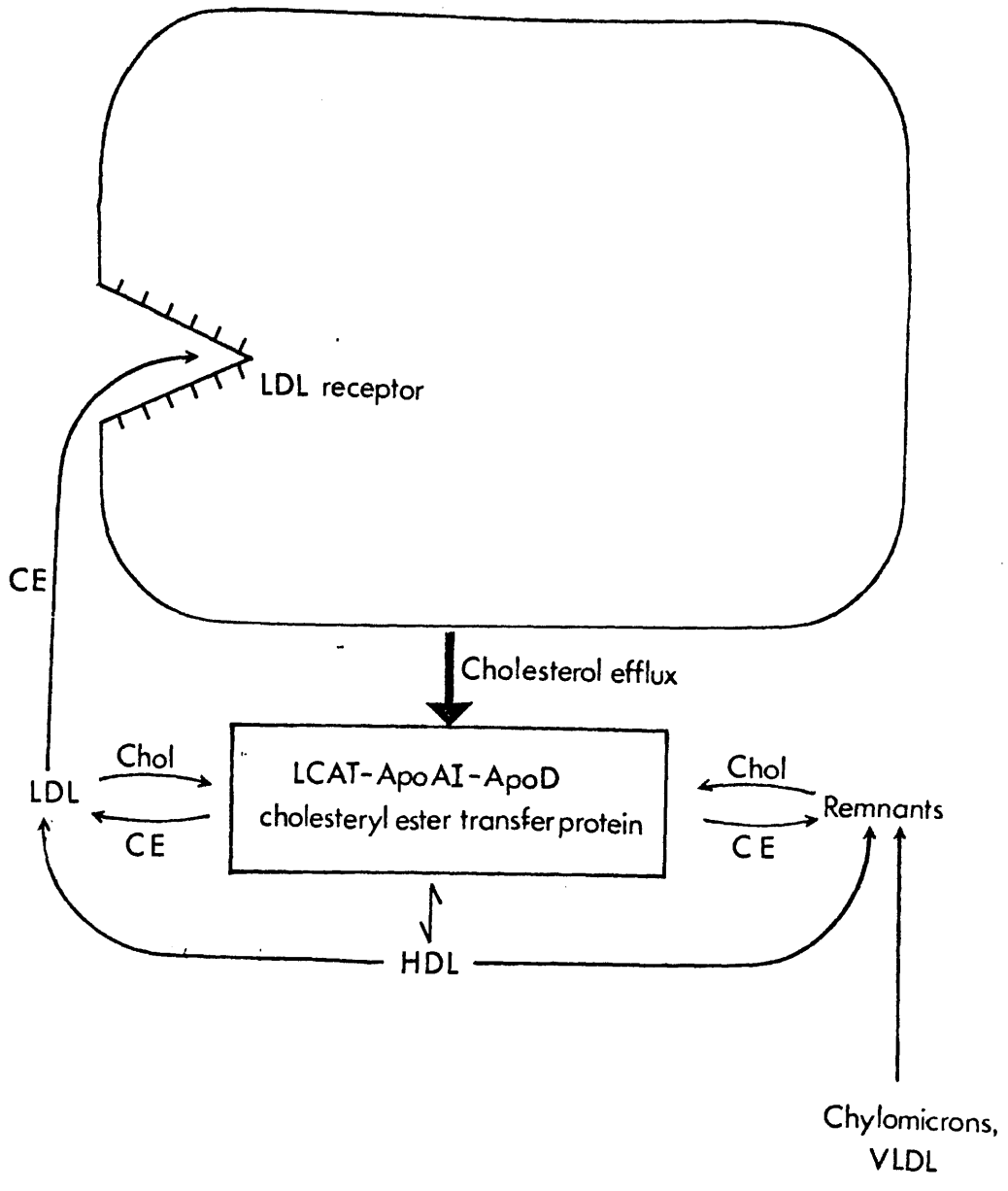
This apoprotein is also known as apoAIII. It is found mostly associated with HDL, though contributes less than five percent of the protein mass. Chajek and Fielding attributed to apoD the role of cholesteryl ester transfer protein (50) which transfers newly synthesised cholesteryl esters from HDL to LDL and other lipoproteins (Fig. 4). This view was later disputed by Morton and Zilversmit (51) after separation of apoD from the cholesteryl ester transfer protein.

ix) ApoE

Human apoE has a molecular weight of 36,000 daltons and contains 299 amino acids. Approximately 10 percent of these are arginine residues and for this reason the apoprotein is often termed "arginine rich apoprotein". In chylomicrons and HDL a portion of apoE is found complexed with apoAII (54). The structure of mature apoE and that of a precursor form, found at the site of synthesis in the liver, is known (52,53). ApoE is a glycopeptide and, after the removal of sialic acid residues, can be resolved into four major bands by isoelectric focusing (55). These bands are designated apoE-1, E-2, E-3 and E-4. It is thought that apoE-1 is the result of post translational modification but that apoE-2, E-3 and E-4 are genetically determined human mutations (56).

ApoE-3 is the "wild type" normal apoprotein being present as apoE3/3 in 51% of the population with a gene frequency of 0.71. ApoE-4, although a mutant, is not functionally different from apoE-3. The mutation of

FIGURE 4:



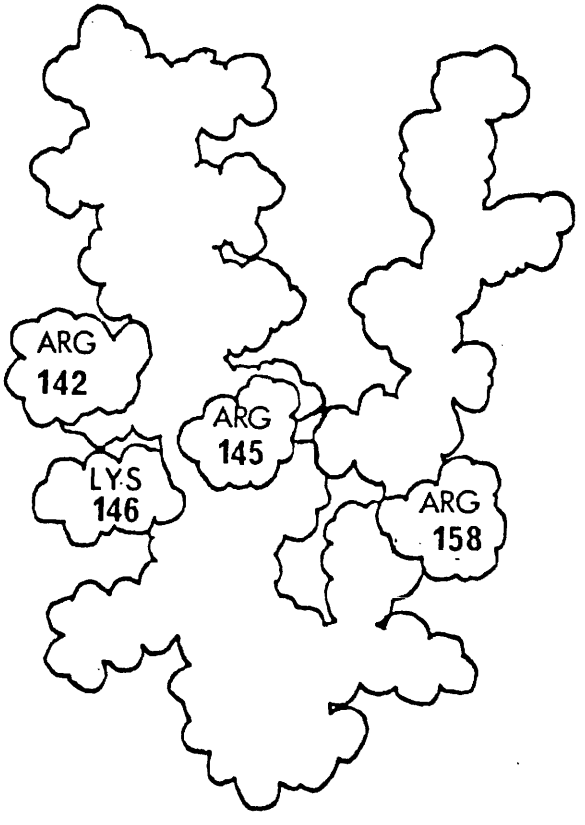
The role of ApoD in the transfer of newly synthesised cholesteryl ester from cells and HDL to other lipoproteins. ApoD is represented here as a separate entity to Cholesteryl Ester Transfer Protein (Zilversmit, ref.51).

apoE-3 which produces apoE-4 is a single amino acid substitution at residue 112 of the primary sequence in which the cystine residue normally present is converted to an arginine residue. The functional normality of apoE-4 reflects the fact that this substitution does not alter the structure of the protein significantly nor is it in such a position as to interfere with binding of apoE to its receptor.

ApoE-2 is functionally different from apoE-3 in the respect that it does not react with the apoE receptor. The first apoE-2 variant was detected using isoelectric focusing and was discovered to contain a single amino acid substitution at residue 158. At this site the arginine residue normally present was replaced by a cystine residue (57) (Fig. 5). Further apoE-2 variants, which are isoelectrically silent because the amino acid substitutions do not cause changes in overall charge, have also been found. The most important of these are apoE-2 in which the lysine residue at position 146 is replaced by glutamine, termed apoE-2 (Lys_{146} ->Gln) and one in which the arginine residue at position 145 is replaced by cystine termed apoE-2 (Arg_{145}->Cys) (58).

ApoE-2, with its inability to react with lipoprotein receptors, has been implicated in the aetiology of the disease known as type III hyperlipoproteinaemia noted for the accumulation of an abnormal VLDL, known as β-VLDL, with a high concentration of apoE-2. Most individuals with this disorder have the

FIGURE 5:



Diagrammatic representation of a Corey-Pauling-Koltun space filling model of residues 132 to 164 of human apoE3. This highlights the binding domain of apoE and the amino acids, important to that binding, which, if substituded, lead to defective apoE-receptor interactions.

E-2/2 phenotype but not all of the people with the E-2/2 phenotype have type III hyperlipoproteinaemia suggesting that other factors are necessary for the manifestation of the disorder (59).

3: Lipoprotein Metabolism

3.1: Lipoprotein Receptors: ApoB and ApoE .

i) The LDL receptor

Since the discovery of the LDL receptor by Goldstein and Brown in fibroblasts (61) it has been found in other cell types, such as arterial smooth muscle cells, adrenal cells, hepatocytes, lymphoid cells and on the membranes of freshly isolated blood lymphocytes and adrenal cortex (62).

The LDL receptor binds both apoB and apoE containing lipoproteins and it has been shown that it is these apoproteins which are responsible for the binding of the lipoprotein to the receptor (63,64).

The receptor itself is a transmembrane protein of the plasmalemma which is synthesised in membrane associated ribosomes (65). It is glycosylated in the Golgi Apparatus before associating with the membrane in a random distribution. The protein has two active domains: a binding domain, to which the lipoprotein attaches, and an internalisation domain on the inner surface of the plasmalemma. The internalisation domain is believed to have a high affinity for clathrin, another membrane protein. Clathrin is found in regions

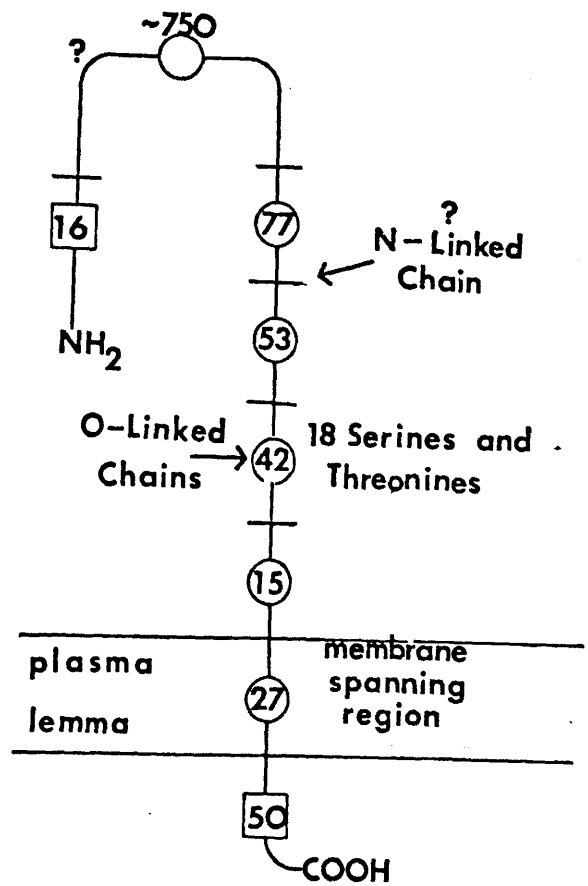
of the membrane known as coated pits which cover no more than two percent of the total cell surface. Although the newly synthesised receptors associate randomly with the membrane, more than 60% of them are found in coated pits, probably due to the affinity between clathrin and the internalisation domain (66).

Endocytosis occurs by invagination of coated pits to form coated vesicles with a half life of approximately 5 minutes. The receptors themselves return to the membrane after the coated vesicle has fused with the primary lysosome (66).

The molecular weight of the receptor protein was originally estimated at 110,000 daltons by Mahley using the radiation inactivation method (67) and at 160,000 daltons by Goldstein and Brown after purification of the receptor from bovine adrenal cortex (68).

The receptor is synthesised as a precursor with an apparent molecular weight of 120,000 daltons, as estimated by SDS-polyacrylamide gel electrophoresis. Within 30 minutes, the precursor undergoes several carbohydrate processing reactions in the Golgi apparatus, which results in the formation of the mature form of the receptor with an apparent molecular weight of 160,000 daltons (69,137). The carbohydrate chains are predominantly 0-linked to a region, near the carboxy terminus, which is rich in serine and threonine residues (Fig. 6) (137). The protein has been divided into regions or domains. The first of these is a cysteine

FIGURE 6:



Working Model for the Structure of the Bovine LDL receptor.

Numbers in circles or squares represent the number of amino acids in the domains.

Russel et al. (G & B)
Cell; 37: 5770585.

rich region of 322 amino acids which is thought to be the ligand binding site. The cysteine residues, which occur approximately every seven amino acids, may be responsible for the stability of the receptor, an important factor as it is recycled between coated pits on the surface and endosomes within the cell. This domain is also rich in aspartate and glutamate residues which, being negatively charged, may be responsible for the interaction with lysine and arginine residues of the ligand proteins; apoB and apoE (137).

Next is a domain of roughly 350 amino acids which has striking homology with the poly protein precursor of mouse epidermal growth factor. The reason for this is unclear but it may be that proteins for nutrient delivery and growth stimulation have a common evolutionary origin (137). The third, threonine and serine rich domain has been discussed and the fourth is the membrane spanning region of 22 hydrophobic amino acids. The carboxy terminal domain is 50 amino acids long and located on the cytoplasmic side of the membrane (137) (Fig. 6).

ii) The Chylomicron Remnant Receptor

This hepatic receptor is responsible for the clearance of apoE-rich lipoproteins which include chylomicron remnants, β-VLDL and a species of HDL, which is rich in apoE, called apoE HDL_C. If the LDL receptor was of major importance in the clearance of apoE containing lipoproteins it might be expected that the lack or

dysfunction of these receptors, such as in familial hypercholesterolaemia, would cause an increase in the plasma concentrations of the apoE rich lipoproteins. This, however, is not the case (138), leading, originally, to the suspicion that the chylomicron remnant receptor might exist.

Further evidence for its existence was obtained in experiments with mature dog hepatic membranes. It was shown that the number of LDL receptors on dog liver declined linearly with age so that at approximately 20 months from birth the dogs had no LDL receptors in evidence on preparations of their liver membranes. The binding of apoB containing lipoproteins, such as LDL, declined colinearly. Chylomicron remnant receptors, as determined by subtracting the amount of apoE HDL_C bound to the LDL receptors from the total amount of apoE HDL_C binding, remained remarkably constant for all ages of dog. It was found that LDL-receptors present on the liver membranes of young dogs could be suppressed by feeding a diet supplemented with cholesterol but the chylomicron remnant receptors could not. In adult dogs, fasting induced the expression of LDL receptor but did not increase the number of chylomicron remnant receptors (71,72).

As with the binding of apoB to the LDL-receptor, the binding of lipoproteins to the chylomicron remnant receptor is protein mediated. Chemical

modification of apoE results in the loss of receptor lipoprotein interaction (70).

iii) The Macrophage Receptor for Acetyl-LDL

LDL that has been modified chemically with acetic anhydride to form acetyl-LDL (ac-LDL) is taken up with extremely high efficiency by macrophages. This uptake can result in a massive cholesterol accumulation within the cells which does not occur with native LDL (139). Studies with radiolabelled ac-LDL showed that the rapid uptake was mediated by a receptor on the cell surface which recognised ac-LDL but not native LDL and that the binding of ac-LDL was abolished on treatment of the cells with low concentrations of trypsin or pronase (139,103), suggesting that the receptor was composed of protein.

Acetylation of LDL removes positive charges from the etta amino groups of lysine residues in the protein moieties of lipoproteins thereby converting them to a strongly anionic species (128) (Fig. 14). It is this increase in overall net negative charge which is responsible for the binding of ac-LDL to the macrophage receptor. Other chemical modifications which abolish the positive charge on lysine residues also convert LDL into a ligand for the ac-LDL receptor. Such ligands include citraconylated LDL (103), maleylated LDL (139), succinylated LDL (139), malondialdehyde-treated LDL (140) and acetoacetylated LDL (141).

Binding to the ac-LDL receptor only occurs when 30 moles of modifying agent has been incorporated

per mole of LDL. Below this threshold value no ligand - receptor interaction takes place and further, below 20 moles of modifying agent per mole of LDL the native LDL receptor begins to bind the ligand.

The acetyl-LDL receptor has been found on macrophages from every source and species investigated including resident peritoneal macrophages from mice, rats and dogs (139,141), Kupffer cells from guinea pigs (139), monocyte derived macrophages from humans (139,140) and on established lines of mouse macrophage tumours such as J774 cells (142).

The bovine receptor has an estimated molecular weight of 283,000 daltons. In contrast to the LDL receptor of non-macrophage cells the acetyl LDL receptor is not down regulated when cellular cholesterol concentration rises. As a result of their failure to suppress the production and utilisation of acetyl LDL receptors, macrophages continuously exposed to medium containing ac-LDL accumulate massive amounts of cholesterol, as much as 80% of which is cholesteryl ester, and are converted into foam cells (139) (section 1.2).

Evidence that the acetyl LDL receptor is expressed *in vivo* comes from experiments done with ^{125}I-labeled chemically modified LDL which was injected into dogs (141) and mice (139). The labeled ligand was cleared from the plasma within minutes by the macrophages of the liver (Kupffer Cells)(144). The hepatic uptake of

intravenously administered ^{125}I-acetyl-LDL in mice was blocked by a simultaneous injection of fucoidin (139), a highly anionic protein which interacts with the acetyl-LDL receptor (139), confirming that the uptake was mediated by a saturable receptor with a specificity similar to the binding site demonstrated in vitro.

The normal ligand for the acetyl LDL receptor in vivo is unknown but several possibilities exist. Malondialdehyde, a biproduct of the oxidation of arachidonic acid by platelets and macrophages, is known to convert LDL into a form recognised by the acetyl LDL receptor (145). There are other substances derived from arachidonic acid which may also modify the etta amino groups of lysine residues removing their positive charge. These include prostaglandin A, 5-hydroxyperoxyeicosatetraenoic acid and leukotriene A_3 (146).

Henriksen et el have shown that cultured endothelial cells can convert human LDL into a form that is recognised by the acetyl-LDL receptor (142,147). Incubation of LDL with one of several types of cultured cells including aortic endothelial cells, umbilical vein endothelial cells or aortic smooth muscle cells can induce the changes in LDL which leads to its recognition by the acetyl LDL receptor (147). They are not, however, induced by incubation of LDL with conditioned medium from these cells or by incubation with fibroblasts. The modified LDL has an increased density, owing to a decreased content of free and esterified cholesterol and

an enhanced electrophoretic mobility, presumably due to an increased net negative charge (147).

Retroendocytosis, a process by which the cells endocytose LDL then return it to the medium without contact with primary lysosomes, is one possible mechanism by which the changes in LDL could occur. Indeed, incubation at 4°C, a temperature at which endocytosis does not occur, fails to modify LDL to a form which is recognised by the ac-LDL receptor (147).

iv) The Macrophage Receptor for β-VLDL

β-VLDL are not normally present in detectable amounts in the plasma of humans or animals but Mahley et al. (74,148) showed them to accumulate in the plasma of a variety of species, including dogs, rats, rabbits and monkeys, when the animals are fed a high cholesterol diet. They are found in the same density range as normal VLDL (0.95-1.006 kg/l) but, in contrast to normal VLDL, have a cholesteryl ester rich core and instead of the pre-β mobility on agarose gel electrophoresis associated with normal VLDL, have a β-mobility (148,82), (see also section 3.3, ii).

β-VLDL particles are thought to represent exaggerated forms of remnant particles which are normally created during the catabolism of chylomicrons and VLDL and are rapidly removed by the liver (62,149). In cholesterol-fed animals the clearance mechanism is overloaded and the remnants accumulate in the plasma,

grow in size and become even further enriched in cholesteryl esters to form β-VLDL (149).

Goldstein et al. (73) reported that mouse peritoneal macrophages express a surface binding site that mediates the uptake and degradation of β-VLDL. The receptor is specific for β-VLDL and does not bind acetyl LDL, normal LDL or normal VLDL (73,74). The β-VLDL receptor is expressed on the cell surface of monocyte derived macrophages from patients suffering from familial hypercholesterolaemia, a genetic disorder in which LDL receptors are not present, suggesting a different genetic locus for the β-VLDL receptor (74).

When the protein moieties of β-VLDL are modified by reductive methylation, a procedure which prevents protein-receptor interaction in other lipoproteins, the binding of β-VLDL to the β-VLDL receptor is also abolished (150). The proteins of β-VLDL consist almost entirely of apoB and apoE yet LDL containing largely apoB and apoE HDL_C containing largely apoE fail to inhibit the degradation of β-VLDL (73,74). Moreover, normal VLDL containing both apoB and apoE, fail to bind to the β-VLDL receptor (73) thus it has not been possible to determine which, if any, of the apoproteins mediates the binding of β-VLDL to its receptor.

The β-VLDL receptor is only partially down regulated by the influx of cholesterol into the cell which may explain the continued accumulation of cholesteryl ester which leads to foam cell formation (73,74).

3.2: Chylomicron Metabolism.

Chylomicrons are large (300-5,000Å diameter) triglyceride rich particles which are synthesised in the mucosal cells of the small intestine. When triglyceride is hydrolysed in the gut the free fatty acids and glycerol are absorbed by the mucosal cells, re-esterified and packaged into chylomicrons (60). Triglyceride accounts for more than 83% of the weight of these lipoproteins, cholesterol for 8% and phospholipid for 7%. Protein in the form of apoAI, AII, AIV and B-48 is present as only 2% of the total particle mass. ApoCI, CII, CIII and apoE are transferred to the chylomicrons only after they enter the plasma (76,77).

The half life of chylomicrons in the plasma is only a few minutes. They are degraded by the action of lipoprotein lipase to core and surface remnants. The surface remnants are rich in phospholipids and free cholesterol as well as apoC and are partially transferred to HDL (78). Core remnants, more commonly known simply as chylomicron remnants, are rich in cholesterol ester and apoE and interact with the hepatic chylomicron remnant receptors (70).

Chylomicrons, therefore, are responsible for the delivery of exogenous cholesterol to the liver through chylomicron remnants and result in the down regulation of cholesterol synthesis by the liver in response to dietary intake (79).

3.3: VLDL Metabolism.

i) Normal VLDL

VLDL, rather than being a single species, is a heterogeneous mixture of particles that have a hydrated density in the range 0.95-1.006 kg/l and exhibit α_2 or pre-β electrophoretic mobility. When VLDL is subjected to electrophoresis on starch block, most particles migrate in the same region as α_2-globulin, hence the designation α_2-VLDL; this corresponds to the pre-β lipoprotein band observed on paper electrophoresis (82,148).

Over 90% of triglycerides found in fasting plasma are present in the form of VLDL which is synthesised and secreted by the liver (80,81). As with chylomicrons, VLDL interacts with lipoprotein lipase by virtue of the presence of apoCII. This results in the formation of IDL from the core remnants of VLDL and the surface remnants are recovered in the HDL density range. The reaction of VLDL with lipoprotein lipase is slower than for chylomicrons resulting in a plasma half-life of 1-3 hours (83). HDL which has received free cholesterol from VLDL surface remnants interacts with lecithin-cholesterol acyltransferase (LCAT) which converts the cholesterol to cholesteryl ester using fatty acids from lecithin. These cholesteryl esters are transferred back to further enrich IDL through the action of cholesteryl ester transfer protein (50) (Fig.4, p.17 and Fig.9, p.38).

Thus VLDL is metabolised to IDL by the hydrolysis and removal of its triglyceride and replacement of this with cholesteryl ester.

ii) β-VLDL

As has been discussed in section 3.1, iv) β-VLDL has a hydrated density in the range 0.95-1.006 kg/l and a β-mobility on starch block electrophoresis (82,148). It appears to be a remnant particle which is rapidly metabolised by hepatic remnant receptors and does not accumulate until this mechanism is saturated, as in the case of cholesterol feeding (74,148). β-VLDL are also found in the plasma of humans with the genetic disease familial dysbetalipoproteinaemia or type 3 hyperlipoproteinaemia, a condition in which the removal mechanism for the remnants appears to be malfunctioning (151).

β-VLDL contains predominantly apoB and apoE with a reduced amount of apoC compared to normal VLDL and although it can interact with the LDL receptor and the chylomicron remnant receptor neither LDL nor apoE HDL_C can inhibit the binding and degradation of β-VLDL by the macrophage β-VLDL receptor (73,74).

Fai naru et al (152) showed that in dogs fed a diet rich in cholesterol and in humans with type 3 hyperlipoproteinaemia the β-VLDL isolated from the plasma could be separated into two fractions by agarose gel chromatography. The larger particles were designated Fraction I (90-300 nm diameter) and had a lower protein-cholesterol ratio than Fraction II (30-70 nm diameter).

On paper electrophoresis Fraction I remained at the origin and Fraction II had mobility. Both fractions contained predominantly apoB and apoE but Fraction I was found to have a mixture of equal amounts of B-100 and B-48 whereas Fraction II contained only B-100. On the basis of these findings Fainaru suggested that Fraction I was of intestinal origin, ie that it was derived from chylomicrons, and that Fraction II was derived from VLDL produced in the liver (152).

Both particles interact with the β-VLDL receptor but Fraction I was 3-15 times more efficient at inducing cholesteryl ester synthesis (152) suggesting that the β-VLDL receptor may preferentially interact with particles of intestinal origin.

In vivo the macrophage receptor may function as a backup mechanism to clear remnant particles which are not dealt with by their normal receptors. Mahley (148) showed that cholesterol-fed dogs develop massive cholesteryl ester deposition in macrophages throughout the body at plasma cholesterol levels exceeding 750 mg/dl. This plasma cholesterol level is also the level at which β-VLDL becomes a predominant lipoprotein (148).

Labelled β-VLDL injected into animals are cleared rapidly from the plasma (84,153), Fraction I more rapidly than Fraction II (152). This rapid clearance is reduced if the β-VLDL are reductively methylated (150) or if the animals have been fed a cholesterol rich diet

(153). These results are clouded by difficulties in interpretation because of the ability of β-VLDL to bind to the LDL receptor of hepatic and other tissues (74,153). Thus it is not certain that the β-VLDL receptors of macrophages are responsible for the removal of β-VLDL from the plasma.

3.4: IDL Metabolism

After the interaction of VLDL with the lipoprotein lipase of capillary walls and the removal of most of its triglyceride the product is a cholesteryl ester enriched lipoprotein designated intermediate density lipoprotein (IDL). These IDL particles are released into the circulation and have two possible metabolic fates: i) conversion to LDL or ii) direct uptake and catabolism by the liver.

There is some evidence to suggest that direct catabolism by the liver is mediated through receptors. IDL contains both apoB and apoE and is thus eligible to interact with either the LDL receptor or the chylomicron remnant receptor (62).

IDL which are not catabolised in this way undergo a further conversion process in which most of the remaining triglyceride and all of the apoproteins except apoB are lost. The resultant particle, which is highly enriched in cholesteryl ester and apoB, is termed low density lipoprotein (LDL). The site of the final

conversion of IDL to LDL is unknown, but it may occur in the liver sinusoids (86).

3.5: LDL Metabolism

LDL carries approximately two thirds of the cholesterol present in the plasma. Its major constituents are cholesteryl ester and apoB and its primary function is to supply cholesterol to extrahepatic tissues and to the liver. This is accomplished via the LDL receptor. After interaction with the receptors the LDL is internalised and degraded in the primary lysosomes. The cholesteryl esters are hydrolysed by an acid lipase and free cholesterol released for use in cellular reaction (87). This cholesterol has a marked effect on cellular cholesterol synthesis and further uptake of LDL by the receptors. The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the enzyme which converts HMG CoA to mevalonate committing it to the de novo synthesis of cholesterol within the cell, is down regulated by cholesterol from degraded LDL particles at this level of enzyme synthesis (88,89). Although hydrolysed in the lysosome, cholesteryl ester is the main storage form of cholesterol within the cell and so must be re-esterified. This is done by acyl CoA:cholesterol acyltransferase (ACAT) which transfers oleate and palmitate onto cholesterol from their coenzyme A forms. ACAT is activated by the influx of cholesterol from LDL (90). Further influx and overloading of the cell by

LDL-cholesterol is prevented by the down regulation of the synthesis of LDL-receptors by cholesterol (Fig. 7) (91).

In individuals metabolising the lipoprotein normally, 33-66 percent of LDL is removed from the circulation by the receptor pathway. The range may seem broad but the actual figure varies among individuals, depending on the plasma concentration of LDL ie the pool size (92-94). In the disease familial hypercholesterol-aemia, which has a genetically determined lack of functional LDL receptors and experiences the gene dosage effect (ie each gene of the pair produces protein hence in the heterozygous form one protein of each type is produced), heterozygous sufferers produce half the number of normally functioning receptors accounting for only 16% of overall LDL catabolism (94). These individuals have a raised plasma LDL cholesterol concentration, typically in the range 8-9 mmol/l (300 mg/dl).

The homozygous form of the disease is manifested in a near complete lack or dysfunction of LDL receptors. This results in a greatly elevated plasma LDL concentration from an early age and typically, a myocardial infarction before the age of fifteen years. These individuals are unlikely to survive their second decade (31).

Thus far three types of the homozygous forms of familial hypercholesterolaemia have been identified,

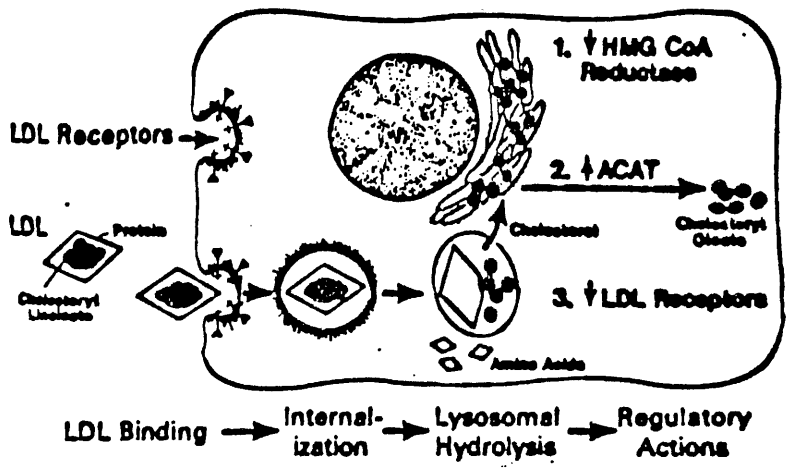


Fig.7 : Sequential steps in the LDL receptor model.

HMG CoA Reductase:- hydroxymethylglutaryl Coenzyme A Reductase

ACAT:- acyl-Coenzyme A: cholesterol acyl-transferase.

each resulting from a different mutation of the receptor gene (92).

i) receptor negative - this, the most common form, appears to result from a complete lack of receptor binding at the cell surface.

ii) receptor defective - in this type less than 20% of normal binding occurs.

iii) internalisation defective - here normal amounts of LDL bind to the receptors but the cells appear to be incapable of internalising the LDL-receptor complex.

Other mutations affecting LDL metabolism by cells have been found. They are autosomal recessive genetic disorders called Wolman's syndrome and cholesteryl ester storage disease. In Wolman's syndrome (95) the absence of lysosomal acid lipase activity results in an inability to hydrolyse cholesteryl esters and triglycerides. The build up of these lipids causes the formation of foamy, lipid laden cells and although sufferers have normal plasma lipid levels, they rarely survive past infancy.

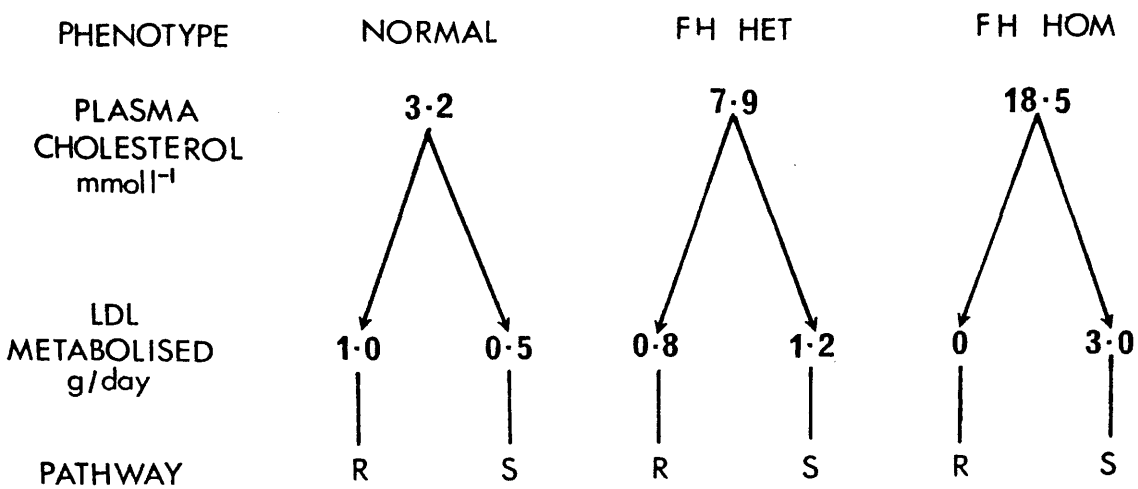
Cholesteryl ester storage disease appears to be similar to Wolman's syndrome in as much as there is accumulation of cholesteryl esters and triglycerides in the absence of hepatic acid lipase activity. It has been suggested by Stone and Fredrickson (96) that the two diseases are caused by mutations of the same gene in which the

cholesteryl ester storage disease lesion is less severe resulting in a longer life expectancy (96).

There are at least two distinct mechanisms for the catabolism of LDL *in vivo*. The better understood of these is the receptor dependent mechanism described above (154) which is responsible for 33-66 percent of LDL clearance. It is therefore clear that a considerable amount of LDL is degraded by another mechanism(s).

Indeed in familial hypercholesterolaemia, discussed above, even more LDL is catabolised daily despite the near total lack of LDL receptors (92) (Fig. 8). This LDL receptor-independent catabolism has been termed the scavenger pathway, but the nature of the pathway has to be elucidated (155). It has been suggested that the cells of the reticuloendothelial system may be a major site of LDL catabolism in the absence of LDL-receptors (102). These cells have a greater capacity for non-specific uptake. Catabolism of LDL at a rate simply proportional to its concentration would be enough to yield preferential uptake by cells of the reticuloendothelial cells at higher plasma LDL levels (ie as found in familial hypercholesterolaemia) (155). Macrophages, an important component of the reticulo-endothelial system, have receptors for negatively charged LDL (139,103, 128,140,141). There is some evidence for the formation of such LDL *in vivo* (145) (section 3.1, iii) by reaction with substances released on the oxidation of arachadonic acid. These negatively charged

FIGURE 8:



LDL metabolism by the receptor pathway (R) and the scavenger pathway (S).

FH HET:- Heterozygous Familial Hypercholesterolaemic.

FH HOM:- Homozygous Familial Hypercholesterolaemic.

species of LDL are more likely to form when LDL remains in the plasma for longer than normal amounts of time (ie in the case of familial hypercholesterolaemia). Retro-endocytosis (a process of endocytosis and receptosome formation followed by exocytosis of the contents of the receptosome without contact with the lysosome), during incubation with cultured endothelial cells, can convert LDL into a form recognised by the same receptor as negatively charged LDL (142,147), again a process which is more effective in cases where LDL has an increased plasma half-life.

A further proportion of this LDL receptor-independent degradation will occur through fluid-phase or bulk endocytosis (156). Here LDL will be taken up by pinocytosis, along with other solutes, at a rate determined by its concentration in the plasma. This, however, does not make a major contribution to LDL receptor independent clearance as determined by Pittman in a rabbit model for familial hypercholesterolaemia using albumin as a determinant for bulk endocytosis (157).

The mechanisms discussed above may form all or more likely part of the LDL receptor-independent degradation of LDL which plays a significant part in the overall catabolism of that lipoprotein.

3.6: HDL Metabolism.

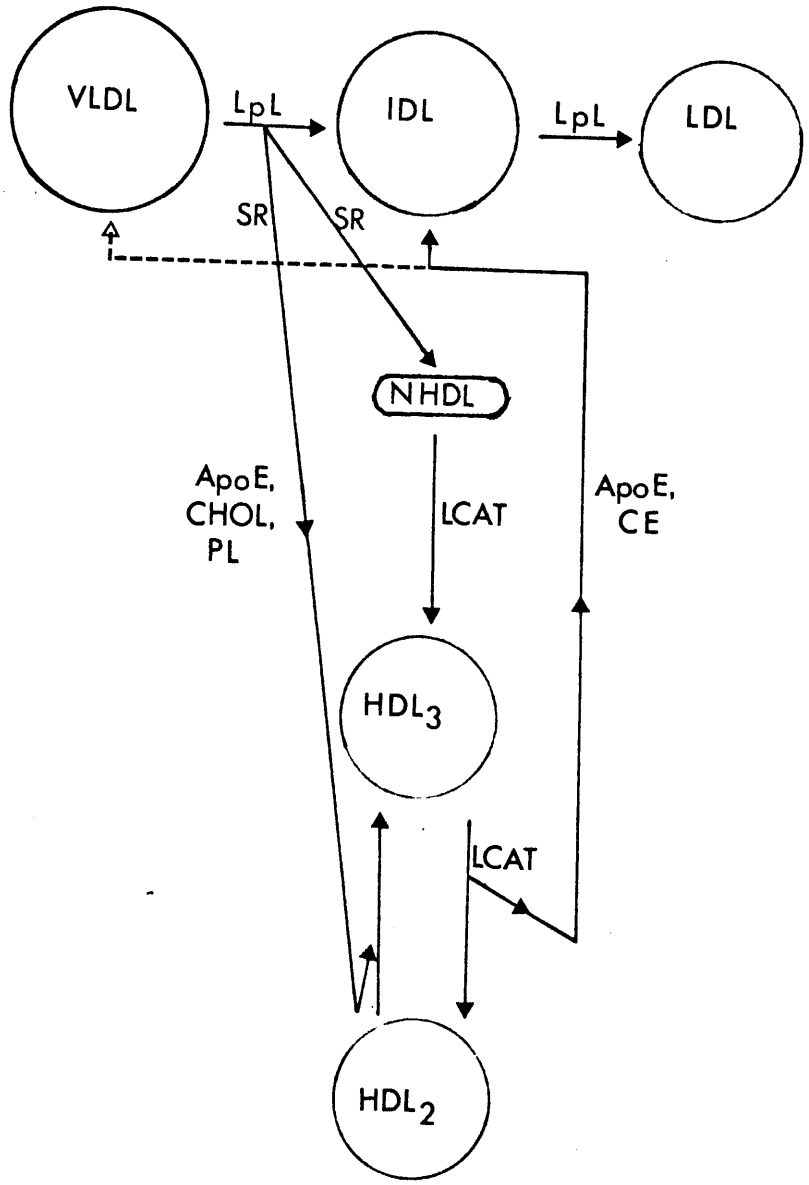
Throughout the conversion of VLDL to smaller, denser

particles by the removal of core triglyceride there is a partial replacement of a portion of the triglyceride with cholesteryl ester probably through the action of lecithin-cholesterol acyltransferase (LCAT) and/or cholesteryl ester transfer protein.

Surface constituents from VLDL appear in the HDL density range. They may become associated with pre-existing HDL or may form new, nascent HDL. Such HDL is likely to exist as the heavier of the two HDL fractions, HDL_3 (105). HDL_3 makes an ideal substrate for LCAT (106) resulting in its transformation to HDL_2. This conversion results in the transfer of cholesteryl ester back to VLDL or IDL with apoE acting as a carrier. As an alternative to the direct formation of HDL_3, surface components from VLDL may interact with HDL_2 to produce HDL_3 which is then metabolised as outlined above. It is conceivable that both mechanisms function simultaneously maintaining the HDL concentration in the plasma (Fig. 9).

Apart from its role as a catalyst in the metabolism of triglyceride rich lipoproteins, HDL has other properties. It can remove and incorporate cholesterol from cells in culture (107,108) and in the presence of acceptor HDL, macrophages hydrolyse cholesteryl esters and excrete cholesterol (109). It has also been implicated as a protective factor in the atherogenic process by inhibiting cellular injury which occurs when LDL is incubated with human endothelial cells (110) and partially inhibiting the uptake and degradation

FIGURE 9:



HDL metabolism and it's catalylic rate in the catabolism of VLDL

LpL	lipoprotein lipase
SR	surface remnants
NHDL	nascent HDL
CHOL	cholesterol
CE	cholesteryl ester
LCAT	lecithin cholesterol acyl transferase

of LDL by cultured smooth muscle cells and human fibroblasts (111,112).

HDL was thought to be catabolised mainly by the liver but there is increasing amounts of evidence that catabolism also occurs in the small intestine (113), adrenal gland (114) and the kidney (115).

4: Reticuloendothelial System

This is still the classically used name for this system but it has been criticised because reticuloendothelial is liable to be interpreted as excluding all but "reticulum cells" and "endothelial cells". The term is, however, generally understood to encompass cells which, by phagocytosis, can take up such substantial quantities of dye particles that their cytoplasm is conspicuously coloured; known as vital staining (158).

While reticulum cells are generally included in the system, endothelial cells, which lack the phagocytic activity which defines reticuloendothelial cells, are not. Reticulum cells are large mononuclear cells associated with the connective tissue fibres of the lymphoid tissues. Because these cells have relatively little phagocytic capacity compared to tissue macrophages there is some doubt about the appropriateness of their inclusion in the reticuloendothelial system.

Tissue macrophages or histiocytes are amoeboid macrophages which are found free throughout the connective tissues of the body. They are probably the

most numerous and certainly the most widespread of the reticuloendothelial cells (158). These cells are undoubtedly the macrophages from which foam cells are formed in atherosclerotic plaques (11,17). The alveolar macrophages of the lungs, peritoneal macrophages and macrophages found in other such cavities of the body may be considered to be tissue macrophages (158).

Endothelial macrophages is the term applied to phagocytes that line the lymph sinuses. They are also found distributed among the simple endothelial cells of blood sinuses (sinusoids) in certain organs, particularly the spleen, liver, pituitary gland and adrenal glands. In the liver these cells are known as Kupffer cells. The phagocytic activity of these macrophages differs from tissue to tissue showing little activity in the adrenals or pituitary compared to the liver and especially the lymph nodes and spleen (158).

Monocytes are of particular importance because they maintain the numerical strength of the reticuloendothelial system throughout the body. The blood borne monocytes are consistently in transit from the bone marrow, their tissue of origin, to make good damage or loss of reticuloendothelial cells. Only when the need for the macrophages is greatly increased and the monocyte replacement insufficient does proliferation of the cells already present occur (158).

The relevance of the reticuloendothelial system to the metabolism of lipoproteins has been discussed in sections 1.3, 3.1 iii), 3.1 iv), 3.3 ii) and 3.5.

5: MATERIALS AND METHODS

5.1: Isolation of Lipoproteins.

5.1.1: LDL

LDL (density 1.030-1.050 kg/l) was isolated from human and rabbit plasma by rate zonal ultracentrifugation (117) through a linear salt gradient of d = 1.00 kg/l to 1.30 kg/l using a Beckman Ti14 rotor in an L5-65 ultracentrifuge. Rotor speed was maintained at 45,000 rpm for one hundred and ten minutes at a temperature of 15°C. Fractions which contained LDL were concentrated by pressure filtration (Amicon Corp., XM 100A filters) and dialysed against Buffer A (0.15 M NaCl containing 0.01% (0.1 g/l) disodium EDTA, pH 8.1). Alternatively the LDL was washed exhaustively in a pressure filtration unit (Amicon) using buffer A until the conductivity of the filtrate equalled that of buffer A (8-9 mMHO).

This method was superior to sequential flotation ultracentrifugation because of its speed and capacity for handling large volumes of plasma.

5.1.2: VLDL

Human and rabbit VLDL (density 0.95-1.006 kg/l) were prepared by one of two methods:

1) Plasma was overlayered with density 1.006 kg/l sodium chloride solution and centrifuged for 18 hours at 39,000 rpm and 20°C using a Beckman Ti60

rotor in an L5-65 ultracentrifuge. The VLDL isolated by this method was washed by a second centrifugation through density 1.006 kg/l sodium chloride solution at 39,000 rpm. The VLDL was finally dialysed against buffer A (section 5.1.1).

2) Plasma subjected to zonal ultracentrifugation, as described in section 5.1.1, yields fractions containing VLDL (Fig. 10). These fractions were collected, overlayered with density 1.006 kg/l sodium chloride solution and centrifuged for 18 hours at 39,000 rpm and 20°C using a Beckman Ti60 rotor in an L5-65 ultracentrifuge. The isolated VLDL was dialysed against buffer A.

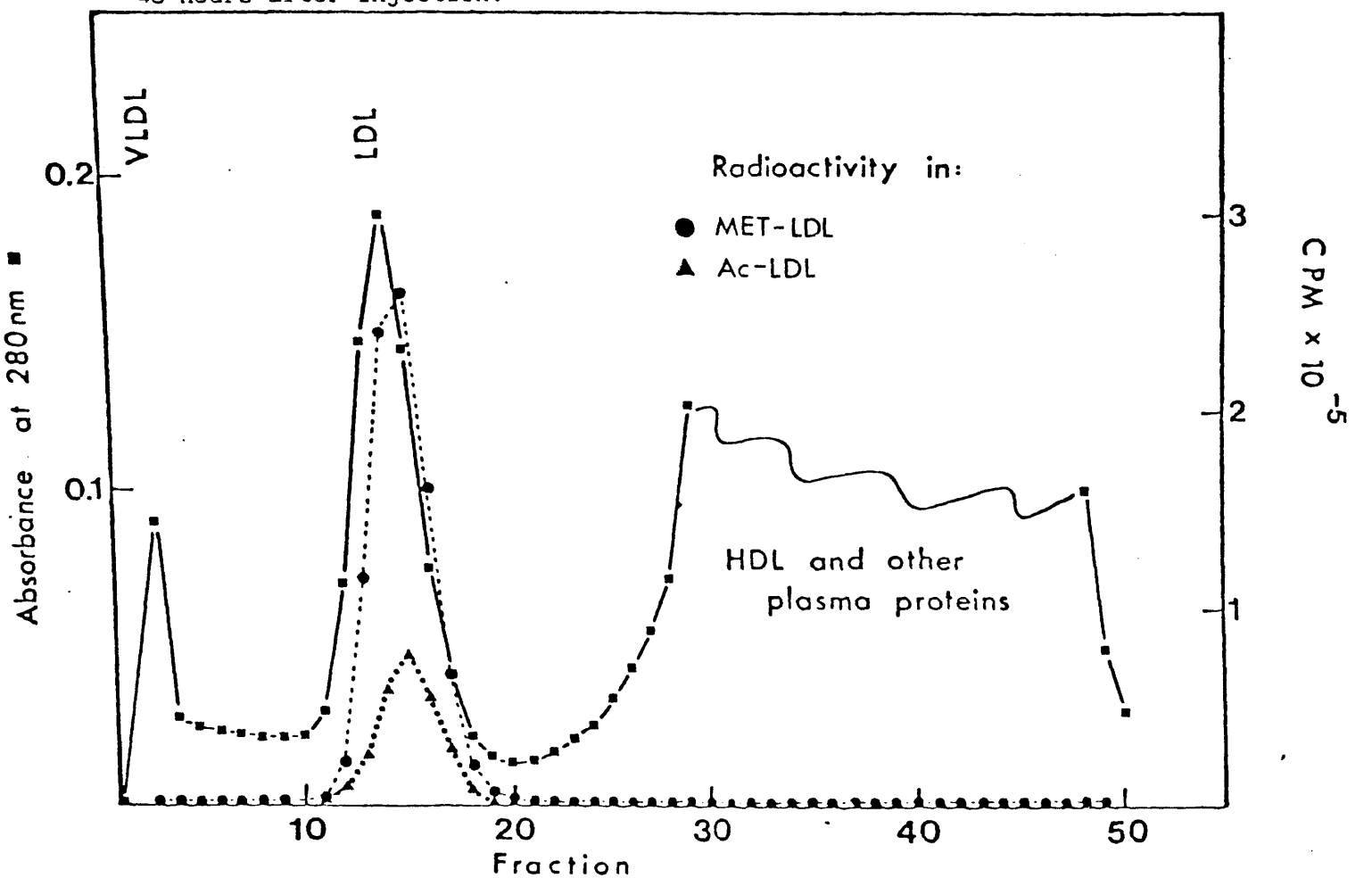
5.2: Electrophoresis of Lipoproteins in Agarose Gels.

Lipoprotein preparations were routinely checked for contamination by other lipoproteins and plasma proteins by agarose gel electrophoresis (118). Preformed gels were employed in a commercial kit system (Corning Eel). Protein and lipid bands were stained with Coomassie Blue (0.05% in 7.5% ethanoic acid) and Fat Red 7B (0.023% in absolute methanol) respectively.

This electrophoretic technique was also used to examine the relative electrophoretic mobilities of lipoproteins sampled from normal animals and those treated with drugs and to check the efficiency of the chemical modification of lipoproteins.

Fig.10 : Stability of lysine- and arginine-modified LDL in rabbits

I^{125} Met/LDL and I^{131} Ac/LDL were reisolated from plasma by zonal ultracentrifugation 48 hours after injection.



5.3: Protein Assay.

The protein concentration in preparations of lipoproteins was measured by the method of Lowry et al. (119) using human serum albumin (1.0 mg/ml) as a standard. ApoB concentration was measured using a modification of this method. The B apoprotein was first isolated by precipitation with an equal volume of pure, redistilled 1,1,3,3,-tetramethylurea (TMU). Lipid in the precipitate was extracted by washing with 5 volumes of chloroform/methanol (2:1 v/v) followed by 5 volumes of diethyl ether. The pellet of apoB was dissolved in 0.5 ml of 0.1 molar sodium hydroxide solution (120). This was then used to perform the Lowry assay.

5.4: Radiolabelling of Lipoproteins, Proteins and Lipids.

5.4.1: The Iodine Monochloride Method

LDL was iodinated with either ^{125}I or ^{131}I (Radiochemical Centre, Amersham, UK) by the method of Macfarlane (121). Radioiodo-LDL prepared by this method has previously been characterised (122). The procedure introduces an atom of iodine into the phenolic ring structure of a tyrosine residue by electrophilic addition. Conditions were adjusted to limit iodination to one mole of iodine per mole of apoprotein, the mass of which was assumed to be 64,000 daltons for LDL and 100,000 daltons for VLDL.

Sample Calculation:

conc. of lipoprotein apoprotein = 5 mg/ml

conc. of ICl solution = 25 nmoles/ml

Molecular weight of protein (LDL) = 64,000 daltons

ICl was used in a 2.5:1 molar ratio with protein.

no. of nmoles of protein per ml of LDL solution = $5.0 \times 1 \times 10^6 / 64{,}000$ = 78.1 nmoles

no. of nmoles of ICl required = 78.1x2.5 = 195.3 nmoles

amount of ICl solution required = 195.3/25 = 7.8 µl.

Therefore to label 1.0 ml of 5 mg/ml LDL solution requires 1.0 mCi of radioiodide and 7.8 µl of 25 nmoles/ml Iodine monochloride.

The product was purified by gel filtration through Sephadex G-25 (Pharmacia (GB) Ltd, London, UK) using buffer A as the eluant. Addition of an equal volume of 20% trichloroacetic acid (TCA) showed that 98% of the radioactivity was found in the precipitate. Lipid was shown to contain less than 5% of the radioactivity by solvent extraction with five volumes of chloroform/methanol (2:1 v/v) and five volumes of diethyl ether.

The iodine monochloride method was also used to iodinate albumin and denatured albumin (assumed molecular weight: 66,000 daltons).

The triglyceride triolein (Sigma Ltd, London, UK) was radioiodinated by a modification of the iodine monochloride method. 0.25 mCi of ^{125}I was added to 1.0 g of triolein and ICl added in a 1:1 molar ratio with the

triolein. The reaction mixture was allowed to stand at room temperature for 5 minutes after thorough mixing then washed repeatedly with 2 ml aliquots of buffer A. The buffer A was drained from beneath the oil layer until no radioactivity was detectable. The purity of the product was determined by thin layer chromatography using heptane/ethyl acetate (9.1 v/v) as the solvent. The single band of product was observed under ultra-violet light. Radiolabelling was confirmed by scraping the band from the plate and counting in a twin channel gamma counter (Packard Instruments, Dowers Grove, IL, USA). This machine was used routinely for the determination of the radioactivity of materials and samples.

5.4.2: Addition of Radiolabelled Cellobiose-Tyramine to Lipoproteins and Albumin.

Cellobiose Tyramine (CT) (ref. 101) was labelled with ^{125}I and ^{131}I by a modification of the iodine monochloride method (123). A 5.0 mmolar (2.4 mg/ml) solution of CT in water was prepared. 50 ul (250 nmoles) of this was mixed with 10 ul of 0.3 molar potassium dihydrogen phosphate (pH 4.5) and 1 mCi of ^{125}I or ^{131}I. 250 nmoles of iodine monochloride (25 nmoles/µl) was then added and the reaction allowed to proceed for 15 minutes at room temperature. After this time 500 ul of distilled water was added and the Iodo-CT purified by anion exchange on Sephadex QAE-A50 anion exchange resin (Pharmacia) using water as the eluant. The iodo CT was

collected in 3 to 4 ml and lyophilised then redissolved in 50 µl of water.

Iodo-CT was linked to the lysyl residue of a protein by a cyanuric chloride bridge (123). 10 ul of the radiolabelled CT solution was mixed with 20 ul of cyanuric chloride dissolved in acetone (2.5 mg/ml acetone), 15 ul of 8 mmolar sodium hydroxide and 10 ul of water. This was allowed to stand at room temperature for 15 seconds then 1.0 ml of 5 mg/ml protein solution (buffer A, pH 8.1) was added rapidly. The reaction mixture was left at room temperature for 3 hours and the protein isolated by gel filtration through Sephadex G-25 (Pharmacia) using buffer A (pH 8.1) as the eluant.

When used to label albumin this method yielded a product in which more than 95% of the radioactivity precipitated in 10% TCA. When used to label lipoproteins less than 5% of radioactivity remained in the solution in 10% TCA and less than 2% of the radioactivity was extracted with the lipid (section 5.4.1). Addition of iodo-CT to lipoproteins did not alter their metabolism (123).

5.5: Chemical Modification of Lipoproteins.

5.5.1: Reductive Methylation

Reductive methylation of lipoproteins was carried out by a modification (125) of the method of Means and Feeney (126) using formaldehyde (methanol) and sodium borohydride (Sigma Chemicals Ltd). The lipoprotein solution

(5 mg/ml) was cooled to 0°C in an ice bath. 0.5 ml of a freshly prepared 2 mg/ml solution of sodium borohydride in 0.2 molar borate buffer (pH 8.0) was added with constant stirring. 1.0 µl of formaldehyde was added at five minute intervals for 30 minutes and the mixture left for a further 30 minutes at 0°C. Methylated LDL (MET-LDL) was purified by gel filtration through Sephadex G-25 (Pharmacia) using buffer A as the eluant.

This procedure modifies the epsilon-amino group of lysine residues forming dimethyl lysine (Fig. 12). Other free amino groups such as the alpha-N-terminal group are presumably also modified. It has been reported (127) that this method modifies more than 75% of free amino groups.

Agarose gel electrophoresis of reductively methylated LDL revealed no change in the mobility of the lipoprotein relative to native LDL (Fig. 11) indicating that the net charge of the particle remained unchanged. This was due to the ability of the dimethyl epsilon amino group $-N(CH_3)_2$ to form the positive ion $-N^+(CH_3)_2H$ in a manner similar to that of the un-modified amino group.

5.5.2: Acetylation of Proteins.

Acetylation of albumin and lipoproteins was achieved by the reaction of acetic anhydride with lysyl residues (128) (Fig. 13). 1.0 ml of a 5 mg/ml solution of protein or lipoprotein was diluted by the addition of an equal volume of saturated sodium acetate. This solution was

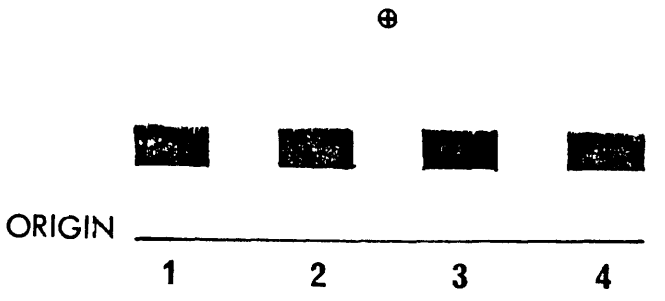


FIGURE 11: Agarose Gel Electrophoresis: comparison of the mobilities of native LDL and reductively methylated LDL (MET-LDL).

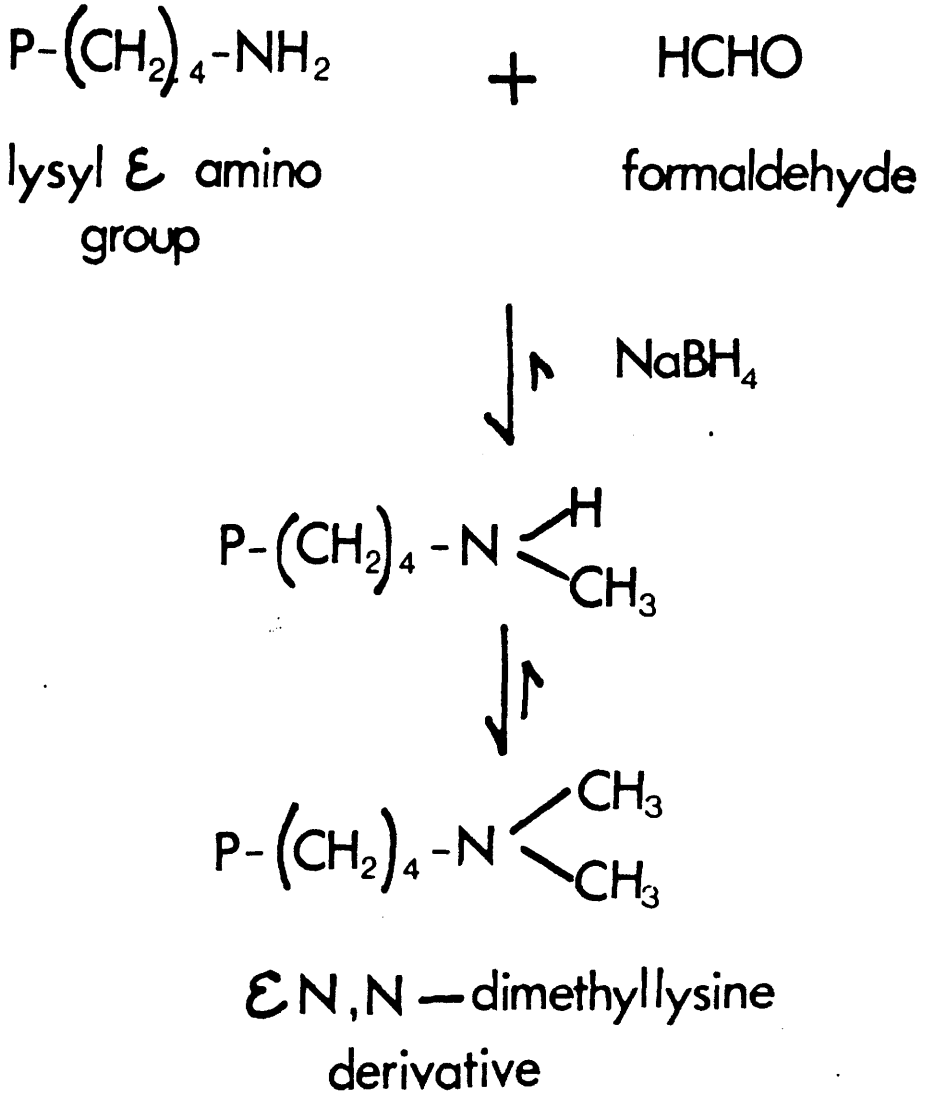


Fig. 12: Reductive methylation of LDL.

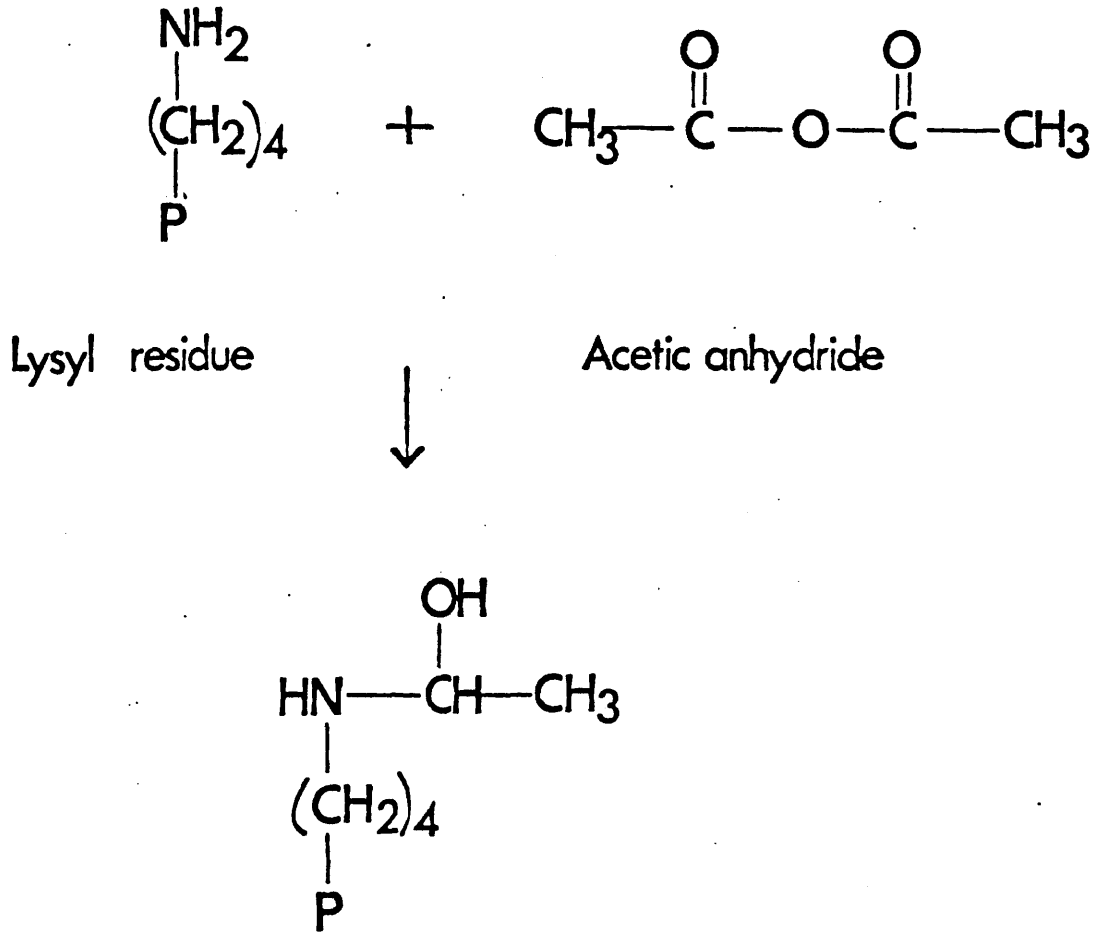


Fig 13 Acetylation of LDL

cooled to 0°C in an ice bath and 1.0 ul of acetic anhydride (Sigma Ltd) added at five minute intervals for 60 minutes with constant stirring. The reaction mixture was left for a further 30 minutes at 0°C and the acetylated protein (Ac-LDL or Ac-BSA) purified by gel filtration in Sephadex G-25 (Pharmacia) and dialysis in buffer A.

This procedure increases the net negative charge on the protein as shown by its enhanced anodic mobility in agarose gel electrophoresis (Fig. 14). This was due to the inability of the acetylated lysine residue ($-NHCHOHCH_3$) to form a positive ion.

5.6: Quantitation of LDL Apoprotein B.

LDL apoprotein B in rabbit plasma was measured by the immunoelectrophoretic method of Laurell (129). The electrophoresis was carried out on a one percent agarose slab gel formed on a glass slide. A two percent agarose preparation was melted and diluted with an equal volume of 0.1 molar barbitone buffer at pH 8.6 and antiserum raised to rabbit apoB in Guinea Pigs was added to a final concentration of two percent. Plasma was diluted 1:10,000 with 0.05 molar barbitone buffer (pH 8.6) and applied to wells cut in the cathode side of the gel. Following electrophoresis at 250 volts and 15°C for 16 hours the plate was washed in saline for a minimum of 3 hours to remove the serum proteins in the gel and stained with one percent amido black in ten percent acetic acid

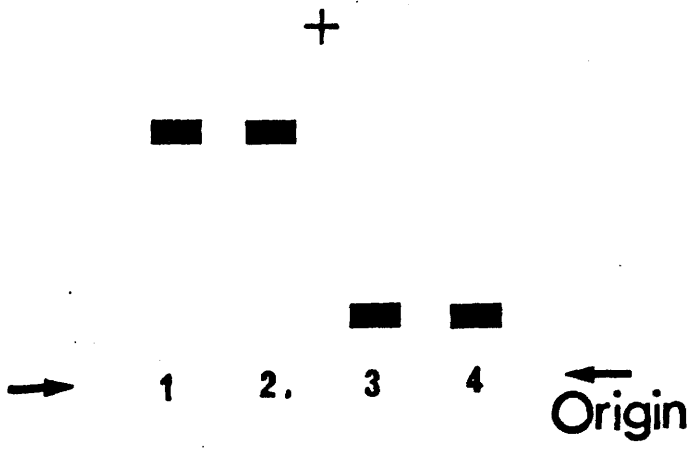


Fig. 14: Agarose gel electrophoresis.

Lane 1 Acetylated LDL

2 Acetylated LDL

3 Native LDL

4 Native LDL

solution. The plate was destained in methanol/acetic acid/water (50:10:40: v/v). The height of the peaks was measured and compared to a standard of known apoB concentration run on the same plate.

5.7: Quantitation of Plasma and Lipoprotein Cholesterol and Triglyceride.

Cholesterol and triglyceride were measured by standard methods (129) in either whole plasma or lipoprotein fractions using commercially available kits (Boehringer Corp.: Cholesterol Kit 236691, Triglyceride Kit 166448). A standard cholesterol was obtained from BDH Ltd, and triglyceride (triolein) from Sigma Chemicals Ltd.

Cholesterol and triglyceride contents of lipoprotein fractions in plasma were obtained as follows: VLDL and LDL were precipitated with heparin/manganese chloride (130) leaving HDL in solution. The lipids in the total plasma were assayed and the lipid concentrations in the HDL supernatant subtracted to give VLDL and LDL lipid values.

5.8: Conjugation of Muramyl Dipeptide to Acetylated BSA.

Muramyl dipeptide (MDP) (Fig. 15A) (kindly provided by Syntex Ltd, Palo Alto, California, USA) was conjugated to albumin, which had previously been acetylated by the method outlined in section 5.5.3, through a carbodiimide link. 5.0 ml of 5 mg/ml acetylated albumin was dialysed

A:

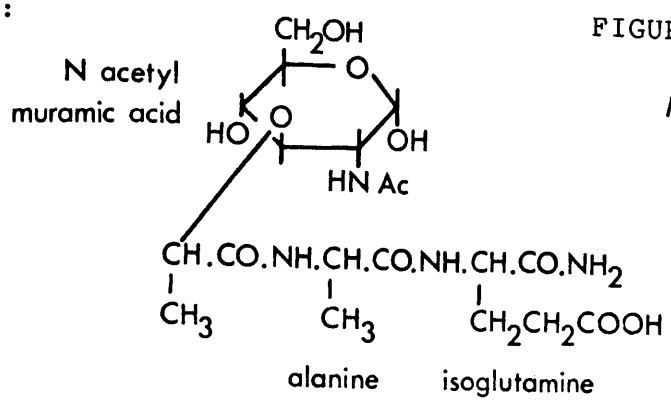
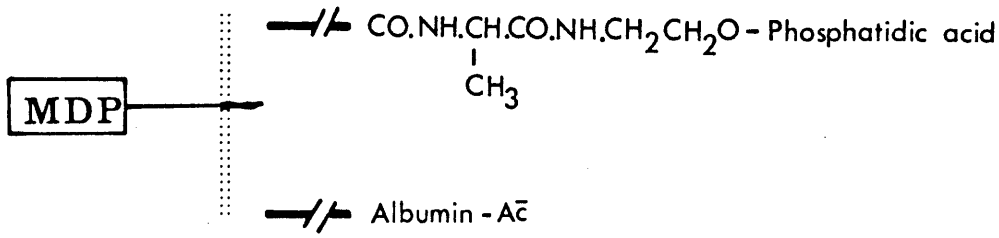


FIGURE 15:

Muramyl dipeptide

B:

Muramyl dipeptide complexes



Muramyl Dipeptide and complexes.

Targeting to the reticuloendothelial system by incorporation via the Phosphatidic acid tail of MTP or conjugation to acetylated albumin.

exhaustively against 0.1 molar phosphate buffered saline at pH 5.5 (Buffer B). 30.0 mg of 1-ethyl-3-(-3-dimethyl aminopropyl)-carbodiimide hydrochloride (Sigma Chemicals Ltd) was dissolved in 500 ul of buffer B and added to the Ac-BSA solution with constant stirring and left for 5 seconds. This was followed by the addition of 20 mg of MDP also dissolved in 500 ul buffer B. The reaction mixture was covered and left at room temperature for 5 hours. After this time it was dialysed against buffer A. The conjugate (Fig. 15B) was stored at -20°C in 1.0 ml aliquots to avoid continuous freezing and thawing. MDP-Ac-BSA was given to experimental animals intravenously at 1.39 mg of Ac-BSA/kg body weight (1.11 mg of MDP/kg body weight).

5.9: Preparation of Ethyl Oleate Emulsion.

Oleic acid ethyl ester (ethyl oleate) (Sigma Chemicals Ltd) was injected intravenously into experimental animals in the form of a stabilised emulsion in saline. 1.0 g of ethyl oleate was measured into a sterile glass tube. 35 ul of Tween 20 detergent (Koch-Light Laboratories Ltd, Bucks, UK) was added with mixing, followed by 4 ml of sterile saline. This was then sonicated in an MSE P6.1199 sonicator for 5 minutes in an ice bath. The emulsion was prepared immediately before use and given in a dose of 0.5 g/kg body weight. Tween 20 detergent had previously been shown to have no effect on the animals in the amounts used in the preparation of the emulsion (102).

5.10: Preparation of MTP-Triolein Emulsions.

Muramyl tripeptide phospholipid (MTP) (Fig. 15B) is a derivative of MDP (section 5.8) in which a phospholipid moiety is linked to the isoglutamine residue of MDP through a second alanine residue. This modification allowed the MTP to be incorporated into hydrophobic liposomes constructed from the triglyceride triolein (Sigma Chemicals Ltd).

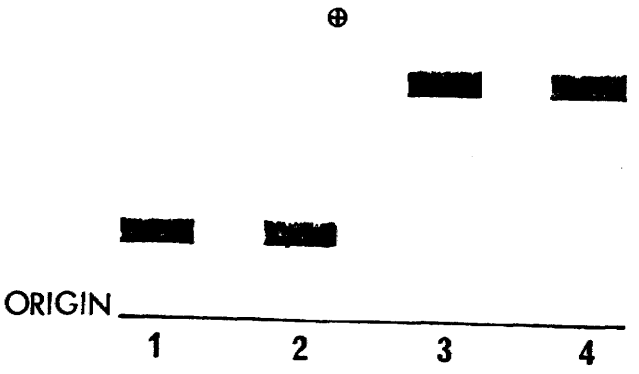
0.25 g of triolein was added to 3 mg of MTP followed by 1.0 ml of saline. 9 µl of Tween 20 (Koch-Light Laboratories Ltd) was added and the mixture sonicated for 5 minutes in an ice bath. The emulsion was injected intravenously at the dose of 1 mg MTP/kg body weight immediately after preparation.

5.11: Denaturation of Albumin.

Albumin was dissolved in 0.05M bicarbonate buffered saline at pH 10.0 (Buffer C) to a concentration of 10 mg/ml. 1.0 ml of this solution was diluted to 10 ml with a 4.4% formaldehyde solution in buffer C yielding a final solution of 1 mg/ml albumin and 4 percent formaldehyde in buffer C. This was left at 4°C for 3 days then dialysed exhaustively against buffer A.

Agarose gel electrophoresis (section 5.2) revealed that the albumin treated in this way had a greater overall negative charge than native albumin (Fig. 16). The formaldehyde denatured albumin was shown

FIGURE 16:



LANE 1. Native albumin
2. Native albumin
3. Formaldehyde denatured albumin
4. Formaldehyde denatured albumin

Agarose gel electrophoresis:

Comparison of the mobilities of native and formaldehyde denatured albumin.

to be an ideal marker of *in vitro* macrophage activity, being rapidly degraded by cultures of the cells.

5.12: Measurement of Macrophage System Activity *In Vivo*.

Macrophage system function was measured *in vivo* using intravenously injected colloidal carbon (100 mg/kg body weight) by standard methods (131). After injection the concentration of colloidal carbon in the plasma was measured in serial blood samples taken over a period of 30-60 minutes by light transmission at 700 nm. The phagocytic index K, defined as the slope of the graph plotted as logarithm of optical density of the plasma at 700 nm versus time, was determined for the animal in the control state and after it had received macrophage system modulating agents. To ensure that the colloidal carbon was not eliciting any effects, K was redetermined two weeks after treatment of the animal with the drugs and compared to K calculated for the animal in the control state.

5.13: Cell Cultures.

5.13.1: *Human Skin Fibroblasts*.

Normal human skin fibroblasts were obtained from Flow Laboratories (Irvine, Scotland, UK). The cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air in Basal Eagles Medium (BEM) (Flow Laboratories). The

BEM was supplemented with 10% new born calf serum, 2 mmolar glutamine, 1% non-essential amino acids (all from Flow Laboratories), 1.68 g/l sodium hydrogen carbonate, 100 units/ml penicillin and 100 mg/ml streptomycin. This complete medium was denoted medium A. The cultures were grown in Nuclon 50 ml plastic flasks (Gibco Biocult, Ltd, Paisley, Scotland, UK) and passaged into fresh medium A when confluent by dissociation with 0.5% trypsin/0.02% disodium EDTA (Gibco Biocult Ltd).

5.13.2: Macrophages.

Peritoneal macrophages were harvested from mice using standard techniques (132). The animals were killed in ether and the skin over the abdomen removed. Ten millilitres of phosphate buffered saline at pH 7.4 was injected rapidly into the peritoneal cavity through a large gauge needle. The needle was oriented so that the bevel faced down and the abdominal wall was elevated to form a tent structure. The fluid, containing the cells, was withdrawn slowly by sterile syringe and transferred to centrifuge tubes. It was centrifuged at 150 g for 8 minutes and resuspended in medium A. After five hours incubation at 37°C the medium was replaced by fresh medium A to ensure minimum contamination by other cell types.

A transformed line of mouse macrophages (J774A) (133) was obtained as a gift from Dr David Hart,

Department of Zoology, University of Glasgow, UK. These cells were maintained in medium A.

5.13.3: Cryogenic Storage of Cell Lines.

Both fibroblasts and macrophages were stored long term in liquid nitrogen (Cryoservice Ltd, Hamilton, Scotland). The cells were dissociated from the growth flasks just before reaching confluence and centrifuged at 150 g for 8 minutes at 20°C. They were resuspended in new born calf serum containing 10% dimethyl sulphoxide (DMSO) (5 ml per flask of cells) and placed, in 1.0 ml batches, in sterile ampoules suitable for storage in liquid nitrogen (Gibco Biocult Ltd). The ampoules were sealed, placed in an insulated container and cooled slowly (1°C/minute) to a temperature of -60°C. They were then transferred to a liquid nitrogen freezer and kept in the liquid phase.

Thawing was carried out rapidly in warm water (40°C) and the liquid immediately poured into 10 ml of medium A (per ampoule). This was centrifuged at 150 g for 8 minutes and the cells resuspended in 5 ml of medium A. The cells were placed in a growing flask and incubated at 37°C for 24 hours at which time the medium was replaced to remove any dead cells which may have been present.

5.14: Binding and Degradation of Lipoprotein In Vivo.

5.14.1: In Vitro Receptor Assay.

All assays of receptor activity in cultured cells were carried out after incubation for 48-72 hours, prior to the assay, in medium B. Medium B was identical to medium A except that it contained lipoprotein deficient serum (LDS) instead of complete serum. LDS was prepared from fresh human serum by ultracentrifugal flotation through a solution of density 1.25 kg/l at 45,000 rpm and 15°c for 48 hours using a Beckman Ti60 rotor in an L565 ultracentrifuge (133).

Excess salt was removed by exhaustive dialysis against buffer A at pH 7.0 and the protein content was adjusted to 50 mg/ml. The final preparation was sterilised by membrane filtration through 0.45 u and 0.22 u filters in series (Millipore Corp, Bedford, MA, US).

Incubation of fibroblasts for 48-72 hours in medium B has been shown to stimulate LDL receptor activity (135).

5.14.2: Measurement of Receptor Binding.

Goldstein et al. (91) described the procedure for the measurement of lipoprotein binding to the LDL receptor. It was found, however, that, especially with macrophages, the cell tended to dissociate from the wall of the

culture flask if incubated at 4°C. A modification of the method whereby cells were incubated at room temperature was employed to overcome this problem.

Cultures were equilibrated at room temperature for 30 minutes after an incubation of 48-72 hours at 37°C in medium B. After this equilibration period, medium B was removed from the cultures and replaced with medium B containing 100 µg/ml of radioiodinated lipoprotein at room temperature. This was left for a further two hours at room temperature, the medium removed and the cells washed six times with 5 ml aliquots of 0.1 molar phosphate buffered saline at pH 7.4 containing 2 mg/ml BSA. The monolayer of cells was then dissolved in 2.0 ml of 0.1 molar sodium hydroxide which was aliquoted for determination of radioactivity and protein.

5.14.3: Measurement of Lipoprotein Degradation.

Lipoprotein degradation in tissue culture was quantified by the measurement of the amount of radioactivity which was not precipitated by trichloroacetic acid (TCA) or extracted as iodine into chloroform ie that present as iodotyrosine, the radioactive break-down product of the protein moiety.

After incubation of 37°C with medium B for 48-72 hours medium B containing 100 to 200 µg/ml (1x106 cpm/ml) of radioactive lipoprotein was added to the cultures. TCA was added to the culture tubes to a final concentration of 25% at selected time intervals up

to 8 hours. The precipitate was removed by low speed centrifugation and the supernatant supplemented with non-radioactive potassium iodide, to a final concentration of 0.4%, to act as a carrier for radioactive iodide. This was oxidised to iodine by the addition of 1 part hydrogen peroxide (30 volume) to 10 parts supernatant and extracted into twice the volume of chloroform. The aqueous layer was then removed and the radioactivity present as ^{125}I or ^{131}I-tyrosine determined. Protein content of the culture tubes was measured in duplicate control cultures which received only medium B with the same amount of unlabelled lipoprotein.

5.14.4: Treatment of Cell Cultures with MDP-Ac-BSA.

Cultures received MDP-Ac-BSA 18 hours before the start of the experiment. The drug was delivered in medium C which was identical to medium B except that it contained two percent LDS and 0 to 500 µg/ml as measured for protein (0-400 µg MDP) MDP-Ac-BSA. In preliminary experiments medium C contained no LDS to prevent degradation of MD-Ac-BSA but it was found that control cultures failed to thrive in this medium. Further experimentation showed that for periods of 24-48 hours medium C supplemented with 2% LDS provided a suitable medium for growth. Control cultures for these experiments received medium C containing no MDP-Ac-BSA for the same time as their experimental counterparts.

5.15: Animals and Diets.

5.15.1: New Zealand White Rabbits.

All studies undertaken in rabbits were carried out using male New Zealand White Rabbits (Cheshire Rabbit Farms, Cheshire, UK) at the age of 4-6 months (3-3.5 kg weight). These animals were maintained on a standard rabbit diet (B Special Diet Services, Witha, Essex, UK) ad libitum, unless otherwise indicated.

5.15.2: Dunkin-Hartley Guinea Pigs.

Antibodies to rabbit apoB were raised in the Dunkin-Hartley strain of Guinea Pigs (Bantin and Kingman, Hull, UK). The animals were maintained on Guinea Pig Maintenance Diet (BP Special Diet Services) supplemented with hay (Finest Meadow Hay, Darleith Farm, Cardross, Scotland).

5.15.3: BALB/C Mice.

All mice used were of the BALB/C strain. They were maintained on Rat and Mouse Maintenance Diet (BP Special Diet Services) ad libitum. The mice were bred in the animal unit under the expert eye of Mr Stuart Saigeman.

5.16: Protocol for In Vivo Turnover Studies.

5.16.1: Injection and Blood Sampling.

Before any injection of radioiodolipoproteins the rabbits were given potassium iodide in the drinking water

(0.1 g/l), two days prior to and throughout each study, to prevent uptake of the radioiodine by the thyroid.

Lipoproteins, isolated from fresh plasma within 48 hours, were labelled with ^{125}I or ^{131}I (section 5.4.1), sterilised by ultrafiltration and injected into the marginal ear vein (10 μCi of ^{125}I, approximately $1x10^7$cpm:18 μCi of ^{131}I, approximately $1x10^7$cpm with a total of 400 μg of protein).

Blood samples were taken, using disodium EDTA as the anticoagulant, from the marginal vein of the other ear at 10 minutes from injection and subsequently at regular intervals over the next 47 hours. The ten minute sample provided an estimate of plasma volume and the zero time point. Radioactivity in each sample was determined in a twin channel gamma spectrometer (Packard Instruments). Plasma decay curves were calculated as a percentage of the 10 minute sample and plotted against time on three cycle semi-log graph paper. No reisolation of LDL from the plasma was necessary since non-protein bound radioactivity in the plasma was less than one percent of the total and apoB does not transfer to other lipoproteins (136) (Fig. 10).

15.16.2: Measurement of Tissue Uptake of Radiolabelled Lipoproteins.

The tissue distribution of each isotope was measured immediately after the final blood sample was taken. In the case of ^{125}I or ^{131}I labelled lipoproteins this

occurred 36-48 hours after injection; in the case of ^{125}I-CT and ^{131}I-CT labelled lipoproteins at 48-60 hours after injection. The rabbits were anaesthetised with an injection of pentabarbitone sodium (30 mg/kg) (Sagital: May and Baker Ltd, Dagenham, UK) followed by an intravenous injection of 500 units/kg of sodium heparin (Weddel Pharmaceuticals Ltd, London, UK) to prevent intravascular coagulation. Exanguination was performed 5 minutes later by cardiac puncture which removed more than 80% of the total blood volume. The organs were dissected out, washed in ice-cold 0.15 molar sodium chloride solution, blotted dry and weighed. Samples of fat, gut and muscle were removed, cleaned and dried.

Accurately weighed, approximately one gram pieces of tissue were taken in quadruplicate and the amount of radioactivity present in each determined. This was corrected to give the amount of radioactivity present per gram of tissue and expressed relative to the radioactivity measured in 1.0 ml of plasma at the time of sacrifice. The results were plotted as a bar graph showing (cpm/g tissue)/(cpm/ml plasma) for each tissue. Total organ uptake was calculated and plotted as above for each weighed organ.

In previous studies each tissue sample was homogenised, precipitated with TCA (final concentration 10%) and radioactivity determined in the washed pellet. This should reflect the tissue content of intact or high molecular weight break down products of LDL. Since at

least 70-90% of radioactivity in the tissue was precipitated and these portions were unaffected by modification of the lipoprotein or by diet, the tissue radioactivities were measured directly.

Tissue uptake of ^{125}I-CT and ^{131}I-CT labelled lipoproteins were determined in the manner outlined above.

5.16.3: Reisolation of LDL.

Radiolabelled LDL (section 5.4.1) was screened in vivo by injecting the lipoprotein into a rabbit. Each isotope (ie ^{125}I and ^{131}I) was injected into a separate animal. The plasma decay of the lipoprotein was followed (section 5.16.1) until the level of radioactivity was approximately ten percent of the initial ten minute sample. The rabbits were then anaesthetised with an injection of sodium pentabarbitone (30 mg/kg) (May and Baker Ltd), given an intravenous injection of 500 units/kg of sodium heparin (Weddel Pharmaceuticals Ltd) and exanguinated by cardiac puncture. VLDL was removed from the plasma by the method given in section 5.1.2(1). The density of the plasma was adjusted to 1.063 kg/l with sodium bromide and overlayered with density 1.063 kg/l sodium bromide solution. This was centrifuged in a Beckman Ti60 rotor using an L565 ultracentrifuge for 24 hours at 49,000 rpm and 18°C. The LDL was dialysed against buffer A to remove excess salt.

Due to the loss of approximately 90% of the radioactivity the initial injection contained between $5x10^7$ and $1x10^8$ cpm and 500 ug of protein. This was achieved by increasing the specific activity of the lipoprotein in the labelling stage. The amount of iodine monochloride used was increased 1.5 fold as was the number of mCi of radioiodine.

5.17: Separation of LDL Subfractions by Continuous Gradient Ultracentrifugation.

LDL from normal rabbits and rabbits treated with ethyl oleate (section 5.9) was isolated by the following method. Plasma from the rabbits was adjusted to a density of 1.019 kg/l by the addition of sodium bromide and overlayered with an equal volume of 1.019 kg/l density solution in a Beckman Ti60 rotor. This was subjected to ultracentrifugation in a Beckman L5 65 ultracentrifuge at 45,000 rpm and 18°C for 18 hours. The VLDL and IDL were removed, the infranatant adjusted to 1.070 kg/l with sodium bromide, overlayered with 1.070 kg/l density solution and subjected to ultracentrifugation as described above. The supernatant LDL was removed and washed in a pressure ultrafiltration unit (amicon) using 1.040 kg/l density solution until the washings had a refractive index of 1.3402, equivalent to a density of 1.040 kg/l for sodium bromide solution. The LDL was then labelled by the ICl method (section 5.4.1)

and exhaustively dialysed against a 1.040 kg/l density solution containing 0.03% disodium EDTA at pH 7.4.

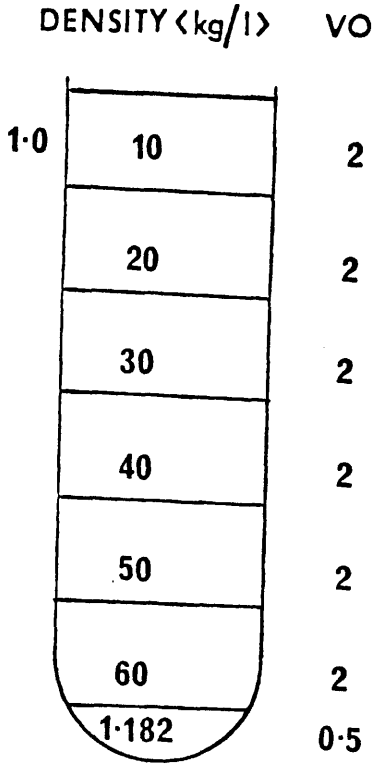
A Beckman SW40 rotor was prepared with a stepped gradient running from 1.060 kg/l to 1.010 kg/l in 0.010 kg/l increments (Fig. 17A), the sample, at 1.040 kg/l, being placed in the centre of the tube. After 18 or 24 hours ultracentrifugation at 39,000 rpm and 19°C in a Beckman L565 ultracentrifuge this step wise gradient formed a continuous gradient, as measured by refractive index (Fig. 17B). Aliquots of 0.5 ml were removed from the top of the gradient and the amount of radioactivity present in each determined.

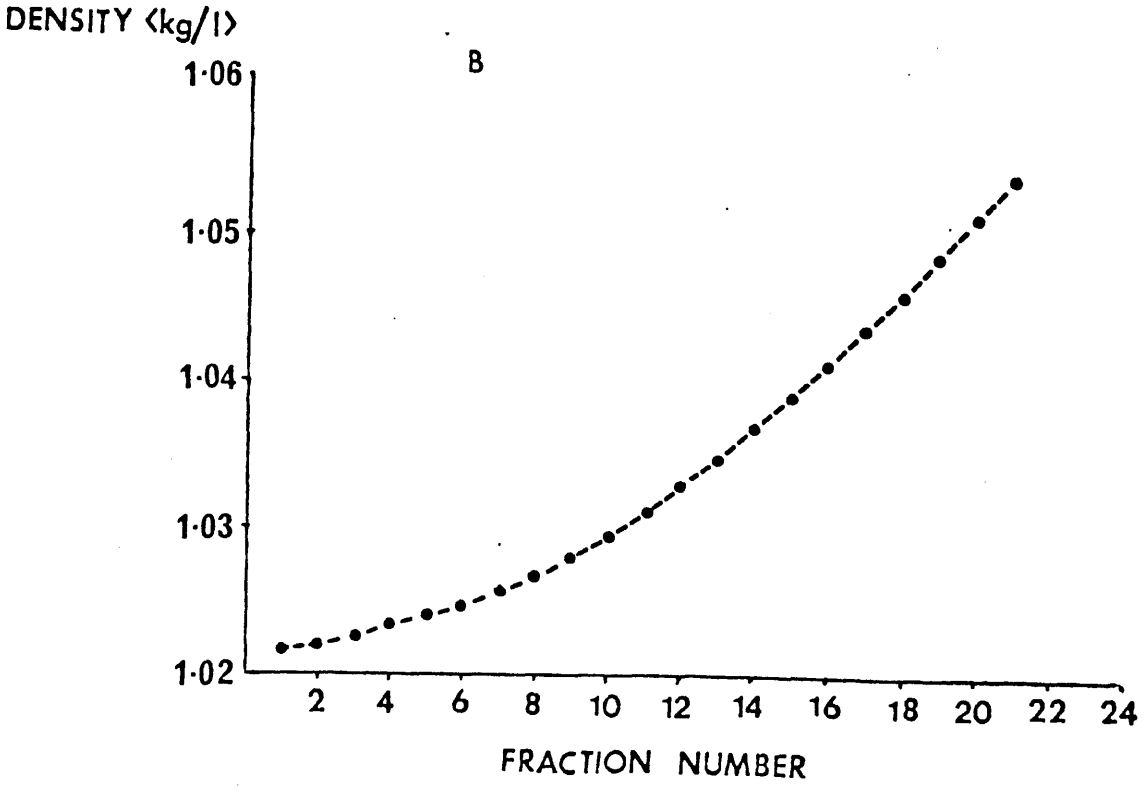
5.18: Separation of LDL Subfractions by Anion Exchange Column Chromatography.

DEAE Sepharose Anion Exchange Resin (CL6B; Pharmacia Fine Chemicals AB, Uppsala, Sweden) was poured to a depth of 8.5 cm in a 1 cm internal diameter column (Pharmacia Fine Chemical AB). This was topped with 2 cm of Sephadex Coarse G-25 (Pharmacia Fine Chemicals AB) and equilibrated for 24 hours with 0.025 M tris buffer at pH 8.0. One millilitre of 0.5 mg/ml LDL in 0.025 M tris buffer at pH 8.0 was layered onto the column and washed in with 10 ml of tris buffer at pH 8.0.

The gradient of increasing sodium chloride concentration was created by a gradient mixer (Pharmacia Fine Chemicals AB, model GM-1) and pumped onto the column at 0.4 ml/minute by a peristaltic pump (LKB Broma

FIGURE 17:





A: Stepwise gradient used to separate species of LDL by density. The radiolabelled LDL was adjusted to d = 1.040 and layered into the gradient.

B: Result of ultracentrifugation of stepwise gradient.

Multiperpex Peristaltic Pump). Eluant was examined for protein content by ultra violet spectrophotometry (ISCO type 6 optical unit) which was recorded on a potentiometric chart recorder. The conductivities of the fractions collected were determined by conductivity meter (Radiometer, Copenhagen, type CDM 2f) and plotted onto the chart recorder trace.

6: RESULTS

6.1.: Target specialty of drug delivery vehicles.

6.1.1.: Lipid Vesicles.

^{125}I-labelled glycerol trioleate (^{125}I-triolein) vesicles were injected into the marginal ear vein of New Zealand white rabbits (NZW rabbits). The plasma clearance rate was determined by serial blood sampling over a 30 minute period (Fig. R1) and expressed as the percent of the zero time radioactivity which remained in the plasma at time of sampling. The zero time radioactivity was calculated from the total amount of radioactivity injected and the plasma volume of the animal. After 30 minutes the animal was anaesthetised, exsanguinated and the tissue uptake of radioactivity determined. Tissue uptake was expressed in two forms; first the relative uptake per gram of tissue (Fig. R2) and second the total uptake by the discrete organ (Fig. R3). Although relative uptake per gram revealed that the spleen was extremely active, total organ uptake showed that liver, due to its size and activity, was responsible for the removal of the majority of ^{125}I-triolein vesicles (39% of the injected dose).

6.1.2.: Acetylated Bovine Serum Albumin (Ac-BSA).

Ac-BSA was labelled with ^{125}Iodine and treated with carbodiimide under the same conditions used for the conjugation of Muramyl Dipeptide (MDP) (section 5.8).

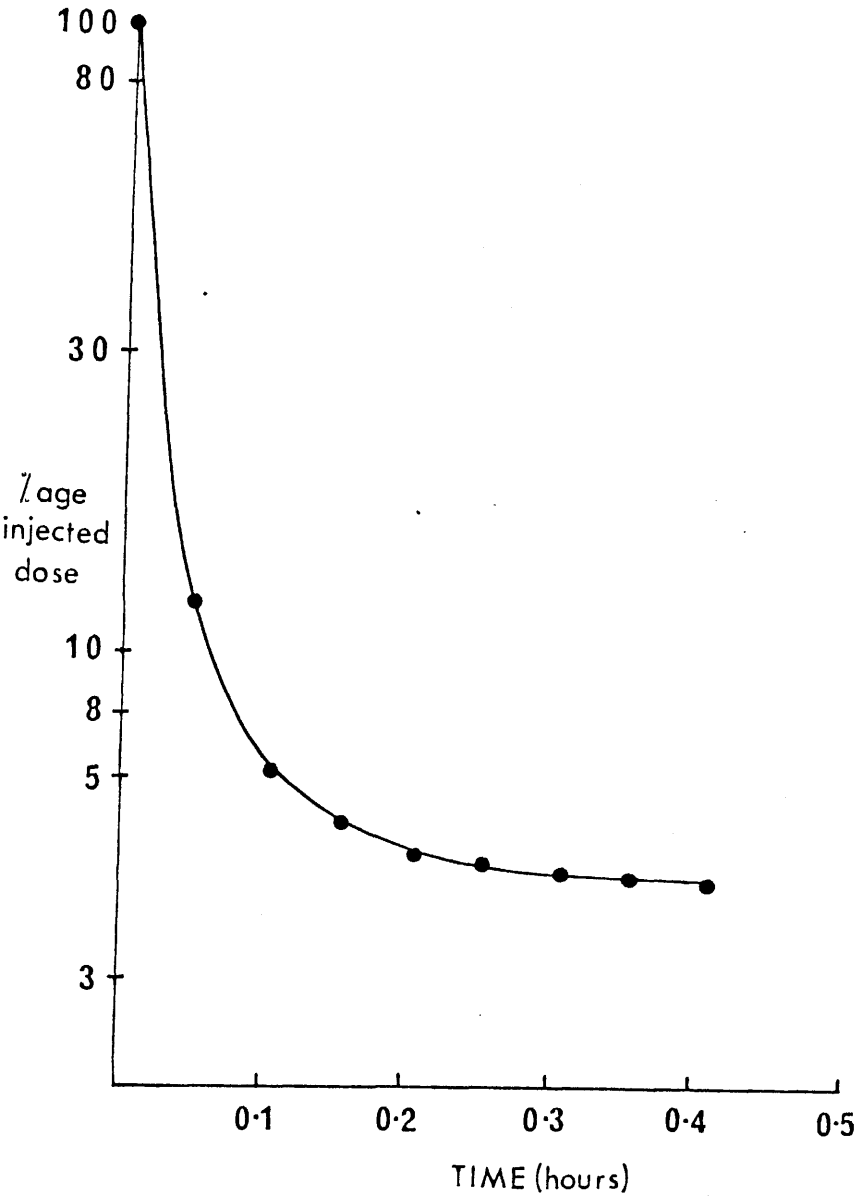


FIGURE R.1: Plasma Clearance of ^{125}I-Triolein Vesicles.

Radioiodinated triolein vesicles were injected into the marginal ear vein of rabbits and the plasma clearance rate determined (n = 2).

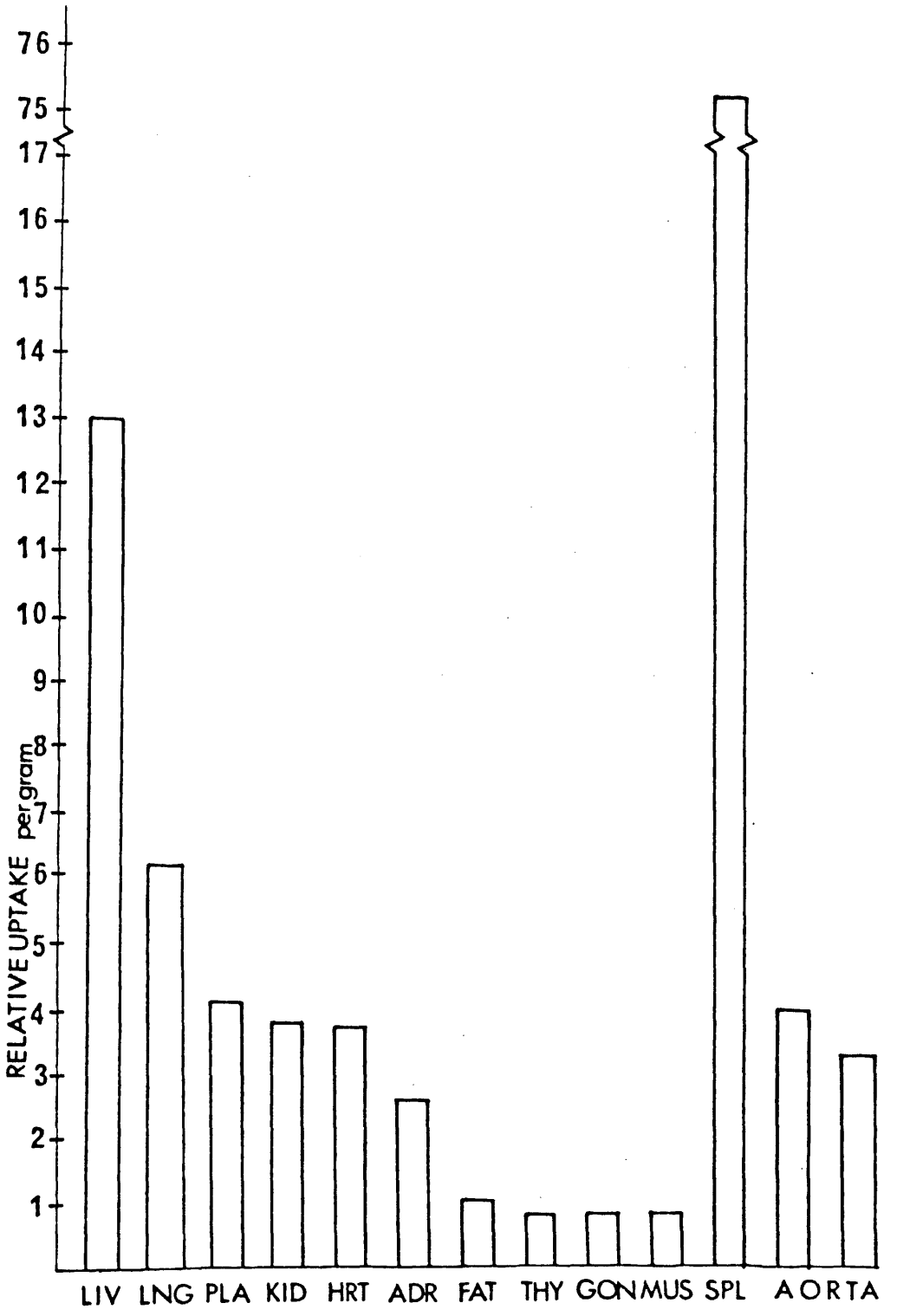


FIGURE R2: Relative Tissue Uptake of ^{125}I-Triolein Vesicles.

Relative tissue uptake of ^{125}I-triolein into the tissues of NZW rabbits was determined 30 minutes after injection of the tracer.

$$\text{Relative Tissue Uptake} = \frac{\text{cpm/gram of tissue}}{\text{cpm/ml of plasma}}$$

(n = 2)

Contractions:- LIV, liver; LNG, lung; PLA, plasma; KID, kidney; HRT, heart; ADR, adrenal; FAT, fat; THY, thymus; GON, gonads; MUS, muscle; SPL, spleen; AORTA, aorta.

The aorta was divided into two sections corresponding to, first, the arch and upper thoracic regions and second the lower thoracic and upper abdominal regions.

These contractions will continue to be used unless otherwise stated.

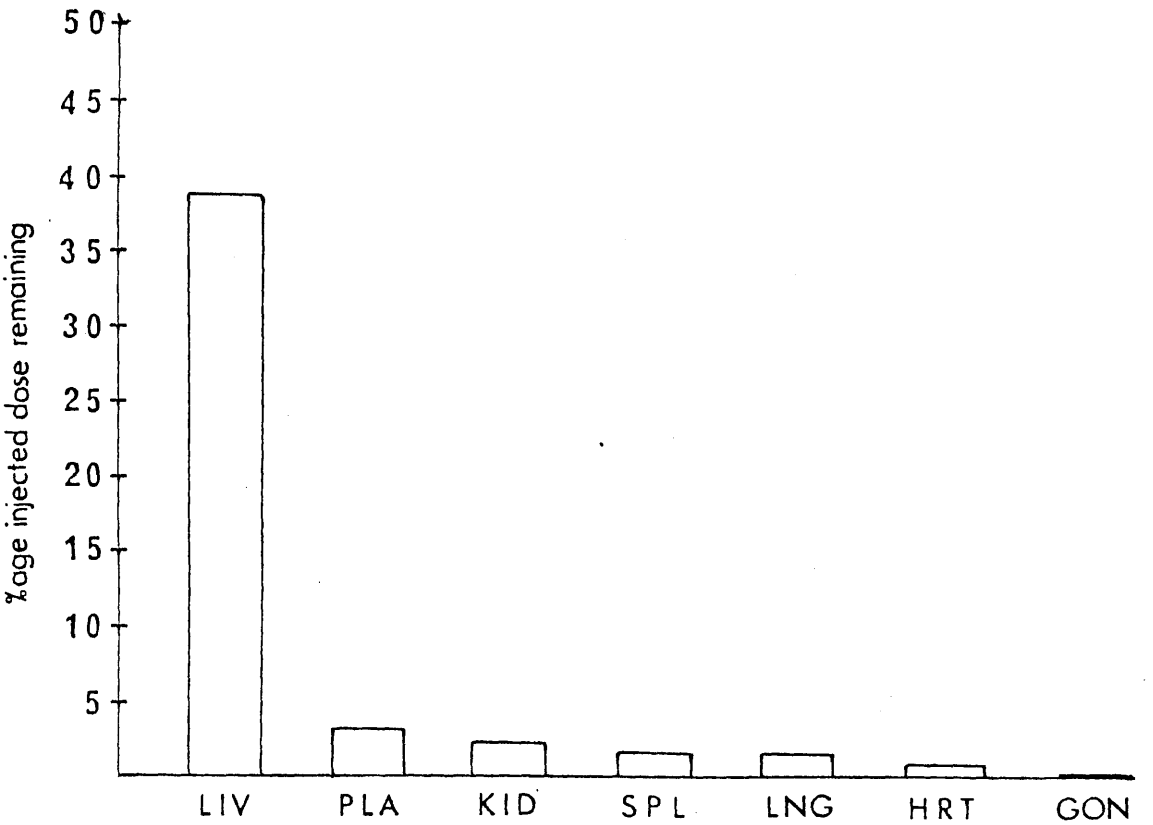


FIGURE R3: Total Organ Uptake of ^{125}I-Triolein Vesicles.

Total organ uptake was calculated from cpm/gram of tissue.

$$\text{Total Organ Uptake} = \frac{\text{cpm/gram of tissue x organ weight}}{\text{cpm in injected dose}} \times 100\%$$

(n = 2)

This was then injected into the marginal ear vein of NZW rabbits. Thirty minutes after administration the rabbits were anaesthetised, exsanguinated and the tissue uptake of radiolabelled tracer determined. The tissue uptakes were expressed as described above (section 6.1.1.). Relative uptake per gram of tissue showed that the liver was as active in the uptake of Ac-BSA as triolein vesicles (Fig. R4), but that the spleen, although the second most active tissue, was less than 10% as efficient in catabolism of Ac-BSA. Whole organ uptake (Fig. R5) revealed that the liver, as with triolein vesicles, removed 40% of the tracer, although approximately twice as much remained in the plasma after 30 minutes.

It is important to note that the amount of radioactivity found in the tissues was that at the instant of death and was not cumulative, ie did not include low molecular weight catabolic products which were excreted back into the plasma and removed by the kidney. However, the figures quoted gave realistic representations of relative uptakes by the tissues.

6.2.: Effects of reticuloendothelial system suppressants on plasma lipids.

6.2.1.: Ethyl oleate emulsion.

NZW rabbits received three serial injections of ethyl oleate emulsion (0.5 g ethyl oleate/kg body weight) at 48 hour intervals to produce sustained suppression of reticuloendothelial (RE) activity (Fig. R6). Following

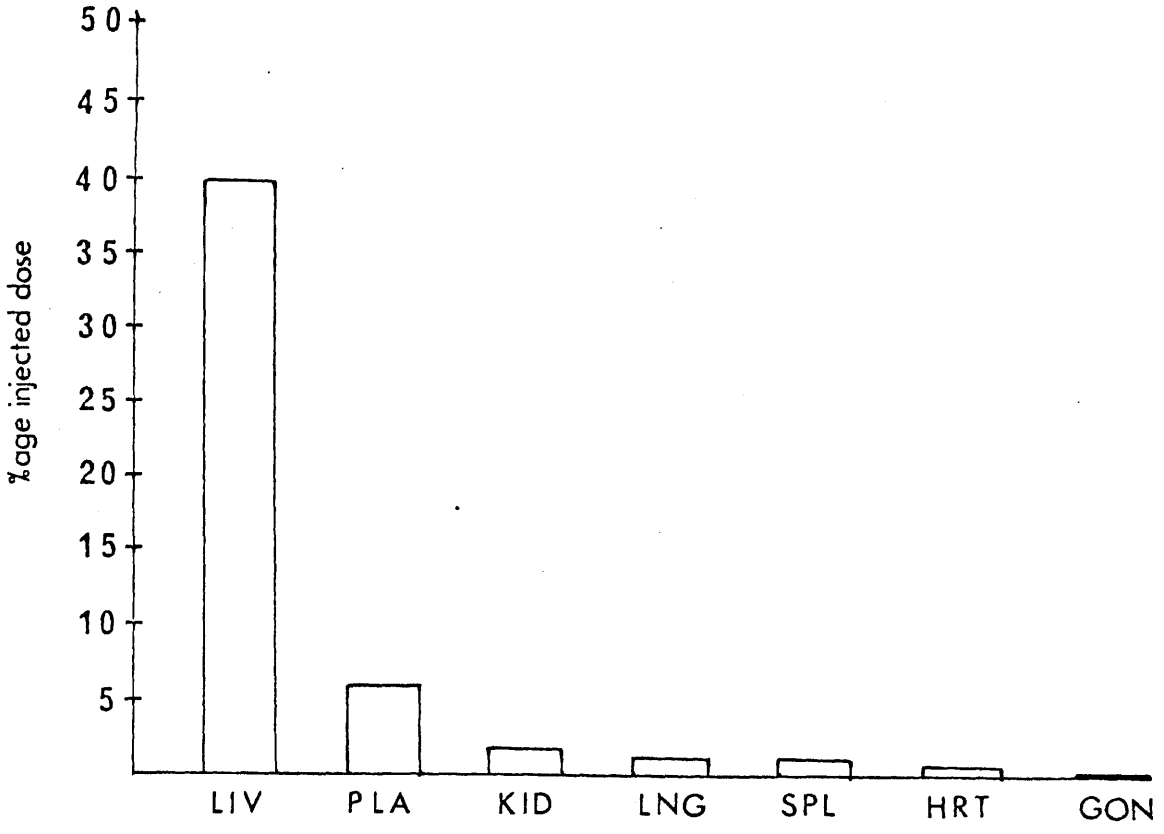


FIGURE R5: Total Organ Uptake of ^{125}I-Ac-BSA

This was calculated from the number of counts per minute in one gram of tissue and expressed as a percentage of total injected dose.

$$\text{Total Organ Uptake} = \frac{\text{cpm/g tissue x organ weight}}{\text{cpm in injected dose}} \times 100\%$$

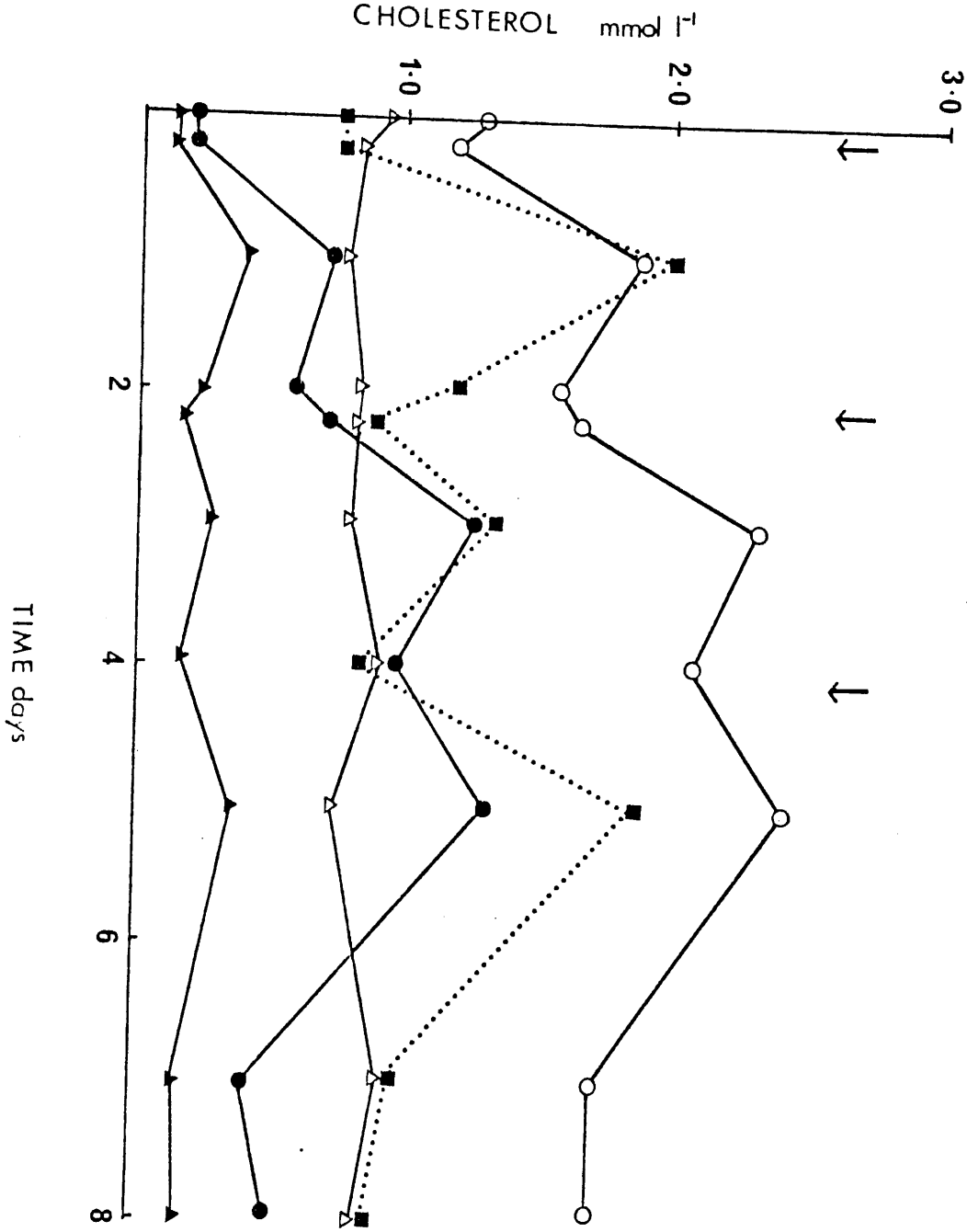


FIGURE R6: Plasma Lipids in NZW Rabbits Treated with Ethyl Oleate.

The animals were given intravenous injection of ethyl oleate emulsion at the times indicated by the arrows (↑) and cholesterol and triglyceride contents of the plasma lipoproteins determined.

- o total cholesterol (mmol/l)
- ● LDL cholesterol (mmol/l)
- △ HDL cholesterol (mmol/l)
- ▲ VLDL cholesterol (mmol/l)
- ■ total triglyceride (mmol/l)

each injection there was a rapid but transient increase in plasma triglyceride levels. This hypertriglyceridaemia resolved within 48 hours and by the end of the study had returned to the base-line value. Total plasma cholesterol concentration increased and, failing to resolve between ethyl oleate injections, reached a value twice that of the control after the third injection. This increase was due to a specific increment in LDL cholesterol, since HDL cholesterol was not affected and VLDL cholesterol increased only slightly (Fig. R6). This experiment was repeated in a second group of animals which received intravenous ethyl oleate emulsion at 24 hour intervals instead of 48 hour intervals (Fig. R7). Again triglyceride levels increased rapidly and resolved between injections. HDL cholesterol remained constant over the period of the study and VLDL cholesterol increased only slightly. Total plasma cholesterol increased reaching a peak of 2.5 times control value after the fourth injection. The increase in total cholesterol, again, was reflected by an increase in the LDL cholesterol component (Fig. R7).

The mean values for total plasma cholesterol and triglyceride for all the animals in this group were calculated (Figures R8 and R9 respectively). Total cholesterol rose, reaching a new steady state by the fourth day at which time the value was significantly different from control values at the 5% level. Triglyceride, however, reached a peak 12 to 24 hours after

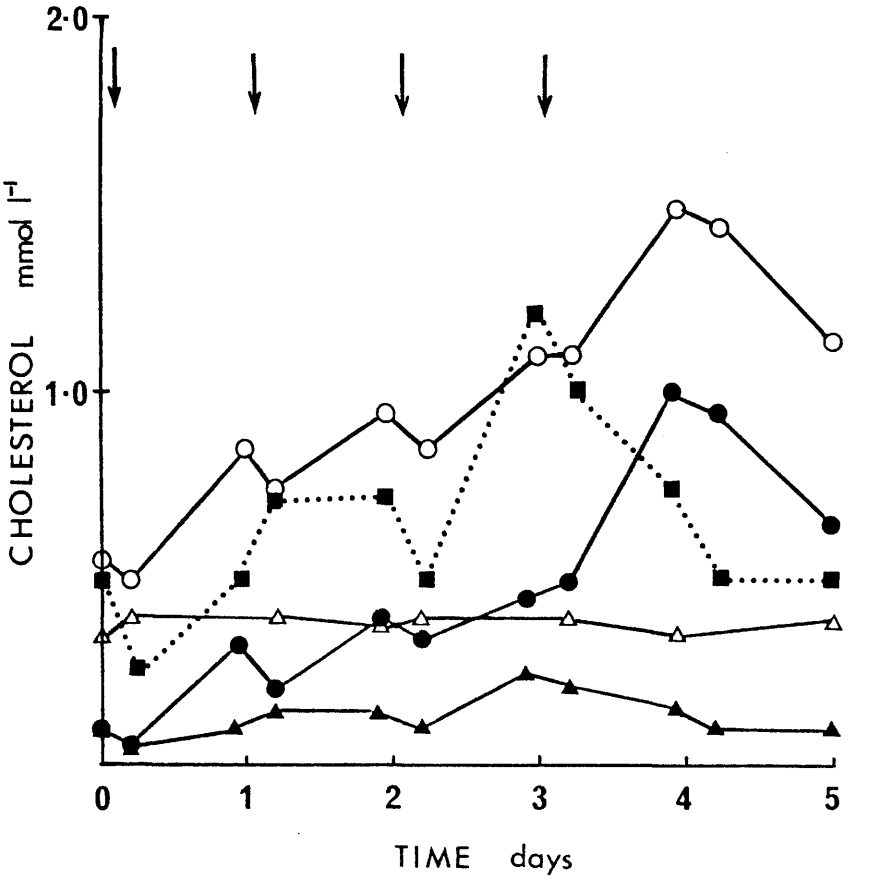


FIGURE R7: Plasma Lipids in NZW Rabbits Treated with Ethyl Oleate.

The animals were given intravenous injections of ethyl oleate emulsion at 24 hour intervals (↑) and the plasma cholesterol and triglyceride levels determined (mmol/l).

- ○ total cholesterol
- ● LDL cholesterol
- △ HDL cholesterol
- ▲ VLDL cholesterol
- ■ total triglyceride

NB: The scalar for cholesterol is the same for triglyceride.

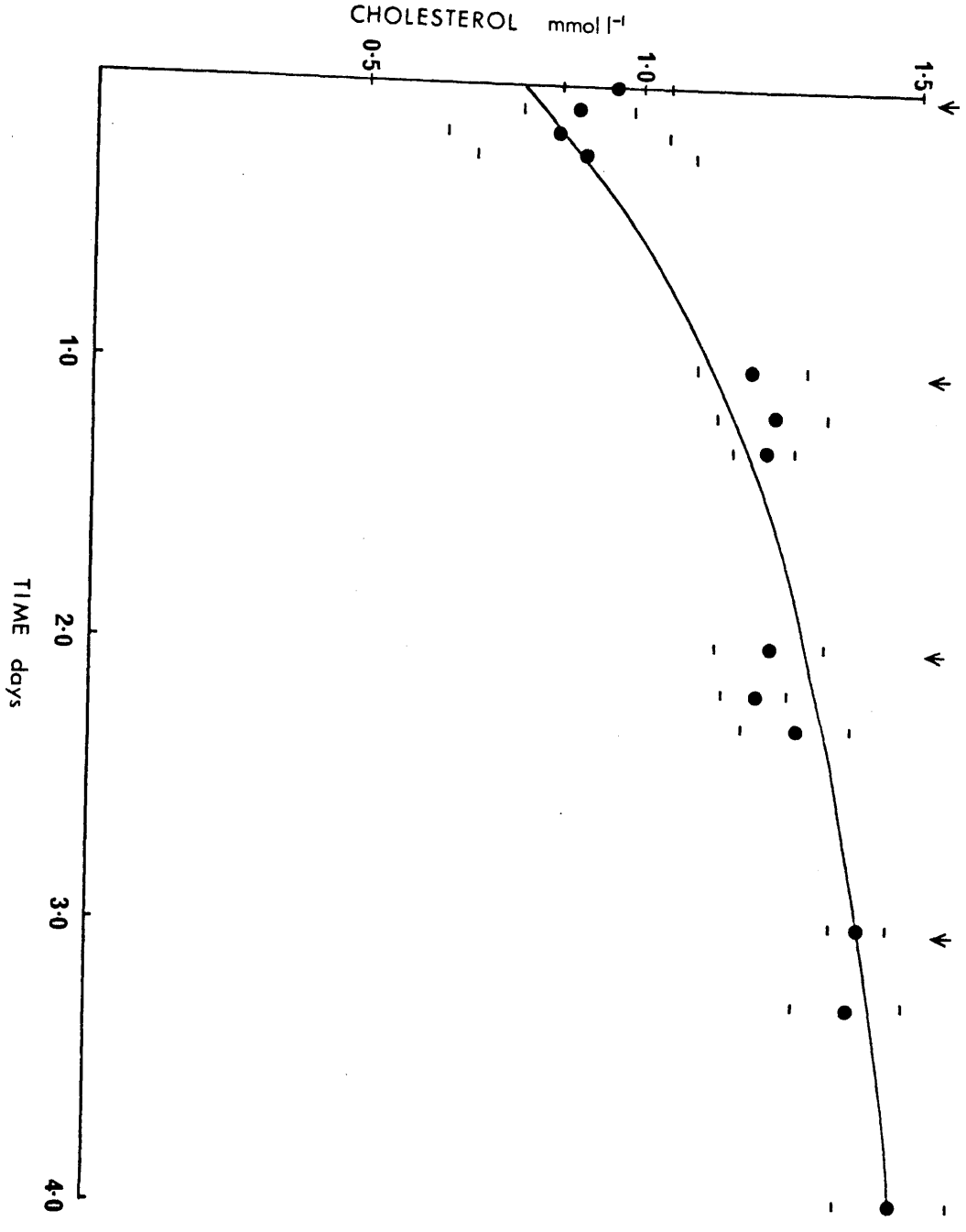


FIGURE R8: Total Plasma Cholesterol in NZW Rabbits Treated with Ethyl Oleate.

The animals were given intravenous injections of ethyl oleate emulstion (↑) and the plasma cholesterol levels determined.

unpaired t-test control vs 4th day $p < 0.05$ ($n = 3$).

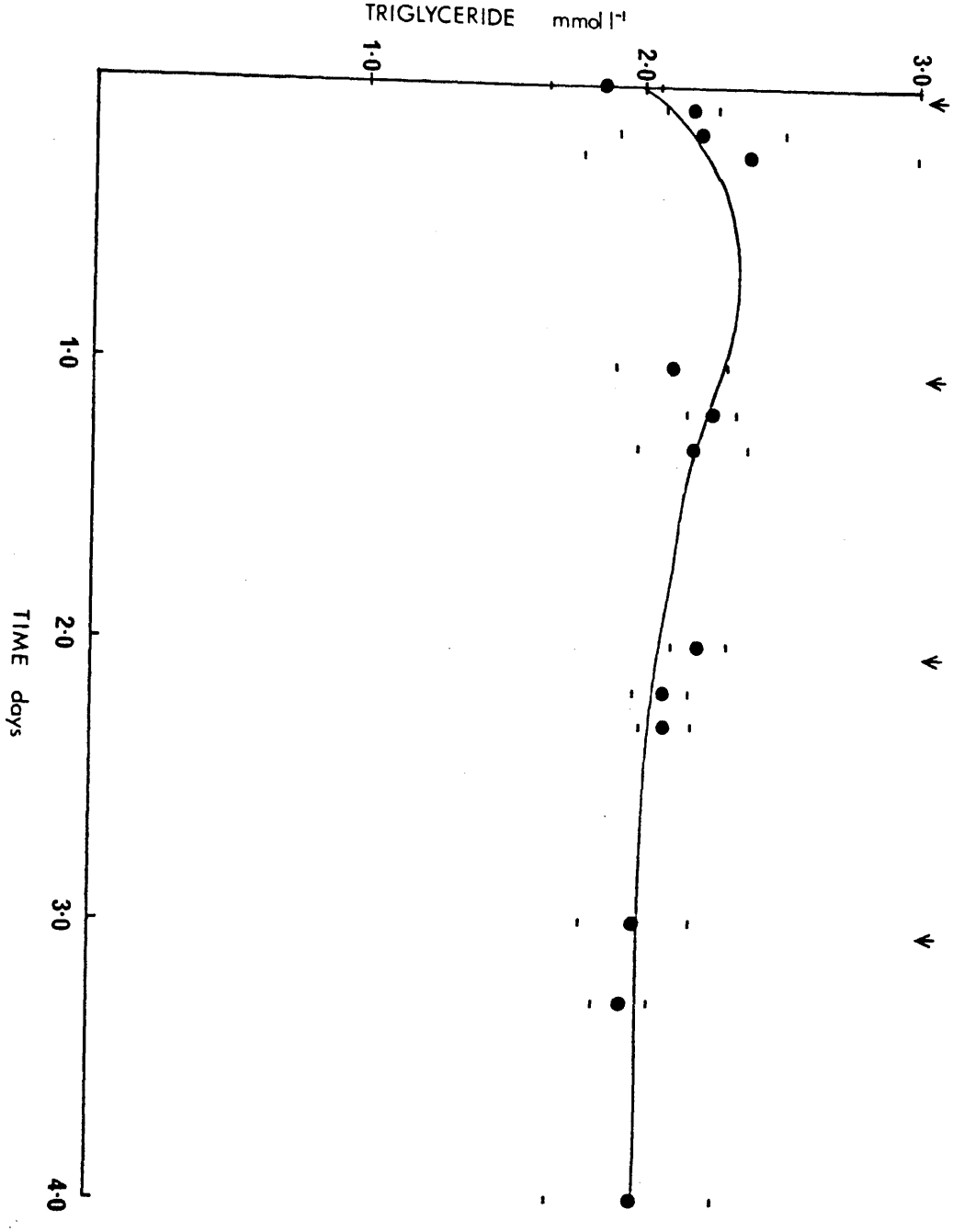


FIGURE R9: Total Plasma Triglyceride Concentration in NZW Rabbits Treated with Ethyl Oleate.

The animals were given intravenous injections of ethyl oleate emulsion (↑) and plasma triglyceride determined.

unpaired t-test control vs 4th day $p < 0.5$ (n = 3).

the first injection and from then resolved to the base-line value by the fourth day.

6.2.2.: Muramyl Tripeptide in Triolein Vesicles (MTP-Triolein).

NZW rabbits received one injection of MTP-triolein (1 mg MTP/kg body weight in a total of 0.25 g of triolein). This single injection produced a marked rise in LDL cholesterol (Fig. R10) which peaked at approximately 30 hours (2.5 times control value) and required a further 42 hours to resolve. HDL cholesterol was unaffected by the manoeuvre but total triglyceride increased dramatically resolving within 30 hours (not shown).

6.2.3.: Muramyl Dipeptide Conjugated to Acetylated BSA (MDP-Ac-BSA).

The rabbits were given one intravenous injection of 1.1 mg MDP per kg body weight (Fig. R11). The pattern of change in lipid levels were similar but not as pronounced as those affected with MTP-triolein. Triglyceride rose rapidly but did not resolve as quickly. The rise in total cholesterol was reflected by an increase in LDL cholesterol and, although VLDL cholesterol increased more, HDL cholesterol remained unchanged.

6.2.4.: Ethyl Oleate and Cholesterol Feeding.

Ten rabbits were fed with normal rabbit chow which was

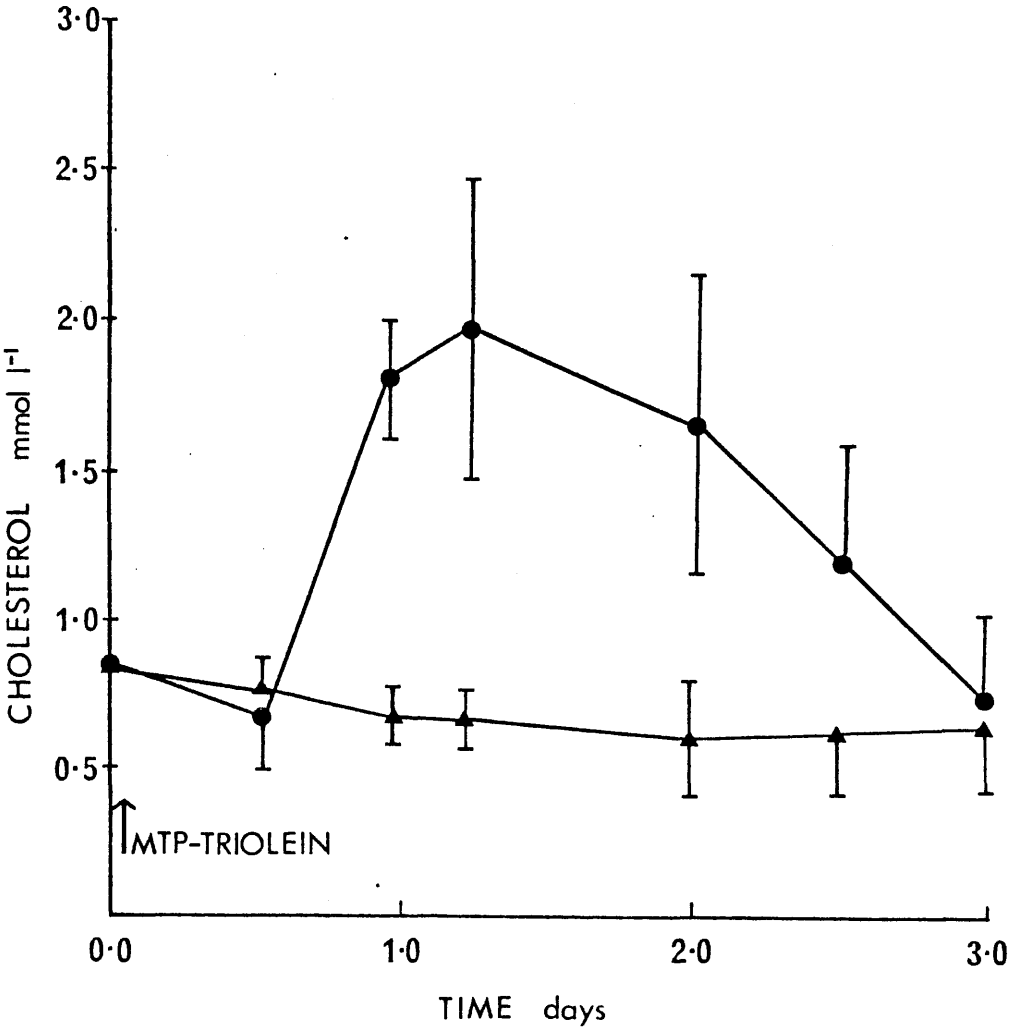


FIGURE R10: Plasma Lipoprotein Cholesterol Levels in NZW Rabbits Treated with Muramyl Tripeptide in Triolein Vesicles (MTP-Triolein).

The animals were injected intravenously (↑) with MTP-triolein (1 mg MTP/kg body weight) and LDL (●) and HDL (▲) cholesterol levels in the plasma measured.

unpaired t-test control vs peak LDL cholesterol level
$p < 0.02$ ($n = 3$).

supplemented with 1% (w/w) cholesterol. Five of the rabbits, group 2, received only this diet and five, group 1, received the diet and were treated with twice-weekly injections of ethyl oleate emulsion (0.5 g ethyl oleate per kg body weight). The animals were maintained on this regimen for 40 days, blood samples for the determination of total cholesterol and triglyceride concentrations being removed twice-weekly. Cholesterol levels increased dramatically reaching as high as 60 mmol l^{-1} (Fig. R12) but no significant difference was observed between the mean values for groups 1 and 2. Triglyceride levels increased to a much lesser extent (Fig. R13) but, again, there was no significant difference in the mean values, despite the apparently higher mean level measured in group 1.

6.2.5.: Ethyl Oleate and Starvation.

Six rabbits were divided into two groups of 3 animals each. Group 1 was given four daily injections of ethyl oleate emulsion and deprived of food during this period. Group 2 was starved for four days. Serial blood samples were withdrawn for the determination of plasma lipid levels. The animals which received ethyl oleate (Fig. R14 panels A and C) showed a rapid but transient increase in triglyceride levels which resolved within 48 hours and a rapid but stable increase in cholesterol levels, typically rising 5 fold over control values. Animals in group 2 (Fig. R14 panels B and D) showed a slight

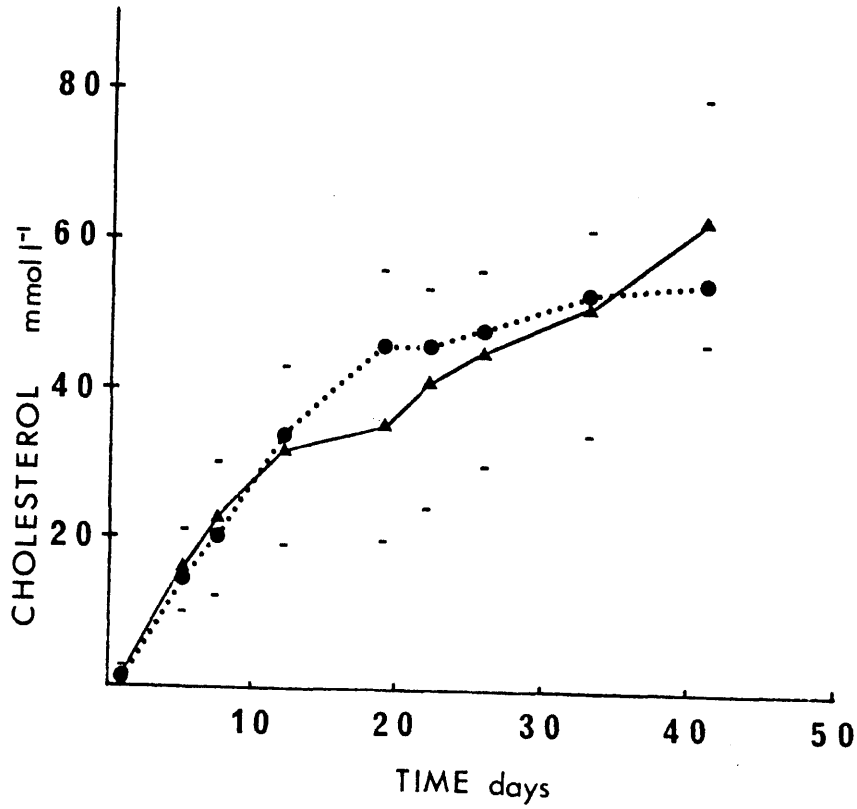


FIGURE R12: Effect of Cholesterol Feeding and Ethyl Oleate on the Plasma Cholesterol Levels in NZW Rabbits.

The animals were fed a diet containing 1% (w/w) cholesterol. Group 1 also received an intravenous injection of ethyl oleate emulsion twice per week. The cholesterol levels in the plasma of the animals in both groups were measured.

●: group 1; cholesterol feeding plus ethyl oleate
▲: group 2; cholesterol feeding only.

unpaired t-test.

control vs end point (group 1) $p < 0.001$ (n = 5)
control vs end point (group 2) $p < 0.001$ (n = 5)
end point group 1 vs endpoint group 2 $p > 0.5$ (n = 5)

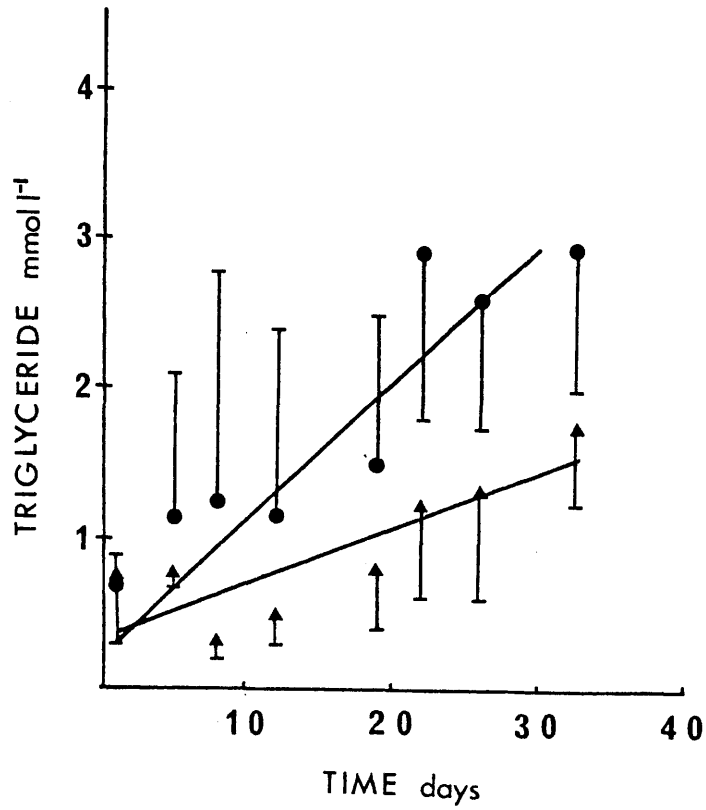


FIGURE R13: Effect of Cholesterol Feeding and Ethyl Oleate on the Plasma Triglyceride Levels in NZW Rabbits.

The animals were fed a diet containing 1% (w/w) cholesterol. Group 1 also received an intravenous injection of ethyl oleate emulsion twice per week. The triglyceride levels in the plasma of the animals in both groups were measured.

●: Group 1; cholesterol feeding plus ethyl oleate (n = 5).
▲: Group 2; cholesterol feeding only (n = 5).

unpaired t-test

control vs end point (Group 1) $p < 0.001$
control vs end point (Group 2) $p < 0.01$
end point group 1 vs end point group 2 $p = 0.05$.

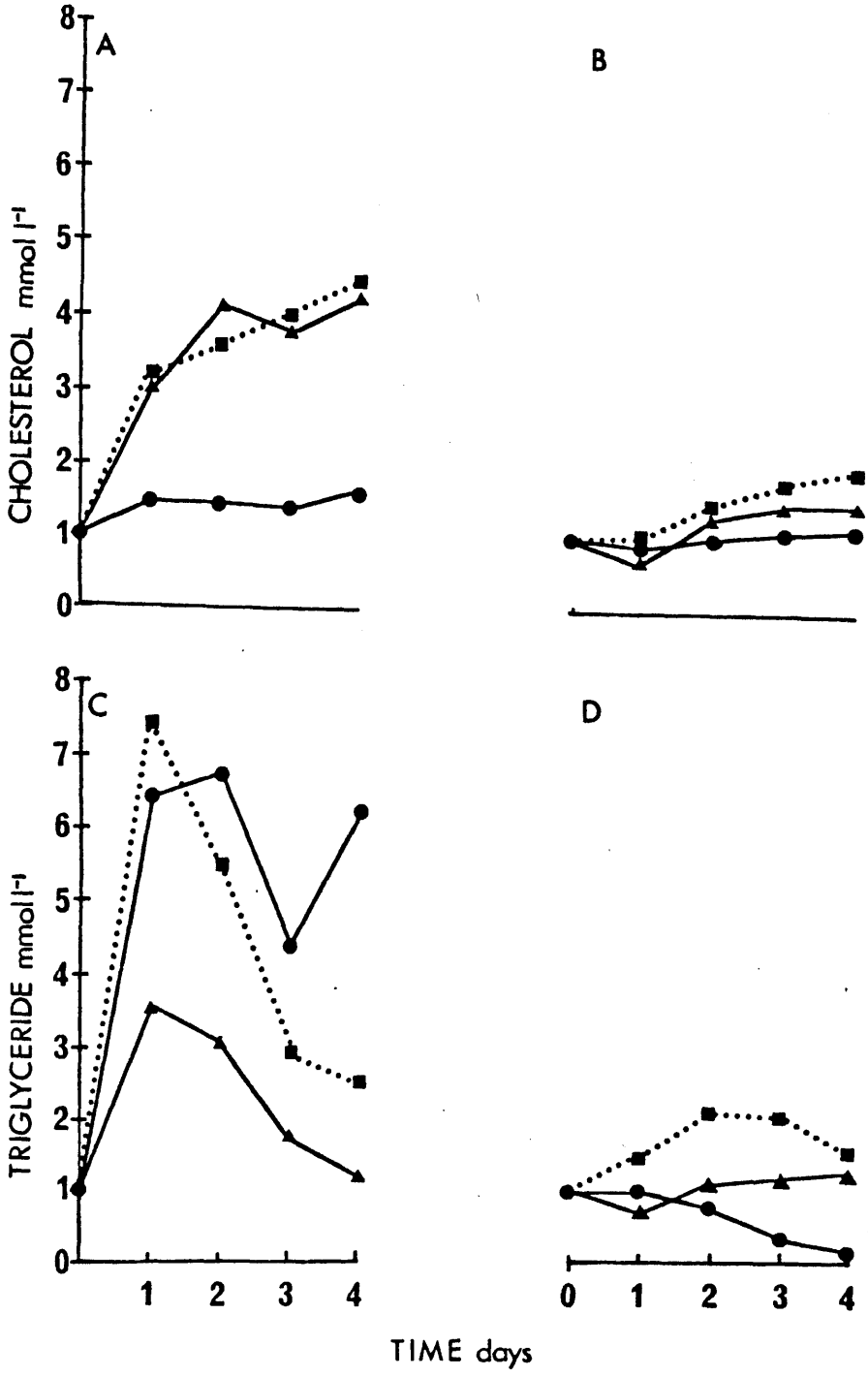


FIGURE R14: Effect of Starvation and Ethyl Oleate on the Plasma Cholesterol and Triglyceride Levels in NZW Rabbits.

The animals were starved for four days, during which time Group 1 received intravenous ethyl oleate emulsion at 24 hour intervals. The plasma cholesterol and triglyceride levels were determined from blood samples removed immediately before the ethyl oleate injections.

A:- Cholesterol levels in Group 1 (starved + ethyl oleate)
B:- Cholesterol levels in Group 2 (starved only)
C:- Triglyceride levels in Group 1 (starved + ethyl oleate)
D:- Triglyceride levels in Group 2 (starved only)

▲ Rabbit 1
● Rabbit 2
■ Rabbit 3

increase in cholesterol concentration and a small fall in triglyceride levels.

Total plasma fatty acid concentrations in groups 1 and 2 were also measured. Group 1, receiving ethyl oleate, showed a two fold increase in stearate (c16:0) (Fig. R15) from 350 µg/ml to 700 µg/ml whereas the concentration of oleate (c18:1) almost halved (400 µg/ml to 220 µg/ml). This observation was significant because animals in this group received a total of 6 grams of ethyl oleate each over a 4 day period. In the control group stearate increased to a peak at 2 days, returning to control values by day 4. Oleate showed a similar but less pronounced pattern, rising to a peak and returning to base-line values in 4 days (Fig. R15).

6.3.: Effects of Reticuloendothelial System Suppressants on Phagocytic Function, as Measured by the Rate of Carbon Particle Clearance In Vivo.

6.3.1.: Ethyl Oleate Emulsion.

Clearance of particulate matter, such as colloidal carbon, from the plasma is a function of the macrophages of the RE system. To ensure that ethyl oleate, and other such agents, elicit their effects through the suppression of that system the Phagocytic Index, K, was determined. K was calculated as follows:

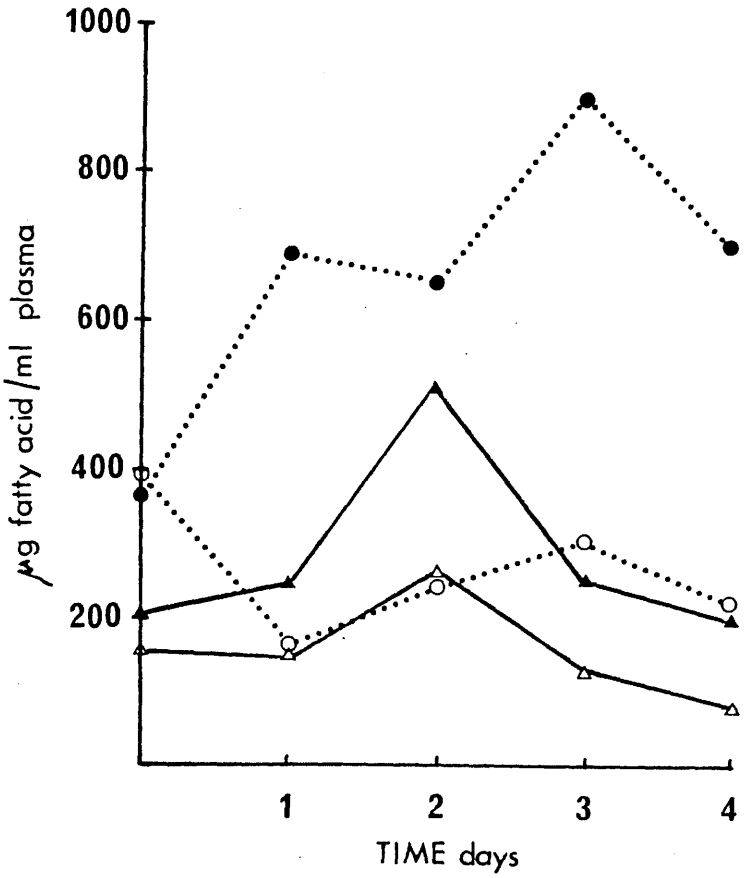


FIGURE R15: Effect of starvation and ethyl oleate treatment on the fatty acid concentrations in rabbit plasma.

- ● Ethyl oleate + starvation on c16:0
- ▲ Starvation only on c16:0
- o Ethyl oleate + starvation on c18:1
- △ Starvation only on c18:1

$$K = \frac{\log_{10} OD_{700}\ P_2 - \log_{10} OD_{700}\ P_1}{t_2 - t_1}$$

where K = Phagocytic Index

OD_{700} = optical density of the plasma at 700mm

P_2 = second selected point

P_1 = first selected point

t_2 = time, in hours, of P_2

t_1 = time, in hours, of P_1

K then was determined for animals in the control state and then after 4 daily injections of ethyl oleate emulsion (Fig. R16). These measurements were carried out two weeks apart to ensure that the phagocytosed carbon particles did not themselves exert an inhibitory effect. Under the influence of ethyl oleate, K increased from -1.08 $hours^{-1}$ to -0.57 $hours^{-1}$ (Fig. R16) thus demonstrating an inhibitory effect on the RE system.

6.3.2.: Muramyl Tripeptide in Triolein Vesicles (MTP-triolein).

The experiment described in section 6.3.1. was repeated in 3 rabbits receiving one injection of MTP-triolein (1 mg MTP per kg body weight) instead of the ethyl oleate. K was determined in the control state then 24 hours after administration of MTP-triolein (Fig. R17) Again, two weeks was allowed for recovery from the colloidal carbon administered for the measurement of control K.

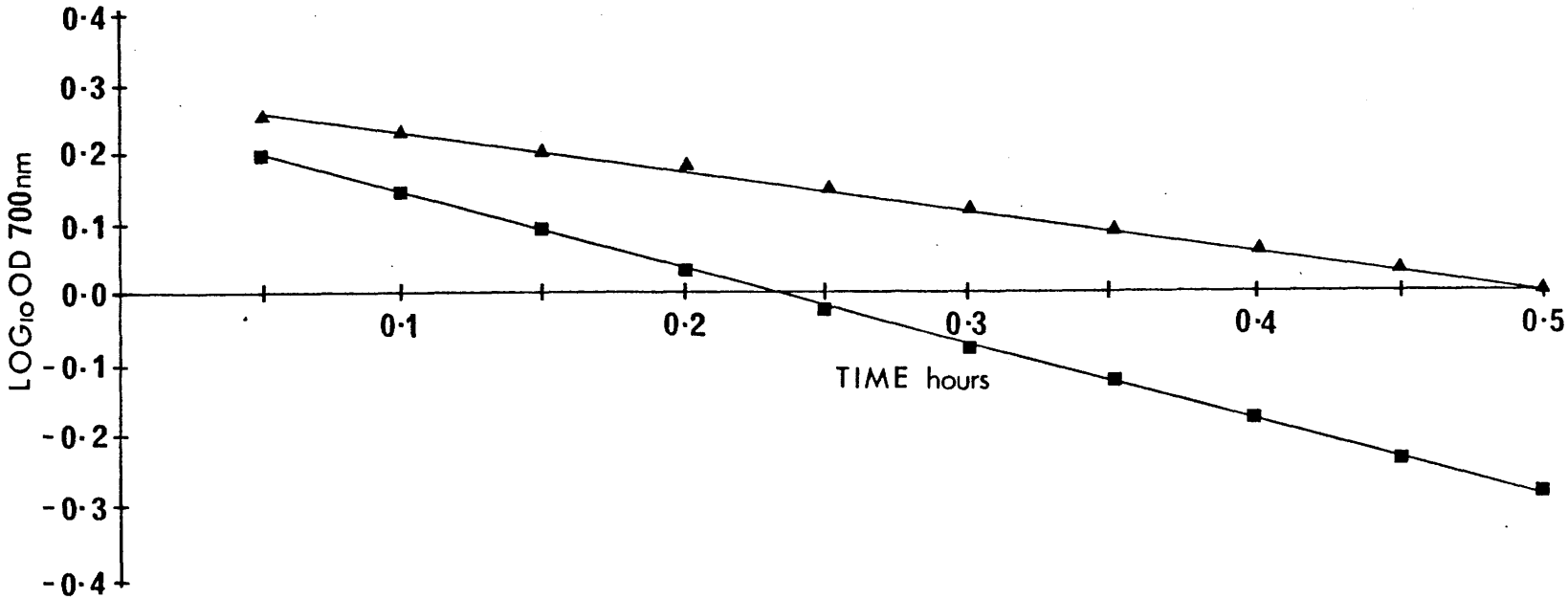


FIGURE R16: Effect of Ethyl Oleate on Plasma Clearance of Colloidal Carbon.

Colloidal carbon clearance was measured in two NZW rabbits. Two weeks later the animals were treated with 4 ethyl oleate injections at 24 hour intervals and the colloidal carbon clearance remeasured.

■ Carbon clearance in untreated animals (n = 2) K = -1.08
▲ Carbon clearance in treated animals (n = 2) K = -0.57

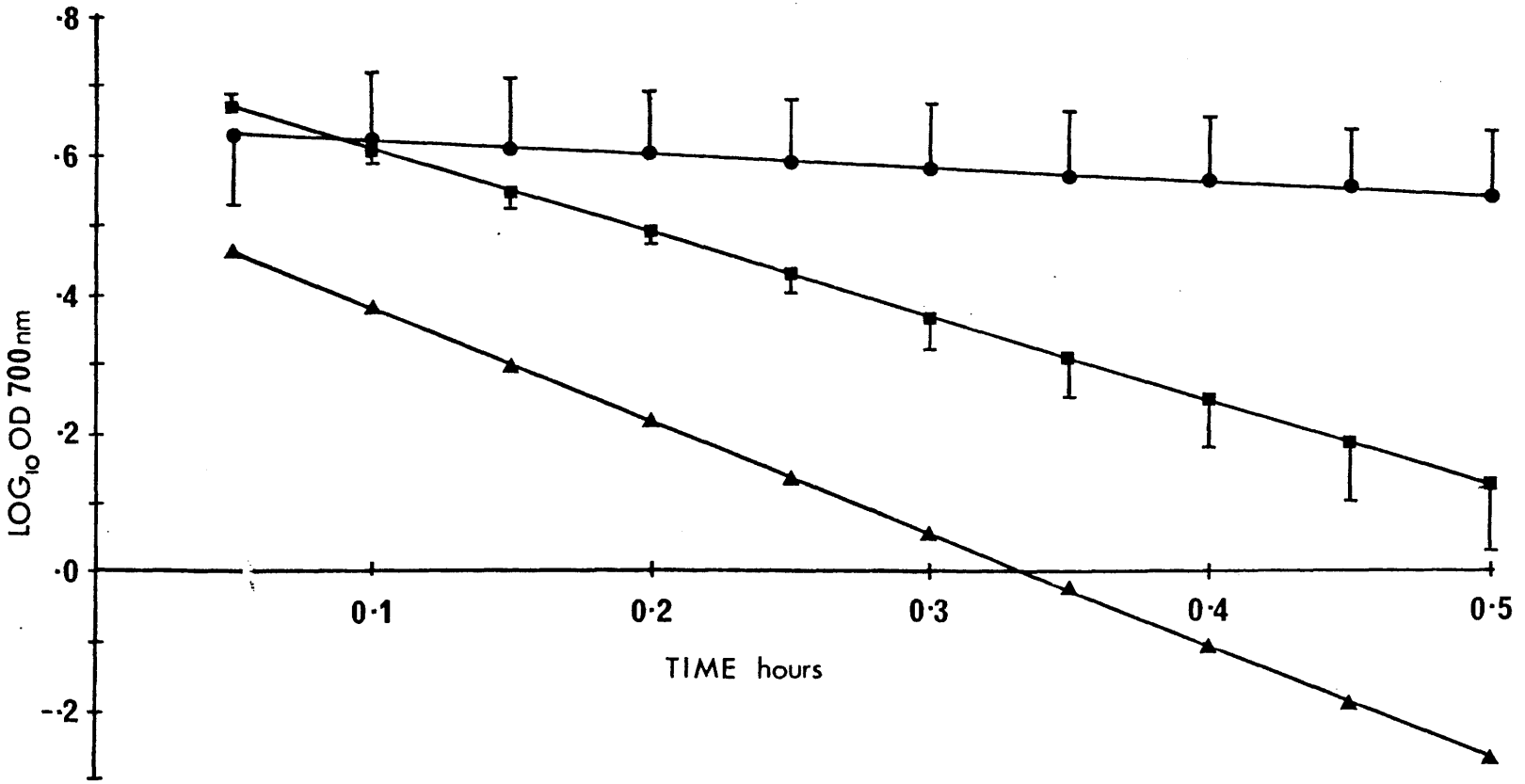


FIGURE R17: Effect of Muramyl Tripeptide in Triolein Vesicles on Plasma Clearance of Colloidal Carbon.

Colloidal carbon clearance was measured in three NZW rabbits. Two weeks later the animals were given 1 injection of MTP-triolein each and the carbon clearance determined after 24 hours. The carbon clearance was remeasured two weeks after the MTP-triolein treatment ensuring that the carbon itself had no inhibitory effects.

■ : carbon clearance in control animals (n = 3) K = -1.20 ± 0.30
● : carbon clearance in MTP-triolein treated animals (n = 3) K = -0.20 ± 0.19
▲ : carbon clearance in recovered animals (n = 2) K = -1.63.

Phagocytic index in the control state was -1.20 ± 0.30 hours^{-1} which compared favourably with control K in section 6.3.1. This increased to -0.20 ± 0.19 hours^{-1} after treatment with MTP-triolein which represented a significant increase ($p<0.02$) over the control value (Table R1). Thus a single injection of MTP-triolein significantly increased the time required to clear particulate matter from the plasma of animals, demonstrating an inhibition of the RE system.

In this experiment K was redetermined two weeks after the MTP-triolein had been given in two of the rabbits. K had decreased from -0.20 hours^{-1} 24 hours after MTP-triolein to -1.63 hours^{-1} two weeks later which, although not identical with the initial value, showed that, if an interval of two weeks was left between consecutive measurements of phagocytic index, no inhibitory effect persisted due to the colloidal carbon. Figure R18 shows the effect of both ethyl oleate and MTP-triolein on the value of K.

6.4: Effect of Reticuloendothelial Suppression on the Degradation of Denatured Albumin by Macrophages In Vitro.

6.4.1.: Muramyl Dipeptide (MDP) and Muramyl Tripeptide (MTP) in Free Solution.

J774 mouse macrophages were exposed to 250 µg MDP/ml or 250 µg MTP/ml for 18 hours. At the end of this period the cells were tested for their ability to degrade

STATE \ RABBIT NUMBER	1	2	3	Mean
[a] CONTROL	-1.39	-1.35	-0.55	-1.20 ± 0.30
[a] MTP	-0.23	-0.38	0.00	-0.20 ± 0.19
AFTER	-1.79	-1.46	ND	-1.63

TABLE R1: Effects of MTP-Triolein on the Clearance of Colloidal Carbon in NZW Rabbits.

a: unpaired t-test control vs MTP triolein treated animals $P < 0.02$.

Values for Phagocytic Index (K) in NZW rabbits in the control state (CONTROL), 24 hours after treatment with MTP-triolein (MTP) and two weeks after treatment with MTP-triolein (AFTER).

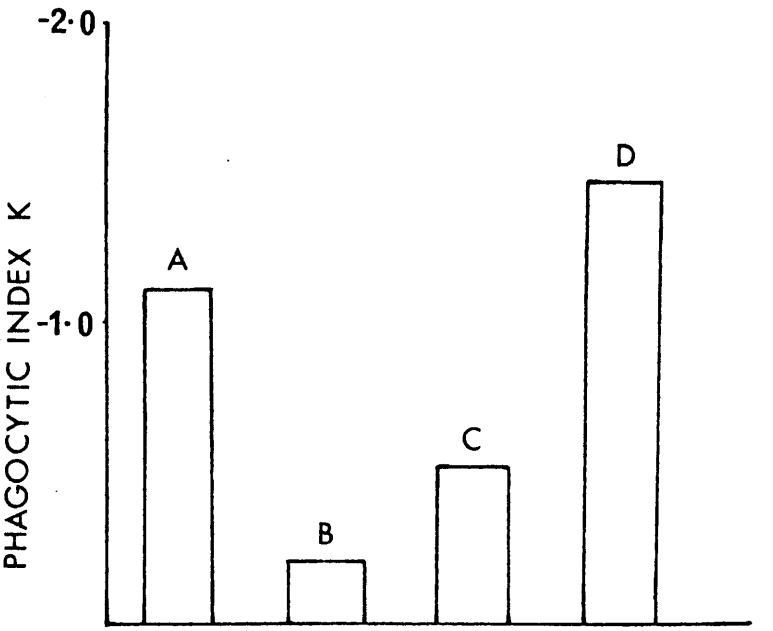


FIGURE R18: Effect of Ethyl Oleate and MTP-Triolein on the Phagocytic Index in NZW Rabbits.

A: Control Animals
B: MTP-Triolein Treated Rabbits
C: Ethyl Oleate Treated Rabbits
D: 2 weeks after treatment.

formaldehyde denatured albumin compared to cells which had received no treatment (Fig. R19). Degradation of the marker was measured as the amount of radioactivity present as ^{125}I-tyrosine (section 5.14.3.) per ug of cell protein in the assay tube. Cells which had been incubated with MDP catabolised only 12.7% as much as the control cells and those incubated with MTP only 3.2% as much. This *in vitro* suppression of the ability of macrophages to degrade the marker supported the results obtained *in vivo* (section 6.3).

6.4.2.: Muramyl Tripeptide in Triolein Vesicles (MTP-triolein).

J774 mouse macrophages were exposed to MTP-triolein or to triolein vesicles for 18 hours. At the end of this incubation the cells were challenged with denatured albumin and their ability to degrade the marker determined as above (Fig. R20). Cells which had been incubated with MTP-triolein catabolised only 3.9% as much marker as control cells. Those which received triolein vesicles only were not significantly suppressed in their ability to degrade denatured albumin.

Again MTP-triolein incubation supported the results obtained *in vivo* and exposure to triolein vesicles showed that the lipid had no significant effect on macrophages.

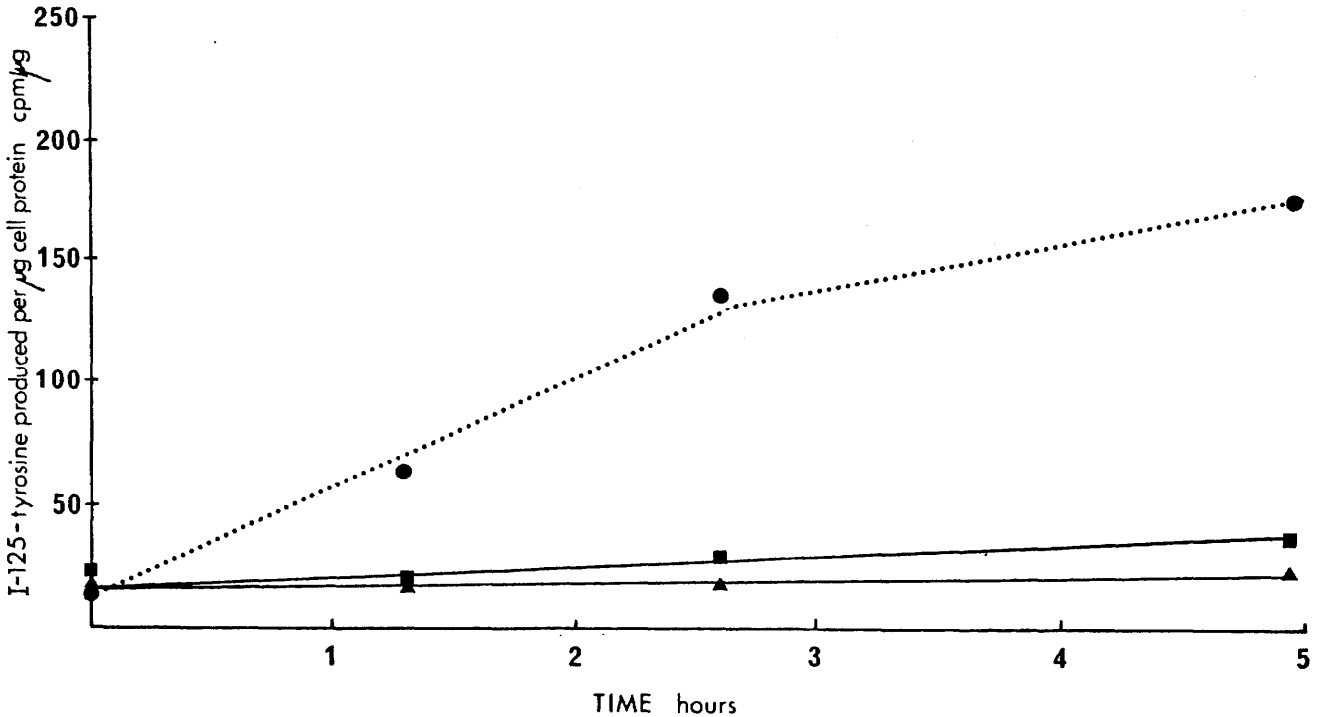


FIGURE R19: Degradation of ^{125}I-Denatured Albumin by Cultured Macrophages (J774).

The cells were treated for 18 hours before the experiment began with either 250 µg/ml MDP or 250 µg/ml MTP.

- ● degradation of denatured albumin by macrophages in the central state.
- ■ degradation of denatured albumin by macrophages treated with MDP.
- ▲ degradation of denatured albumin by macrophages treated with MTP.

Each point was the mean of 2 experiments.

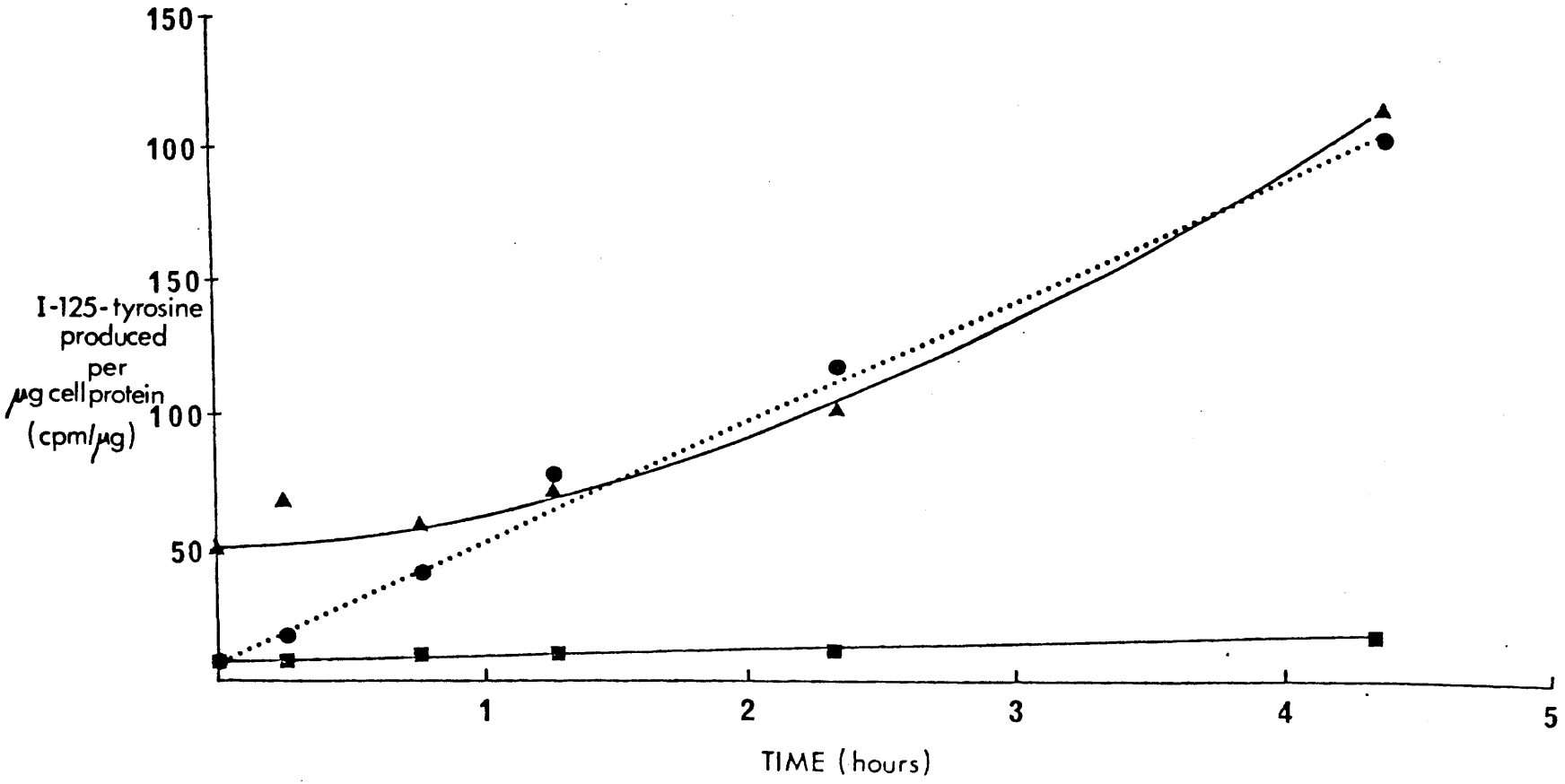


FIGURE R20: Degradation of ^{125}I-Denatured Albumin by Cultured Macrophages (J774).

The cells were treated for 18 hours before the experiment began with either 1 ml of culture medium containing 10 µg of triolein as vesicles or 1 ml of culture medium containing 10 µg of triolein + 250 µg of MTP as vesicles.

- ● degradation of denatured albumin by control cells
- ▲ degradation of denatured albumin by cells treated with triolein only
- ■ degradation of denatured albumin by cells treated with MTP-triolein.

6.5: Effect of Reticuloendothelial System Suppression of Low Density Lipoprotein (LDL) Metabolism In Vivo.

6.5.1.: Muramyl Dipeptide Conjugated to Acetylated Albumin (MDP-Ac-BSA).

The effect of MDP-Ac-BSA on the metabolism of LDL in vivo was determined in rabbits. The animals were injected with ^{125}I-LDL and ^{131}I-reductively methylated LDL (^{131}I-met-LDL) and the plasma clearance rates of the two tracers followed until they had reached terminan exponential (Fig. R21). At that point MDP-Ac-BSA was administered (1.1 mg MDP/kg body weight) intravenously and within seven hours the plasma clearance rate slowed to 43.7% of control rate for native-LDL and 41.9% of control rate for Met-LDL (Table R2). These slower rates of clearance of the tracers persisted for a further 24 hours both returned to control values.

6.5.2.: Muramyl Tripeptide in Triolein Vesicles (MTP-Triolein).

The effect of MTP-triolein on the metabolism of LDL in vivo was determined in rabbits. The animals were injected with ^{125}I-LDL and ^{131}I-Met-LDL and the plasma clearance rates plotted against time until the decay was monoexponential (Fig. R22). At this time, MTP-triolein (1 mg MTP/kg body weight) was administered intravenously and within 5 hours the plasma clearance rate for native-

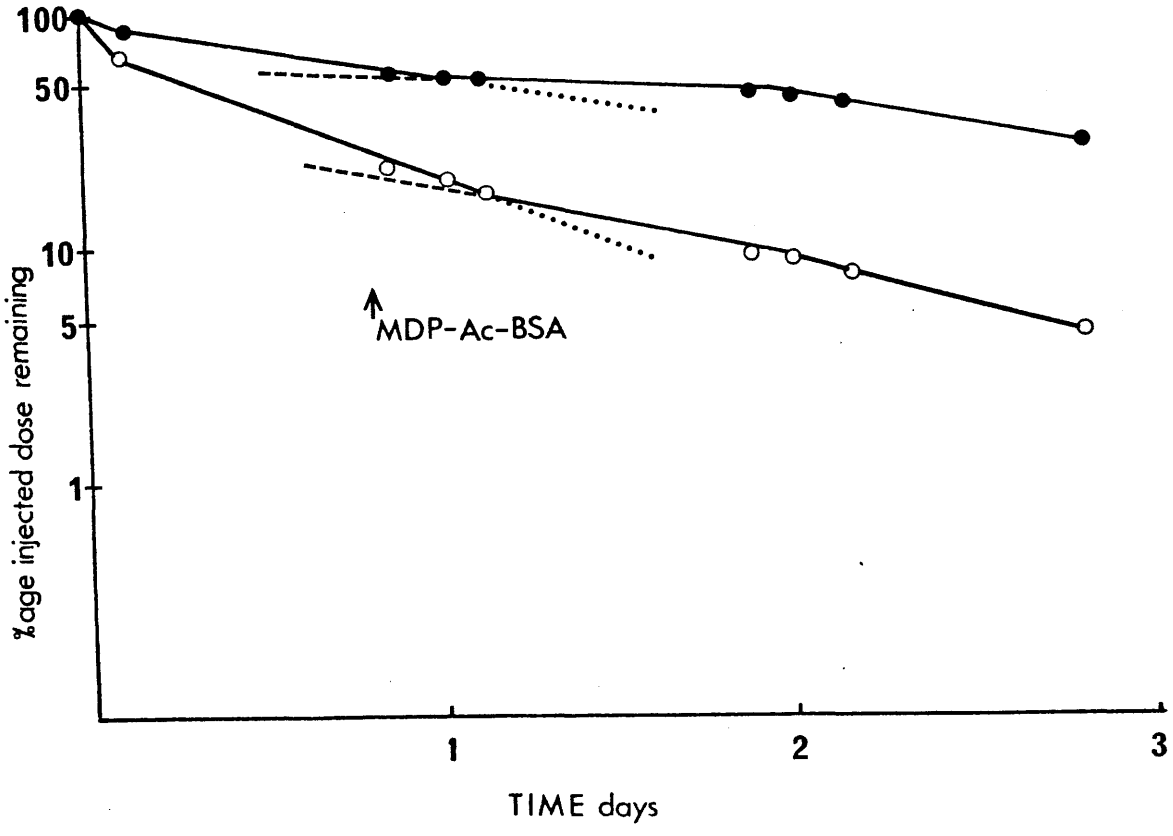


FIGURE R21: Acute Effects of MDP-Ac-BSA on LDL Catabolism in NZW Rabbits.

The plasma clearance of native LDL (o) and reductively methylated LDL (met-LDL; ●) was followed for 24 hours, by which time the decay was monoexponential. MDP-Ac-BSA (1.1 mg MDP/kg body weight) was then administered intravenously, as indicated by the arrow (n = 2).

TRACER: / DRUG ADMINISTERED	LDL CONTROL	LDL MDP-Ac-BSA	LDL MTP TRIOLEIN	MET-LDL CONTROL	MET-LDL MDP-Ac-BSA	MET-LDL MTP TRIOLEIN
1	0.62	1.13		1.31	2.82	
2	0.64	1.25		1.38	2.59	
3	0.66		1.85	1.16		2.00
4	0.26		0.95	0.87		2.13
5	0.40		1.35	1.00		1.92
6	0.55		1.06	1.05		1.87
MEAN	0.52 ± 0.16	1.19	1.44 ± 0.38	1.13 ± 0.19	2.70	1.98 ± 0.11

Table R2: Effect of MDP-Ac-BSA and MTP-Triolein of the Plasma Half-Lives of Native and Met-LDL.

unpaired t-test

native LDL vs native LDL after MTP-triolein: $p < 0.0\text{-}1$
native LDL vs met-LDL : $p < 0.001$
met-LDL vs met-LDL after MTP-triolein : $p < 0.001$
both tracers after MTP-triolein : $p < 0.05$

The half-life was calculated for the monoexponential phase of the clearance of native and met-LDL then recalculated for the slower decay obtained after injection of MTP-triolein or MDP-Ac-BSA ($t_{\frac{1}{2}}$ was measured in $days^{-1}$).

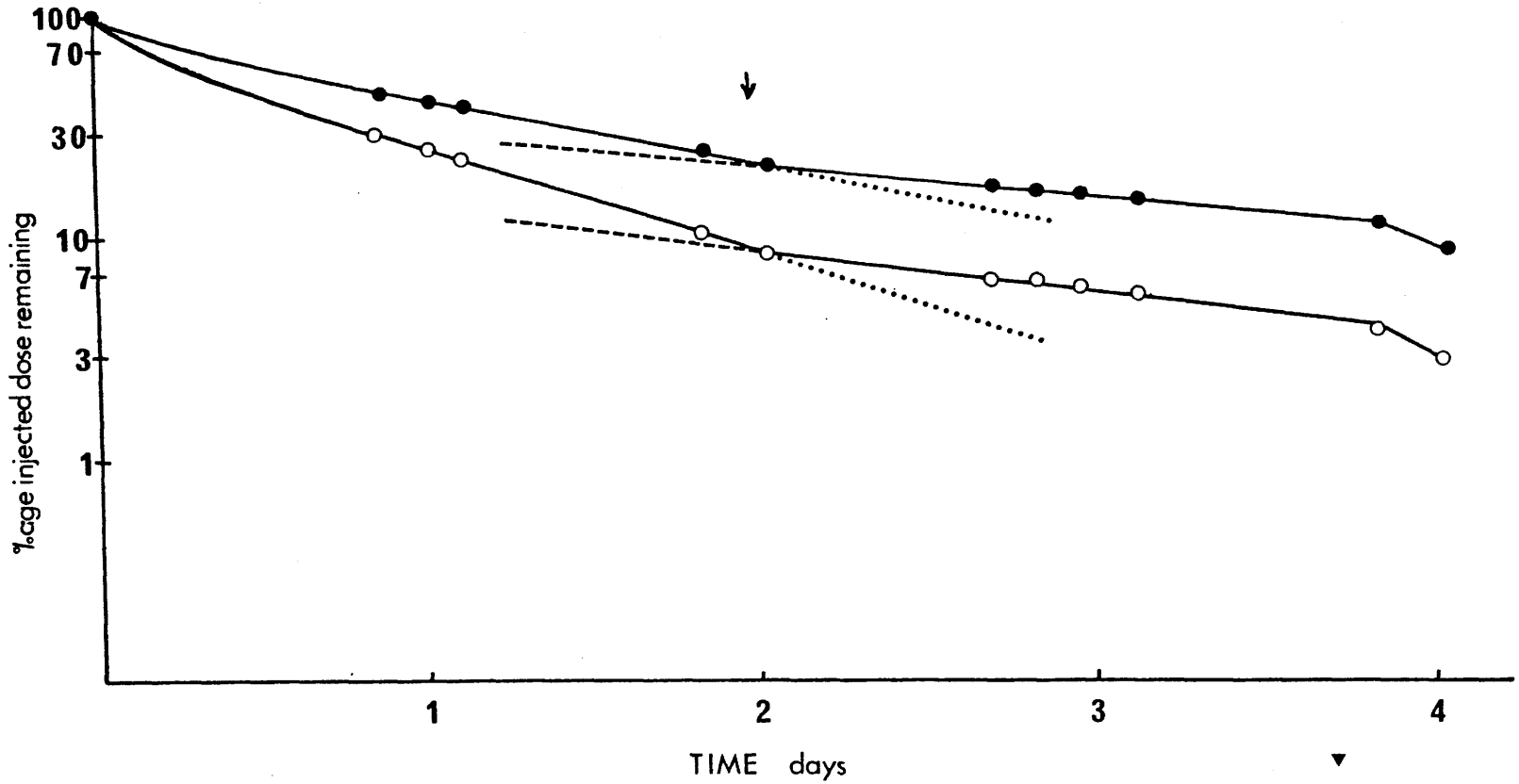


FIGURE R22: Acute Effects of MTP-Triolein on LDL Catabolism in NZW Rabbits.

The plasma clearance of native LDL (o) and reductively methylated LDL (met-LDL; ●) was followed in NZW rabbits for 48 hours, by which time the decay was monoexponential. MTP-triolein (1 mg MTP/kg body weight) was then administered intravenously, as indicated by ↓ , and the plasma clearance followed for a further 48 hours.

LDL had slowed to 36.1 ± 11.0% (n = 4) of the control rate and to 51.75 ± 7.7% (n = 4) for met-LDL (Table R2). These slower rates of clearance of the tracers were significantly different from the control rates at the 0.1 percent level and persisted for a further 40 hours before remitting.

6.6: Effects of Reticuloendothelial System Suppressants on the Degradation of Lipoproteins by Cultured Macrophages.

6.6.1.: Effect of MDP-Ac-BSA on the Degradation of Native and Met-LDL.

J774 mouse macrophages were treated with 0, 100, 250 and 500 µg/ml of MDP-Ac-BSA. These concentrations were measured in terms of the amount of albumin present and were equivalent to 0, 80, 200 and 400 µg/ml of MDP. After 18 hours preincubation with the above concentrations of MDP-Ac-BSA the cells were challenged for their ability to degrade native and met-LDL (Fig. R23 and Fig. R24 respectively). After 6 hours of preincubation with 250 ug MDP-Ac-BSA the amount of native LDL degraded was reduced to 79% of the control value and 500 µg MDP-Ac-BSA/ml to 60% of control value (Fig. R23). 100 µg MDP-Ac-BSA/ml reduced the amount of catabolism by as much as 250 µg/ml. The reason for this was unclear but the effect was observed in other parameters measured during the experiment and was perhaps a real effect of dose dependence.

The catabolism of met-LDL was reduced by 42.2% and 46.7% by 250 and 500 μg MDP-Ac-BSA/ml respectively (Fig. R24) in 6 hours. 500 μg Ac-BSA/ml of culture medium, however, produced a reduction in degradation of the markers of only 6% (Fig. R23) demonstrating that the carrier was not significantly affecting cell catabolic function. Dose response curves were constructed for MDP-Ac-BSA (Fig. R25) and showed that, for both native and methylated LDL, there was an efficient suppression at 100 μg MDP-Ac-BSA/ml and there after increasing amounts produced a steady but slower decline in the ability of the macrophages to degrade the tracers. Cell protein was measured in each concentration group and expressed as mg of cellular protein/ml of culture medium (Fig. R26). Treatment with the suppressant produced a significant reduction in the amount of protein present, with the most marked drop occurring after incubation with 100 ug MDP-Ac-BSA/ml (64.5% of control value). Ac-BSA alone did not reduce the amount of cellular protein present ($p > 0.5$).

The decrease in cellular protein was likely to reflect a reduction in the number of cells present, perhaps by inhibition of cell replication and this was compensated for in calculation of the ability of macrophages to degrade the LDLs (ie degradation was expressed as the amount of ^{125}I or ^{131}I-tyrosine produced per ug of cellular protein).

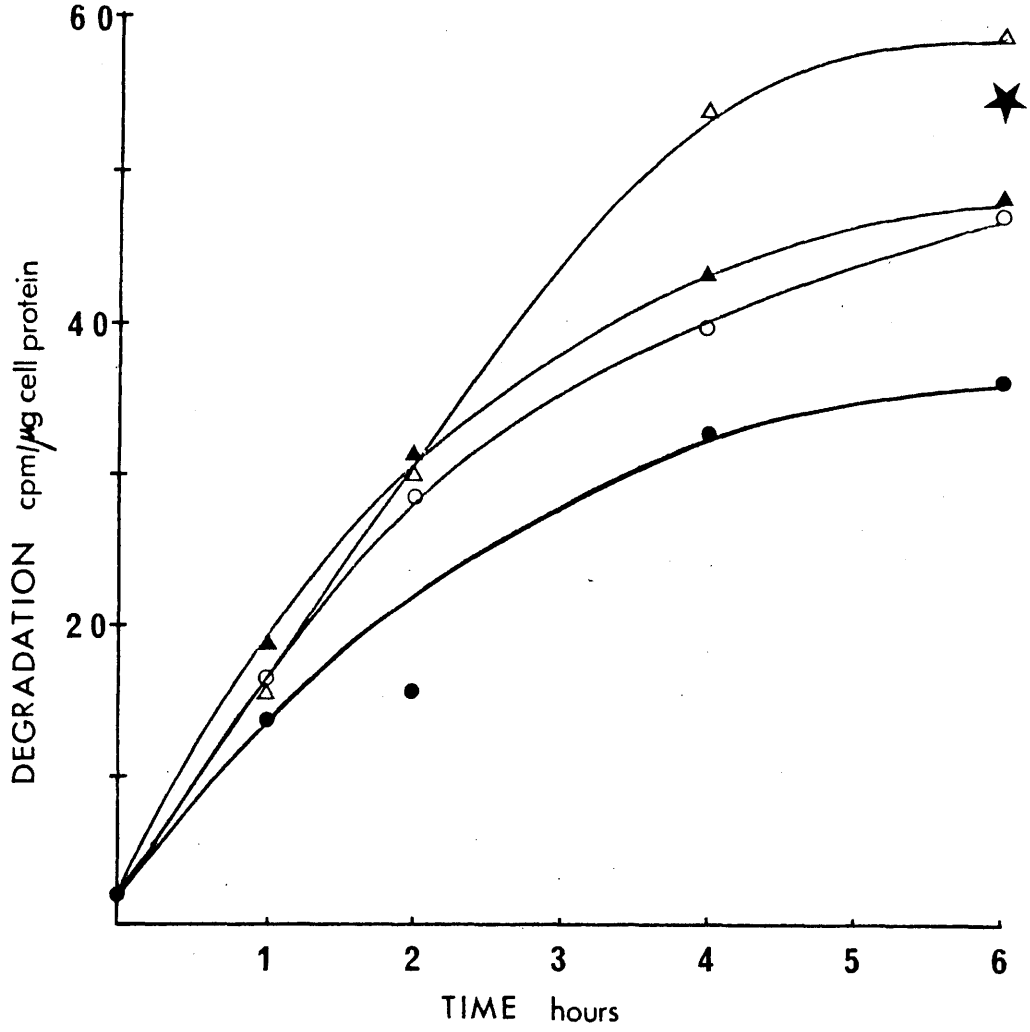


FIGURE R23: Degradation of ^{125}I-LDL by Cultured Macrophages (J774) after Exposure to MDP-Ac-BSA.

The cells were exposed to MDP-Ac-BSA for 18 hours then challenged with 150 µg/ml (1 x 10^6 cpm/ml) of ^{125}I-LDL.

△ : control

▲ : cells preincubated with 100 µg/ml MDP-Ac-BSA (80 µg MDP)

o : cells preincubated with 250 µg/ml MDP-Ac-BSA (200 µg MDP)

● : cells preincubated with 500 µg/ml MDP-Ac-BSA (400 µg MDP)

The macrophages were also treated with 500 µg/ml Ac-BSA (★).

(each point was the mean of duplicate experiments)

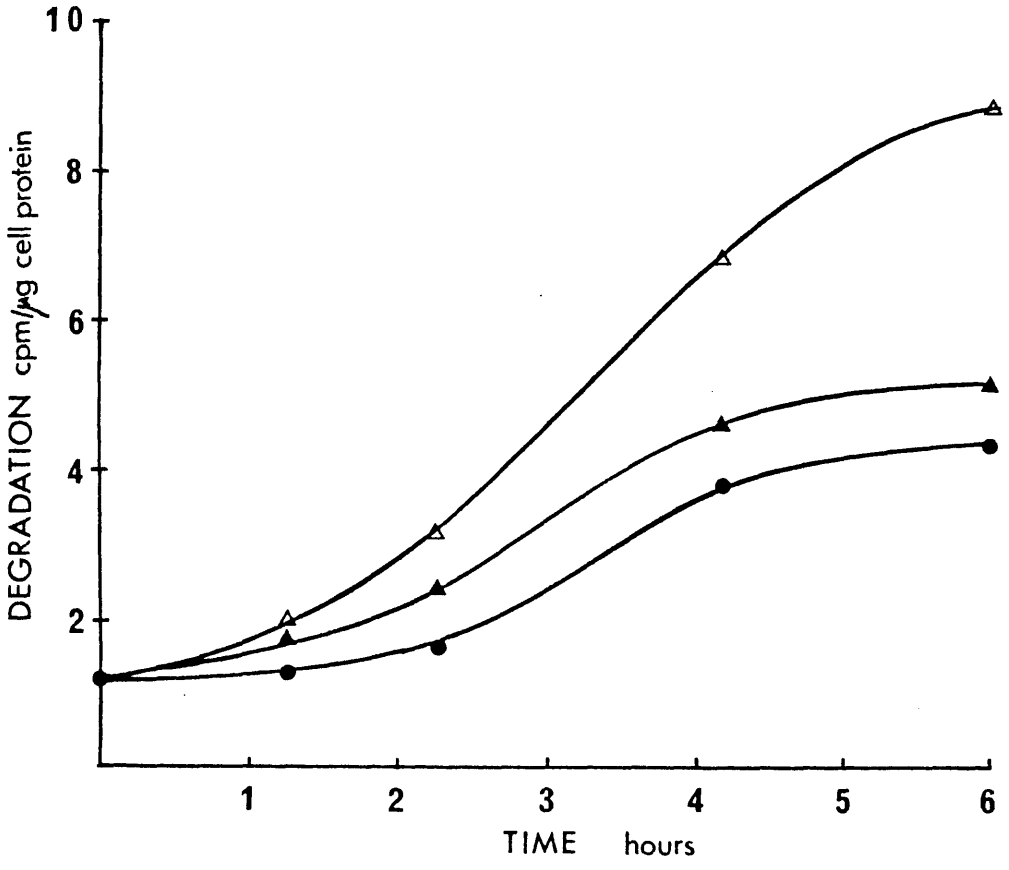


FIGURE R24: Degradation of ^{131}I-Met-LDL by Cultured Macrophages (J774) after Exposure to MDP-Ac-BSA.

The cells were exposed to MDP-Ac-BSA for 18 hours then challenged with 150 µg/ml (1 x 10^6 cpm/ml) of ^{131}I-Met-LDL.

△: Control

▲: cells preincubated with 250 µg/ml MDP-Ac-BSA (200 µg MDP)

●: cells preincubated with 500 µg/ml MDP-Ac-BSA (400 µg MDP)

(each point was the mean of duplicate experiments).

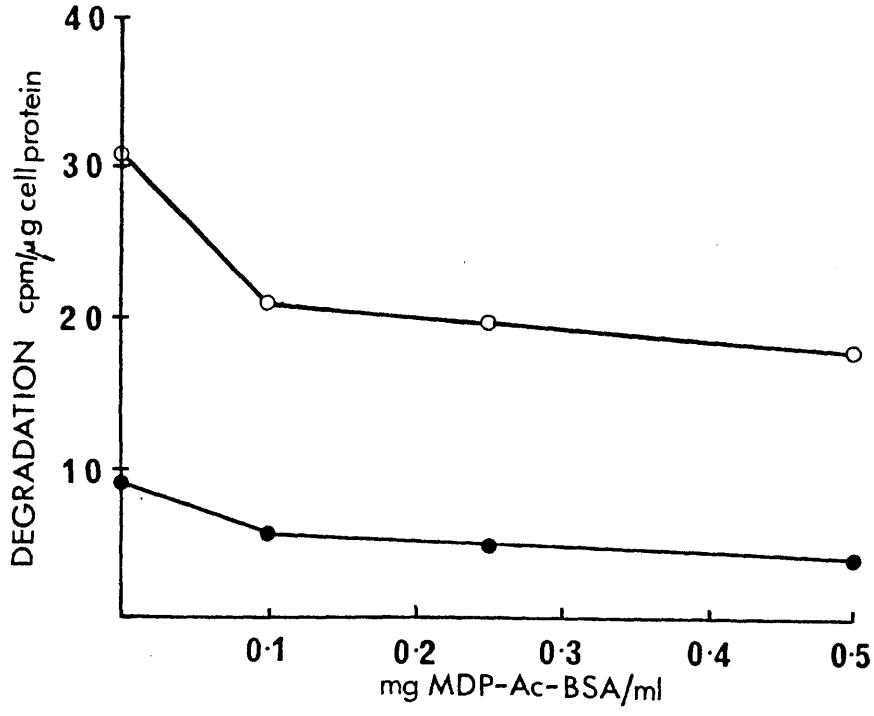


FIGURE R25: Dose Response Curve for Increasing Concentration of MDP-Ac-BSA in Cultured Macrophages (J774).

o: Effect of increasing dose of MDP-Ac-BSA on the degradation of ^{125}I-LDL.

●: Effect of increasing dose of MDP-Ac-BSA on the degradation of ^{131}I-Met-LDL.

The concentration of MDP-Ac-BSA was determined as BSA equivalents. 500 µg of MDP-Ac-BSA measured as BSA equivalents contains 400 µg MDP. Degradation was calculated 6 hours after experiment began.

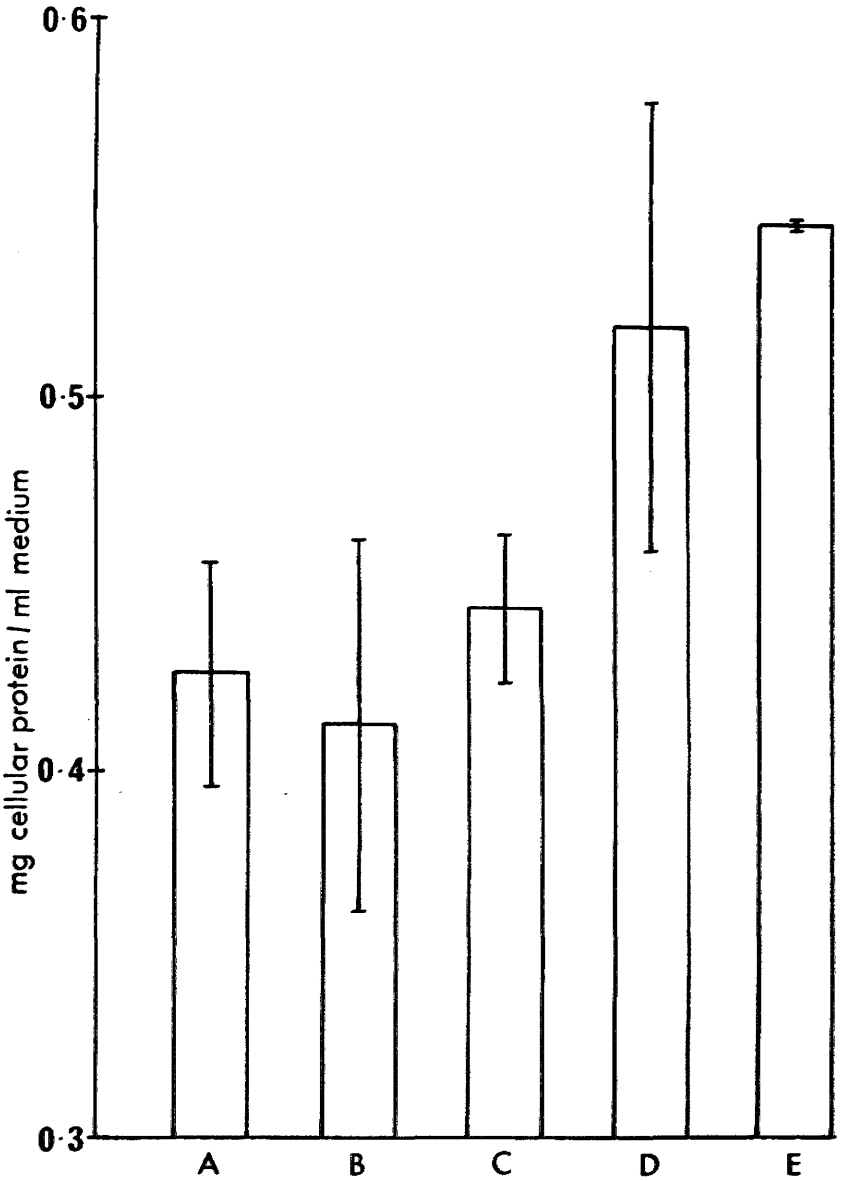


FIGURE R26: Effect of Increasing Concentration of MDP-Ac-BSA on Cellular Protein.

A: 500 µg/ml MDP-Ac-BSA (n = 8)
B: 250 µg/ml MDP-Ac-BSA (n = 8)
C: 100 µg/ml MDP-Ac-BSA (n = 7)
D: control (0 µg/ml MDP-Ac-BSA) (n = 8)
E: 500 µg/ml Ac-BSA (n = 3)

unpaired t-test

control vs A $p < 0.01$
control vs B $p < 0.01$
control vs C $p < 0.01$
control vs E $p > 0.5$

6.6.2: Effect of MDP-Ac-BSA on the Degradation of β-VLDL by Cultured Macrophages

J774 mouse macrophages treated with 500 ug of MDP-Ac-BSA per ml culture medium were shown to degrade β-VLDL at a much reduced rate compared to that of control cells and those treated with 500 ug Ac-BSA/ml (Fig. R27). LDL degradation, measured at the same time, was also reduced and although much more β-VLDL than LDL was degraded the reduction after treatment with MDP-Ac-BSA was similar (55% for β-VLDL and 51% for LDL; Fig. R28).

6.7: Examination of Control Rabbit LDL and LDL from Rabbits Treated with MDP-Ac-BSA or Ethyl Oleate by Ion Exchange Column Chromatography.

Control rabbit LDL (LDL) subjected to ion exchange column chromatography with an increasing sodium chloride concentration gave a single protein peak (Fig. R29) which eluted when the conductivity of the NaCl solution was 6.50 ± 0.45 mMHO (Fig. R32).

LDL from rabbits treated with MDP-Ac-BSA (MDP-LDL) produced two peaks under the conditions described above (Fig. R30). The peak which eluted first (P_1-MDP-LDL) was collected in sodium chloride solution with a conductivity of 6.85 ± 0.37 mMHO which was not significantly different from that of LDL (Fig. R32), however, the second peak eluted in sodium chloride

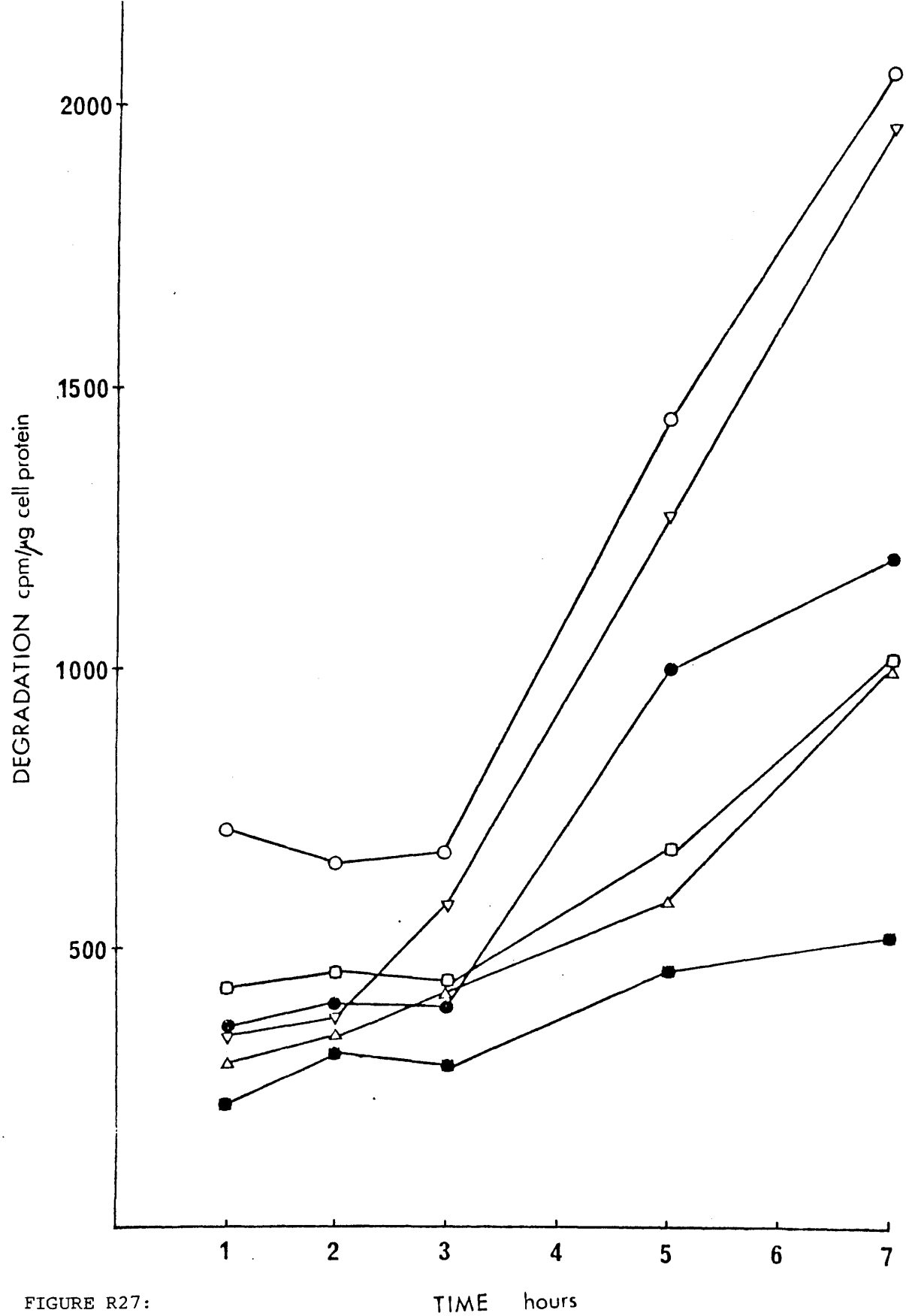


FIGURE R27:

Degradation of LDL and β-VLDL by Cultured Macrophages in the Control State and After Treatment with MDP-Ac-BSA.

- o β-VLDL control
- □ LDL control
- ▽ β-VLDL + 500 µg/ml Ac-BSA
- △ LDL + 500 µg/ml Ac-BSA
- ● β-VLDL + 500 µg/ml MDP-Ac-BSA
- ■ LDL + 500 µg/ml MDP-Ac-BSA

(each point was the mean of duplicate experiments)

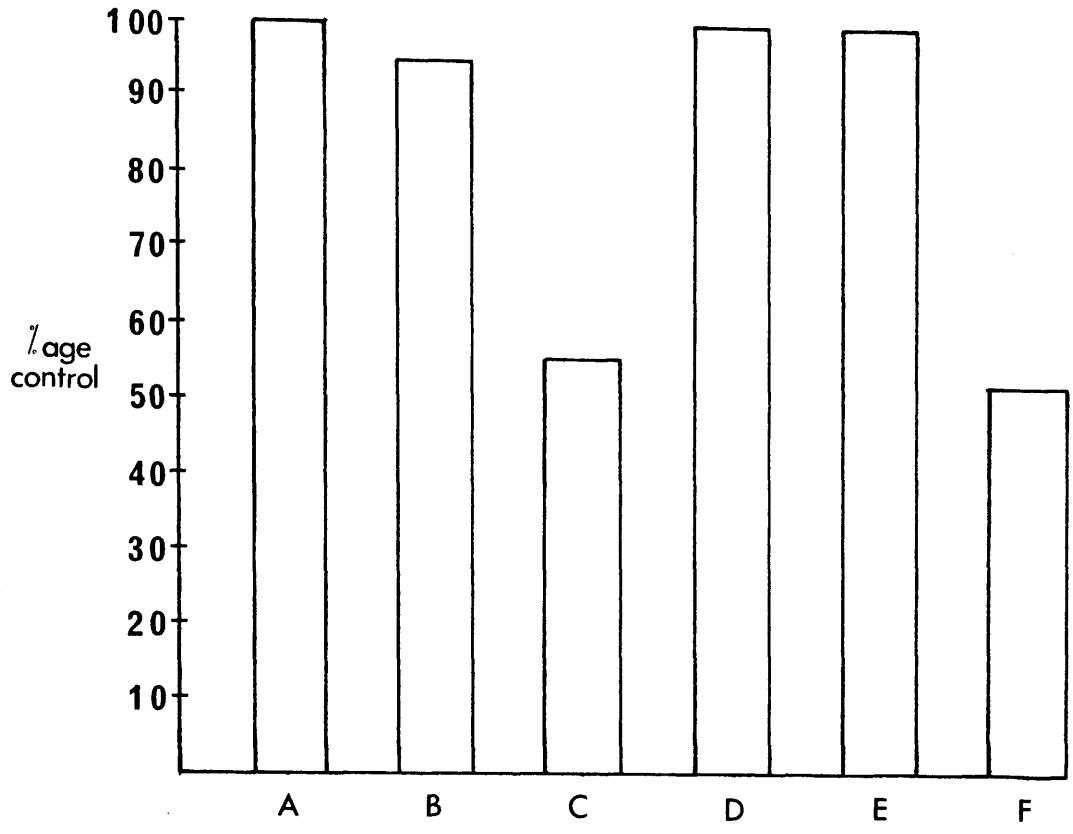


FIGURE R28: Effect of MDP-Ac-BSA on the Degradation of βVLDL and LDL by Cultured Macrophages (J774).

Percent of the amount of degradation of VLDL or LDL occurring in the control state after 7 hours incubation.

A: βVLDL control
B: βVLDL after treatment with Ac-BSA
C: βVLDL after treatment with MDP-Ac-BSA
D: LDL control
E: LDL after treatment with Ac-BSA
F: LDL after treatment with MDP-Ac-BSA

(each point is the mean of duplicate experiments)

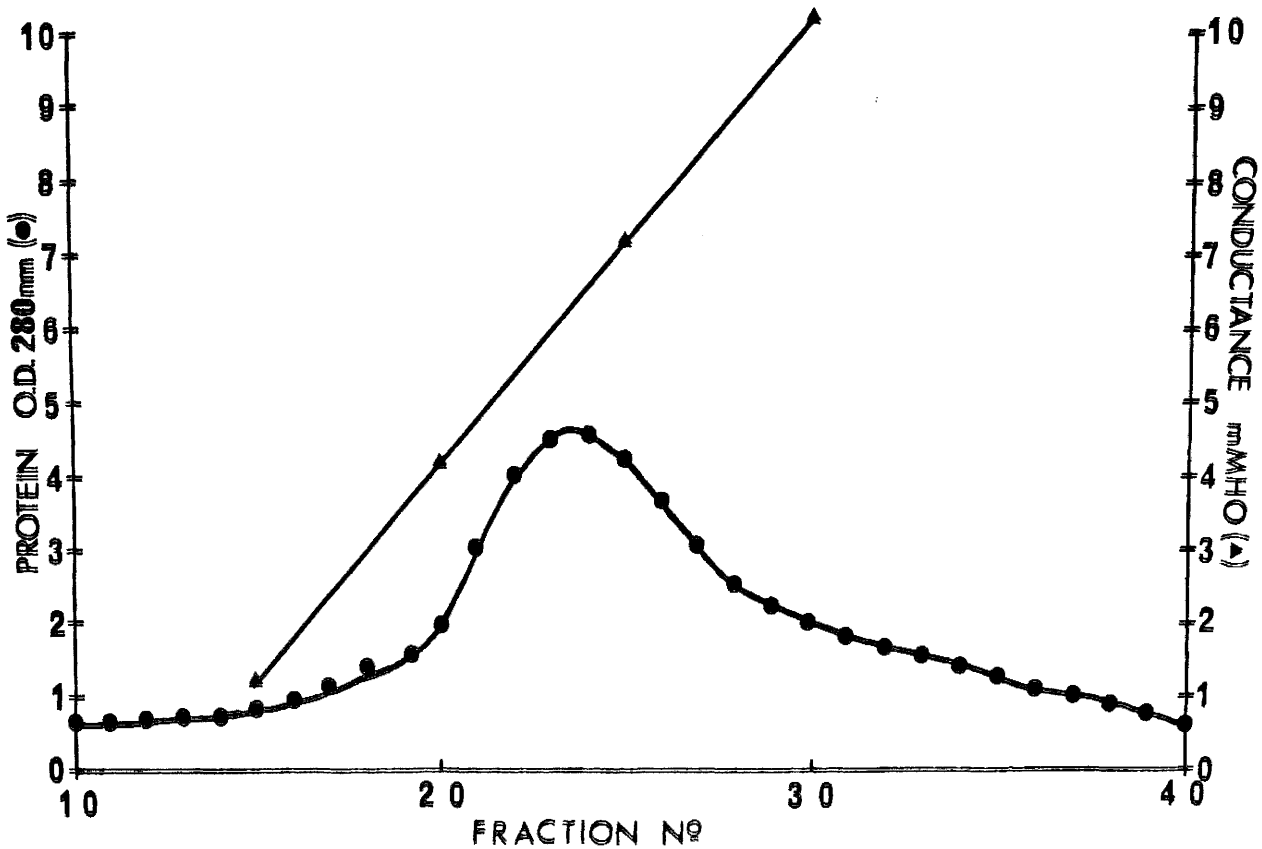


FIGURE R29: Anion Exchange Column Chromatography: Control LDL.

Eluted protein concentration (●) was measured spectrophotometrically at 280 nm and plotted against fraction number. The conductance of each fraction was measured in mMHO (▲) and used to determine the conductivity of the peak protein fraction.

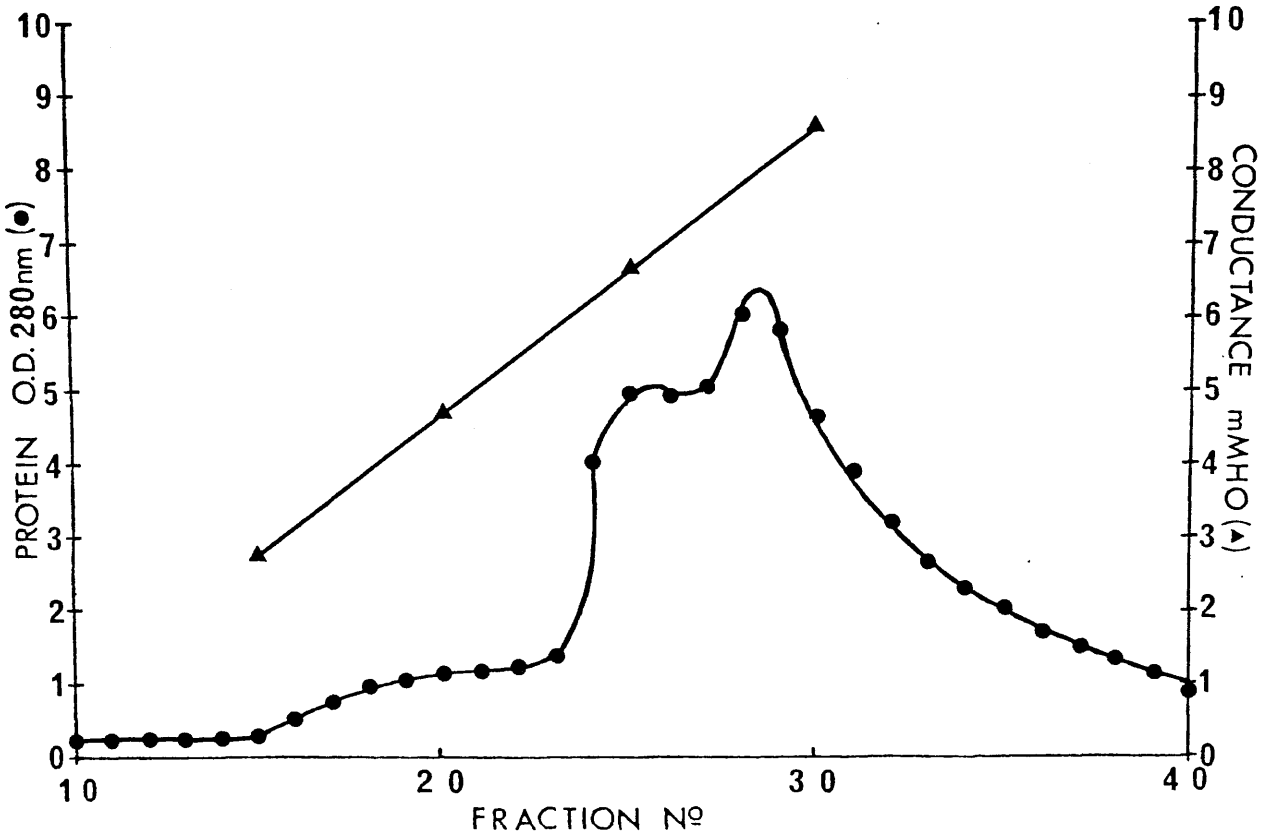


FIGURE R30: Anion Exchange Column Chromatography: MDP-Ac-BSA Treated Rabbit LDL.

Eluted protein concentration (●) was measured spectrophotometrically and the conductance of each fraction (▲) used to determine the conductivity of the protein peaks.

solution of conductivity 7.90 ± 0.35 mMHO which was significantly different from LDL ($p<0.001$; Fig. R32).

LDL from rabbits treated with ethyl oleate (PE-LDL) yielded one protein peak in NaCl solution of conductivity 7.17 ± 0.38 mMHO (Fig. R31) which was significantly different from LDL at the 2% level (Fig. R32).

The difference between the MDP-LDL and PE-LDL may arise from various sources, for example rabbits treated with MDP-Ac-BSA are in the acute phase of RE suppression whereas rabbits receiving ethyl oleate are injected over a period of days and are in a new steady-state, chronic phase. As has already been shown (section 6.1.2.) uptake of MDP-Ac-BSA by the RE system does not follow the same pattern as uptake of lipid vesicles and this may also give rise to differences.

6.8: Examination of Control Rabbit LDL and LDL from Rabbits Treated with Ethyl Oleate (PE-LDL) by Continuous Gradient Density Ultracentrifugation.

Control LDL and PE-LDL were labelled with ^{131}I and ^{125}I respectively and centrifuged at 39,000 rpm for 18 or 24 hours in a gradient of sodium bromide from 1.010 - 1.060 kg/l in 0.010 kg/l steps. At the end of that period the gradient was continuous, as determined by refractometry, and was sampled in 0.5 ml aliquots which were then assayed for their content of radioactive

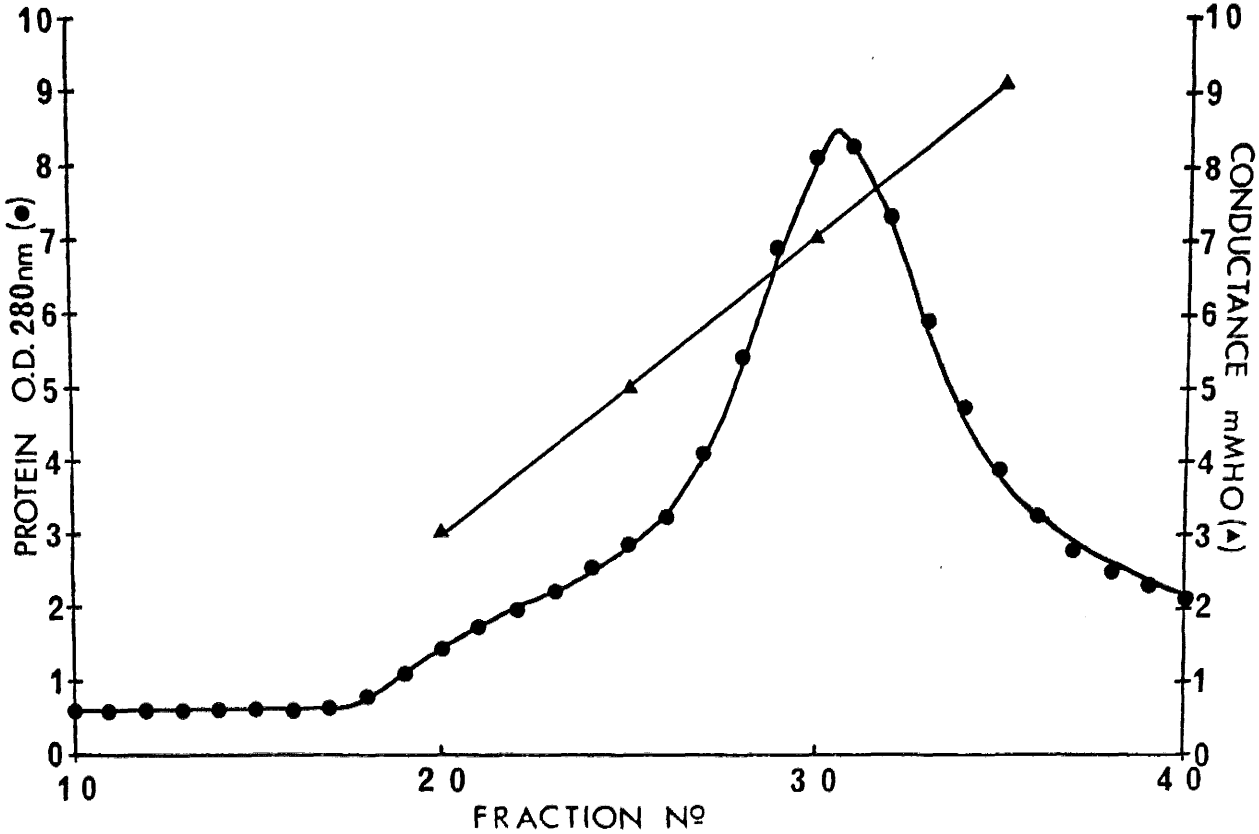


FIGURE R31: Anion Exchange Column Chromatography: Ethyl Oleate Treated Rabbit LDL.

Eluted protein concentration (●) was determined spectrophotometrically at 280 nm and the conductance of each fraction (▲) used to determine the conductivity of the protein peak.

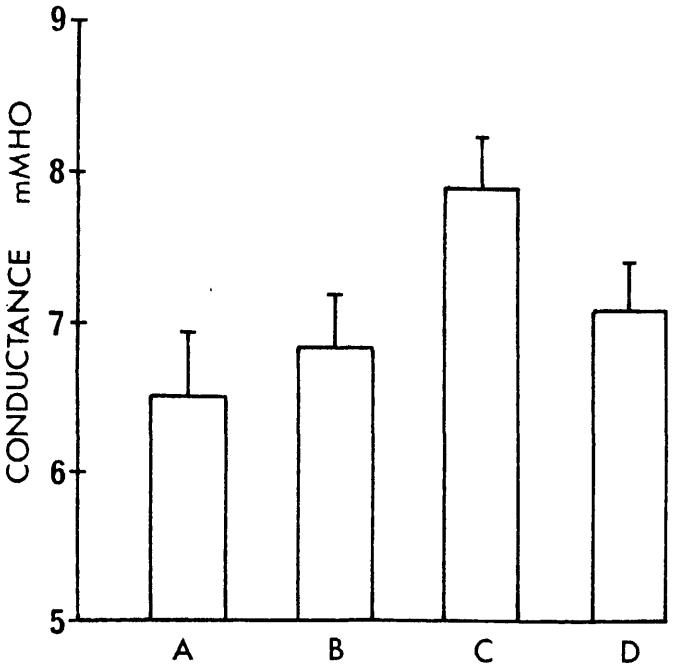


FIGURE R32: Protein Peak Conductivities for Control LDL, and LDL from Rabbits Treated with MDP-Ac-BSA or Ethyl Oleate.

A: Control LDL:- 6.50 ± 0.45 mMHO
B: First eluted peak from MDP-Ac-BSA treated Rabbit LDL:- 6.85 ± 0.37 mMHO.
C: Second eluted peak from MDP-Ac-BSA treated Rabbit LDL:- 7.90 ± 0.35 mMHO.
D: Ethyl oleate treated Rabbit LDL:- 7.17 ± 0.38 mMhO.

unpaired t-test

control LDL (n = 6) vs B (n = 4) $p > 0.10$
control LDL (n = 6) vs C (n = 4) $p < 0.001$
control LDL (n = 6) vs D (n = 7) $p < 0.02$.

lipoprotein. ^{131}I-LDL yielded a single radioactive band which was most concentrated at a density of 1.0298 ± 0.0013 kg/l (Fig. R33; Table R3). ^{125}I-PE-LDL was found to have two radioactive bands (Fig. R34), the first (P_1-PE-LDL) was concentrated in sodium bromide solution of density 1.0274 ± 0.0010 kg/l, which was significantly less than control LDL ($p < 0.02$; Table R3). P_1-PE-LDL was also significantly different form the second band which had a mean density of 1.0415 ± 0.0010 kg/l at the 0.1% level. P_2-PE-LDL was significantly denser than control LDL ($p < 0.001$; Table R4).

Ethyl oleate treated rabbit LDL, then, appears to contain two species separable by density but not by ion exchange column chromatography. These bands equilibrate in solutions of density 1.0274 and 1.0415 kg/l because centrifugation for 24 hours instead of 18 hours does not alter the fractions in which they are found (results included in calculation of mean values).

6.9: Cholesterol to Protein Ratio in Control Rabbit LDL and in LDL from Rabbits Treated with Ethyl Oleate (PE-LDL).

Protein content of the LDLs was determined and expressed in mg/dl, cholesterol concentration was also expressed in these terms and the ratio of cholesterol to protein content of each species of LDL calculated. Control LDL had a cholesterol/protein ratio of 0.70 ± 0.13 which was

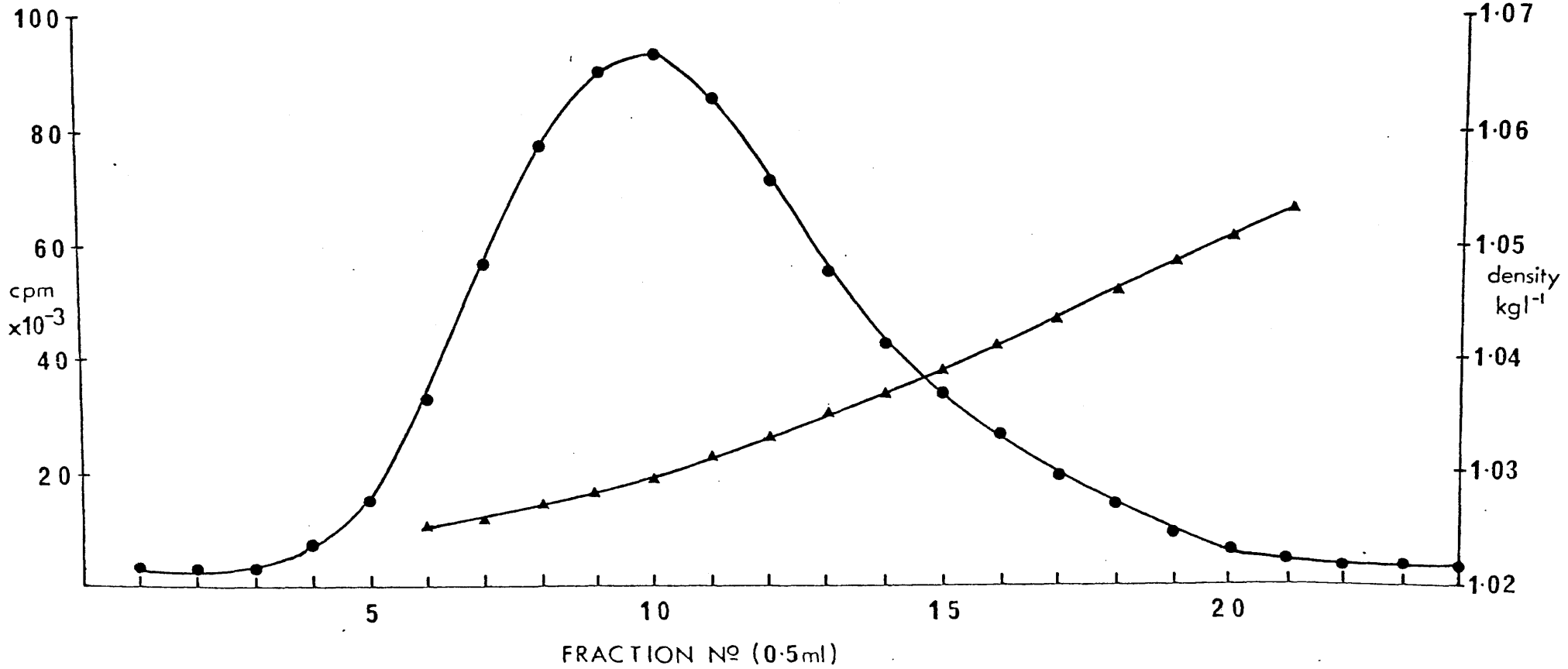


FIGURE R33: Density Gradient Ultracentrifugation Profile:- Control LDL.

The continuous gradient (▲) was formed from a step wise gradient from 1.010 - 1.060 kg/ml. Radioactivity in each fraction was determined and plotted against fraction number (●).

	CONTROL RABBIT LDL	ETHYL OLEATE TREATED RABBIT LDL PEAK 1	ETHYL OLEATE TREATED RABBIT LDL PEAK 2
1	1.0315	1.0267	1.0420
2	1.0290	1.0287	1.0420
3	1.0290	1.0276	1.0400
4	1.0315	1.0267	1.0420
5	1.0290	-	-
6	1.0290	-	-
MEAN	1.0298±0.0013	1.1274±0.0010	1.0415±0.0010

TABLE R3: Density in kg/l of the Peak Fraction from Control Rabbit LDL and LDL from Rabbits Treated with Ethyl Oleate.

unpaired t-test

Control LDL vs Peak 1 from ethyl oleate treated rabbit LDL $p < 0.02$
Control LDL vs Peak 2 from ethyl oleate treated rabbit LDL $p < 0.001$
Peak 1 vs Peak 2 ethyl oleate treated rabbit LDL $p < 0.001$

Control LDL n = 6
Ethyl oleate LDL n = 4

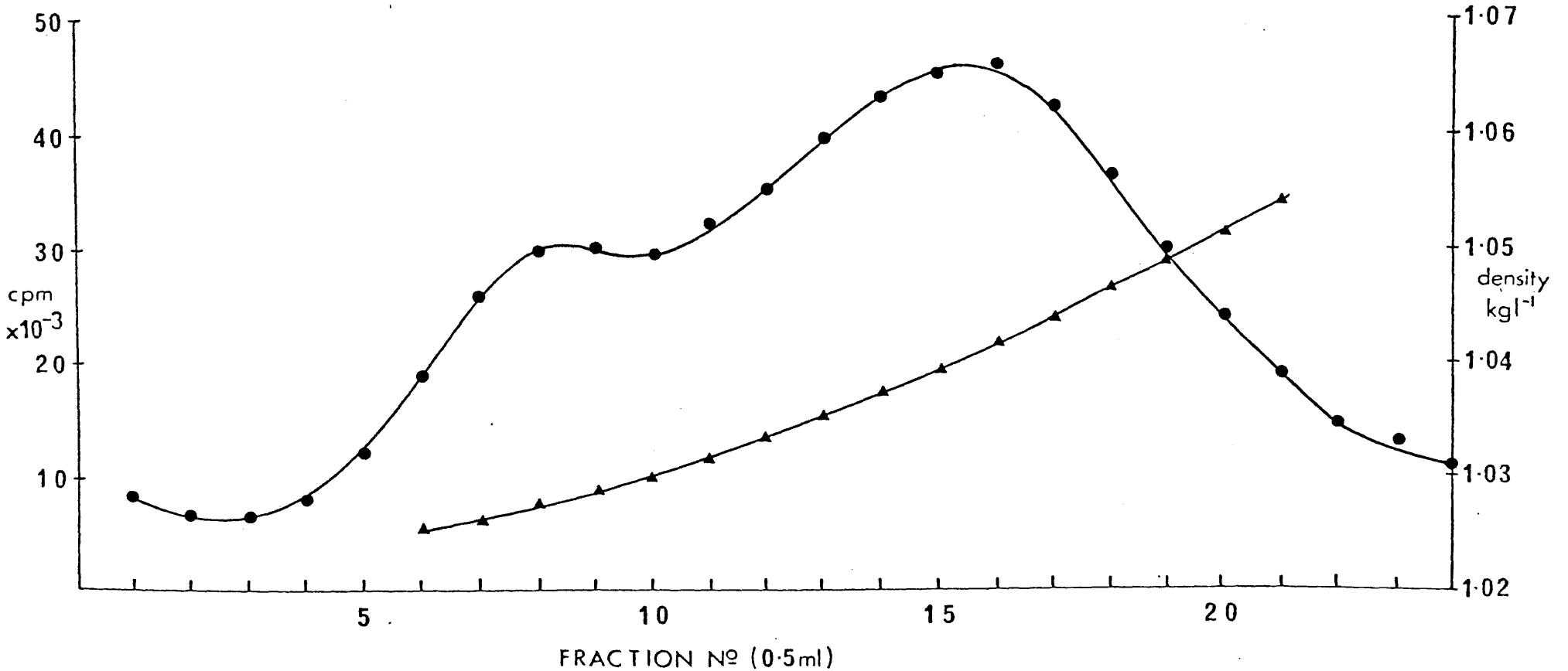


FIGURE R34: Density Gradient Ultracentrifugation Profile:- Ethyl Oleate Treated Rabbit LDL.

The continuous gradient (▲) was formed using a step wise gradient from 1.010 - 1.060 kg/l. Radioactivity in each fraction was determined and plotted against fraction number (0.5 ml volume) (●).

	PROTEIN mg/dl	CHOLESTEROL mg/dl	CHOLESTEROL / PROTEIN
CONTROL RABBIT LDL			
1	76.4	50.3	0.66
2	83.5	49.9	0.60
3	75.6	63.9	0.87
MEAN			[a] 0.70 ± 0.13
ETHYL OLEATE TREATED RABBIT LDL			
1	171.2	195.4	1.14
2	79.0	86.7	1.10
3	67.0	71.6	1.07
MEAN			[a] 1.10 ± 0.04

TABLE R4: Cholesterol to Protein Ratio in Control LDL and Ethyl Oleate Treated Rabbit LDL.

unpaired t-test, control LDL vs ethyl oleate treated rabbit LDL $p < 0.01$

significantly lower than that for total PE-LDL at 1.10 ± 0.04 ($p < 0.01$; Table R4).

6.10: Plasma Clearance of Control LDL and LDL from Ethyl Oleate Treated Rabbits (PE-LDL) in Control Rabbits.

^{131}I-LDL and ^{125}I-PE-LDL were injected simultaneously in control rabbits and the decay of radioactivity from the plasma recorded (Fig. R35). PE-LDL was removed faster than control LDL, the fractional clearance rate (FCR) for PE-LDL being 3.41 ± 0.31 pools per day and the FCR for control LDL being 1.83 ± 0.26 pools per day ($p << 0.001$; Table R5).

After 30 hours the animals were anaesthetised, exsanguinated and uptake of radioactive tracer into a range of tissues determined (Fig. R36). This tissue uptake of radioactivity was expressed as the difference between the relative uptake of control LDL and PE-LDL. The liver, spleen and adrenals showed the largest difference when this parameter was calculated per gram of tissue, catabolising larger amounts of PE-LDL. Difference in relative uptake for whole organs revealed that the liver was the most important organ in the degradation of PE-LDL (Fig. R37), being more than 350 fold as active as the spleen. It was noted that the tissues showing larger uptakes of PE-LDL over control LDL were rich in macrophages of the RE system (section 4, 158).

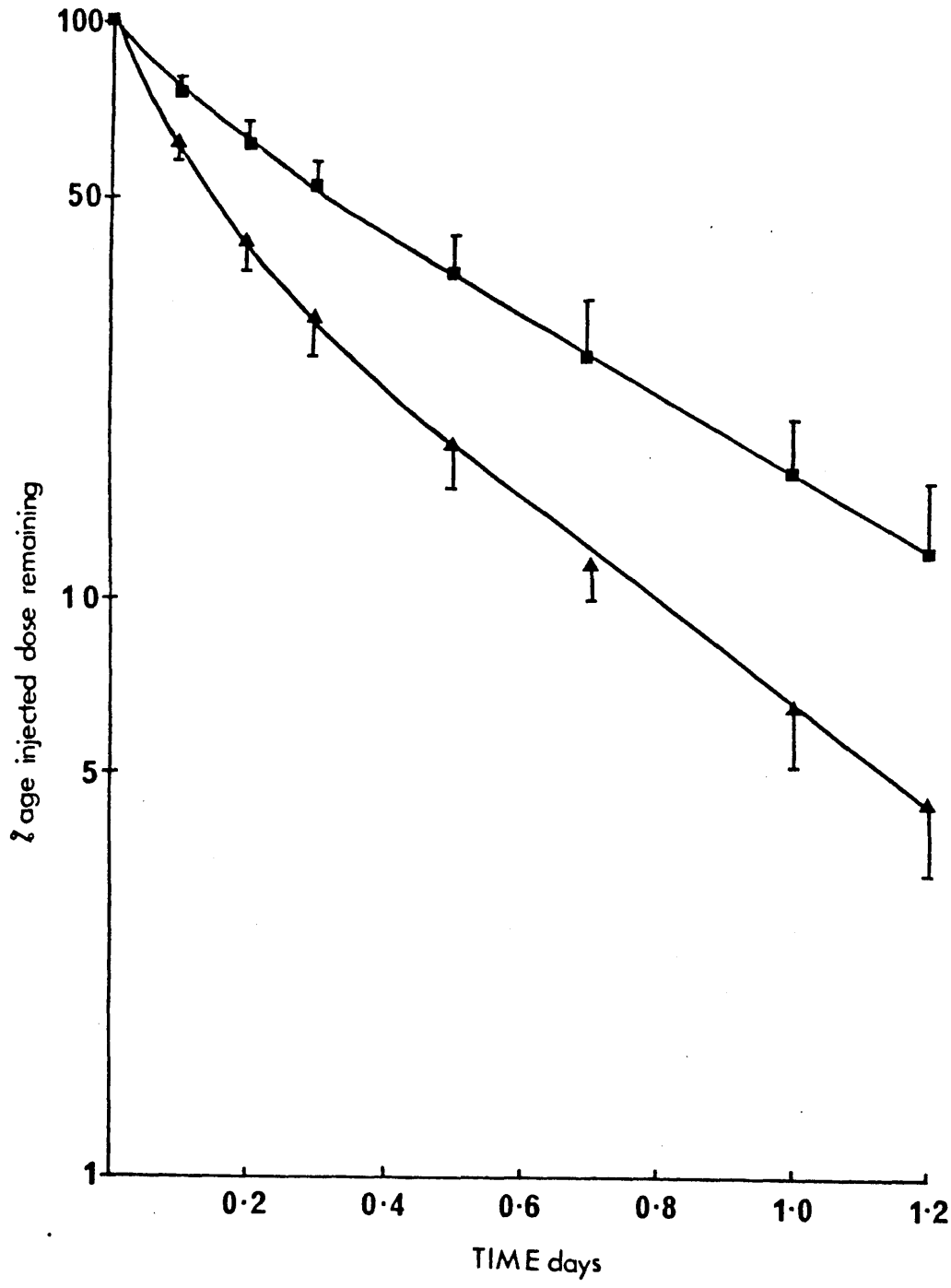


FIGURE R35: Plasma Clearance Rates of Control Rabbit LDL and LDL from Rabbits Treated with Ethyl Oleate in Control Rabbits.

500 ug (1 x 10^7cpm) of ^{125}I-LDL and ^{131}I ethyl oleate treated rabbit LDL (^{131}I-PE-LDL) were injected into control rabbits. The plasma clearance rates of each tracer was followed for 30 hours and expressed as percent injected dose.

■ : control LDL
▲ : PE-LDL

TABLE R5:

FCR CONTROL LDL (pools/day)	FCR PE-LDL (pools/day)
1.57	3.17
1.65	3.40
2.00	3.43
2.11	3.43
	3.96
	3.42
	2.86
	3.16
	3.47
	3.76
1.83 $\pm$ 0.26	3.41 $\pm$ 0.31

Unpaired t-test vs control $p << 0.001$

Table shows fractional catabolic rate (FCR) in pools per day for clearance of control LDL and ethyl oleate treated rabbit LDL (PE-LDL).

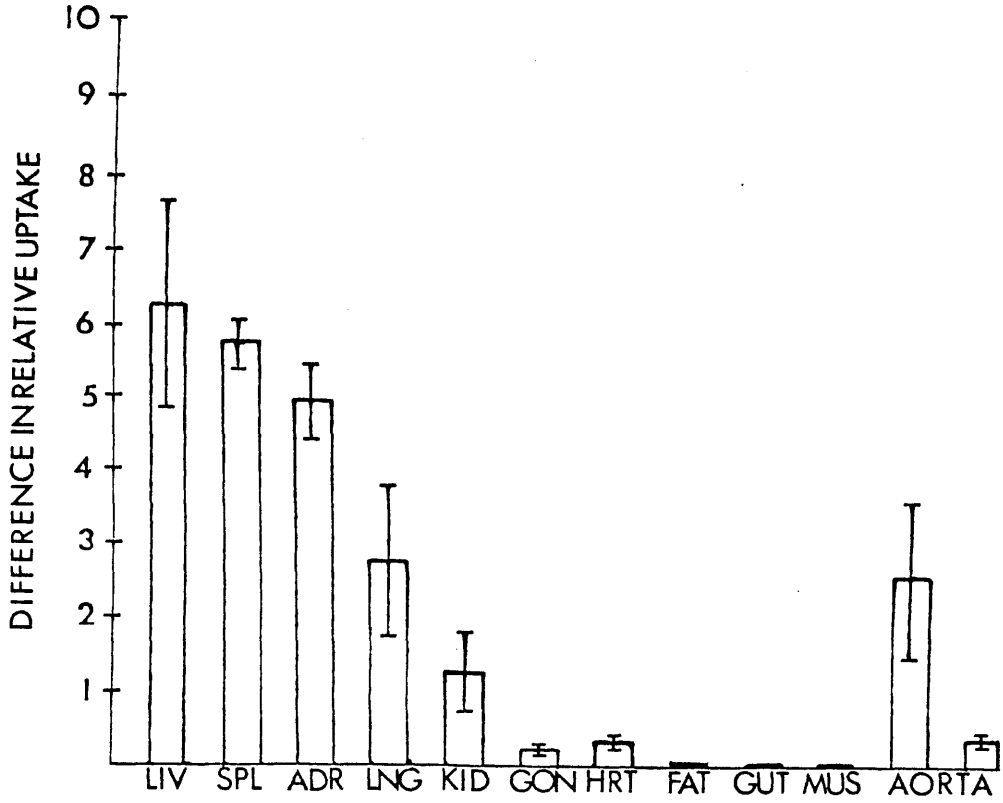


FIGURE R36: Difference in Relative Tissue Uptakes of ^{125}I-LDL and ^{131}I-PE-LDL in NZW Rabbits.

30 hours after the injection of the tracers the animals were exanguinated and the relative tissue uptakes were calculated.

$$\text{Relative Tissue Uptake} = \frac{\text{cpm/gram tissue}}{\text{cpm/ml plasma}}$$

The RTU for control LDL was subtracted from the RTU for PE LDL (n = 4) to demonstrate those tissues which had the highest affinity for PE LDL. The aorta comprised two sections which were:

1) the arch and upper thoracic and
2) the lower thoracic and upper abdominal.

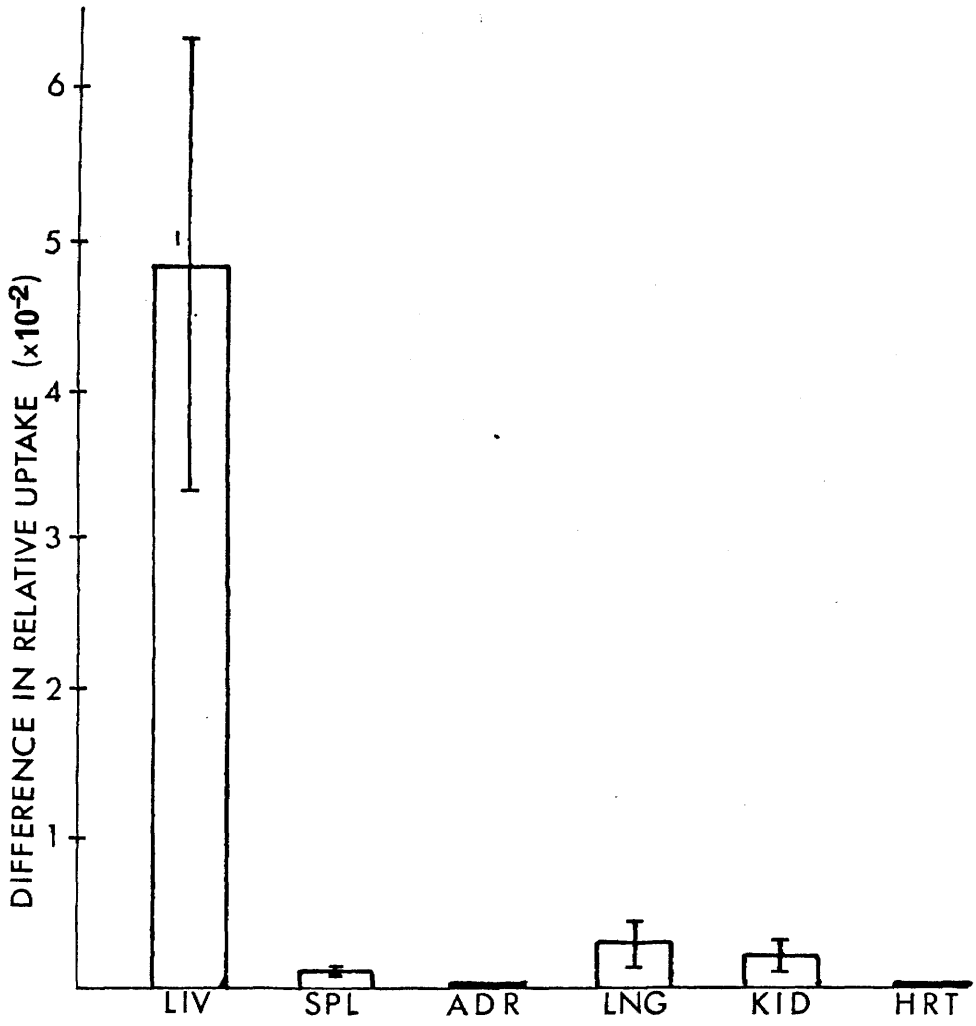


FIGURE R37: Difference in Relative Uptakes of ^{125}I-LDL and ^{131}I-PE LDL in the organs of NZW Rabbits.

Total organ uptake was calculated from the relative organ uptake of each tracer times the weight, in grams, of the organ. The difference between ^{131}I-PE LDL and ^{125}I-LDL was then determined.

6.11: Competition for the Acetyl-LDL Receptor on Macrophages by Control Rabbit LDL, Human LDL and LDL from Ethyl Oleate Treated Rabbits (PE-LDL).

^{125}I-Acetyl-LDL degradation was measured in J774 mouse macrophages and compared to the amount of degradation which occurred in the presence of measured amounts of competitor. Increasing amounts of human and control rabbit LDL reduced Ac-LDL catabolism by only 20% and 30% respectively when 1,000 µg LDL protein/ml was present (Fig. R38 and R39). PE-LDL, however, was able to reduce the amount of Ac-LDL degradation to less than 20% of the control value at a concentration of 1000 ug LDL protein/ml (Fig. R38 and R39). EDTA at a concentration 10 fold greater than working concentrations had no effect on the degradation of Ac-LDL.

The results obtained in these experiments were plotted to demonstrate increasing inhibition of Ac-LDL degradation with increasing concentrations of the LDLs (Fig. R40).

Fibroblasts were challenged with ^{125}I-LDL and ^{131}I-PE-LDL and the degradation of the two markers determined as described. These cells did not degrade PE-LDL faster than control LDL (Fig. R41), supporting further the evidence that macrophages of the RE system are responsible for the catabolism of PE-LDL.

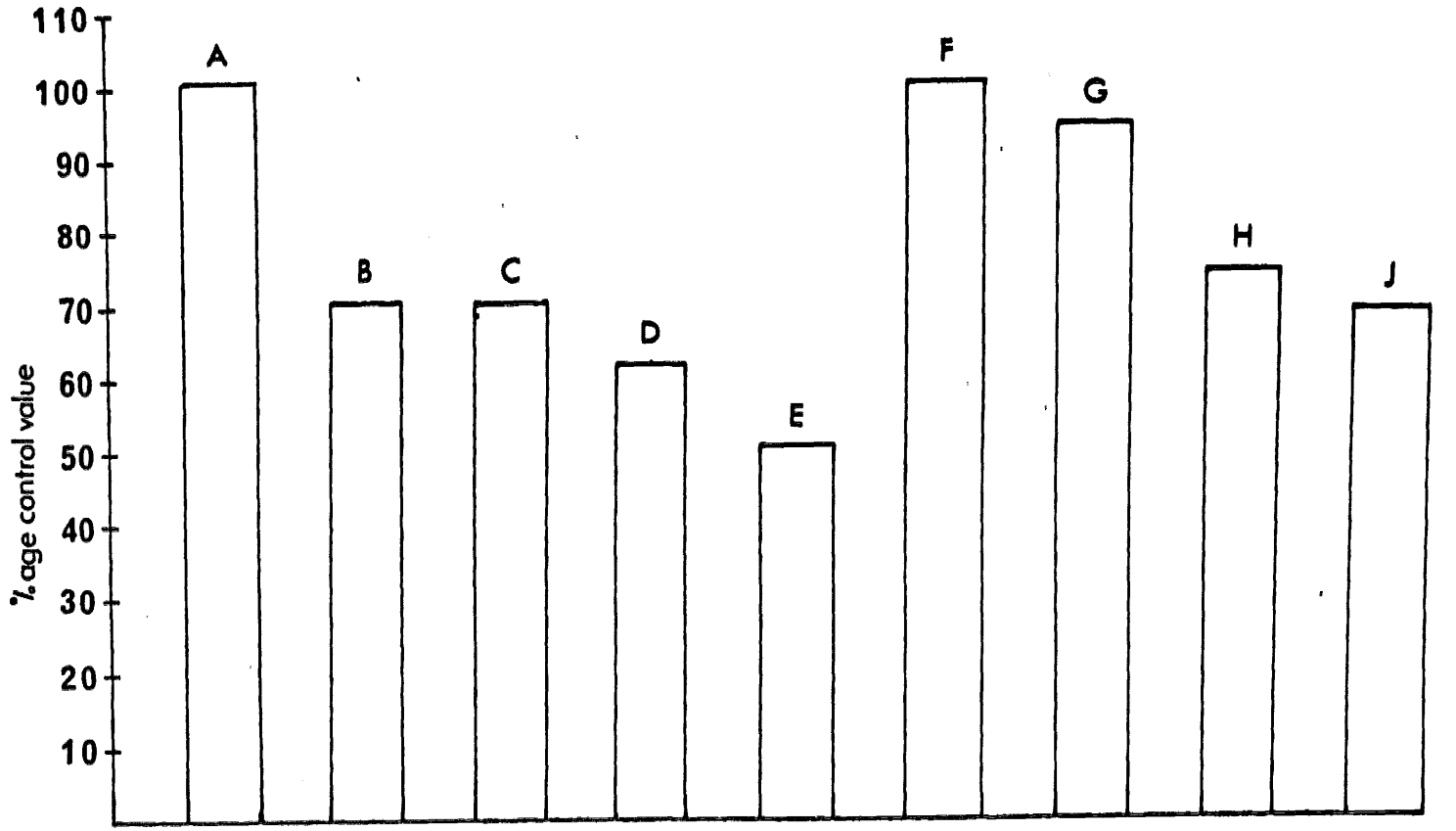


FIGURE R38: Competition with ^{125}I-Ac-LDL by PE-LDL and Human LDL for Degradation by the Acetyl LDL Receptor on Cultured Macrophages.

A:	control (no addition)	F:	control (no addition)
B:	40 µg/ml PE-LDL	G:	200 µg/ml Human LDL
C:	81 µg/ml PE-LDL	H:	500 µg/ml Human LDL
D:	164 µg/ml PE-LDL	J:	1000 µg/ml Human LDL
E:	273 µg/ml PE-LDL		

(each was the mean of duplicate experiments)

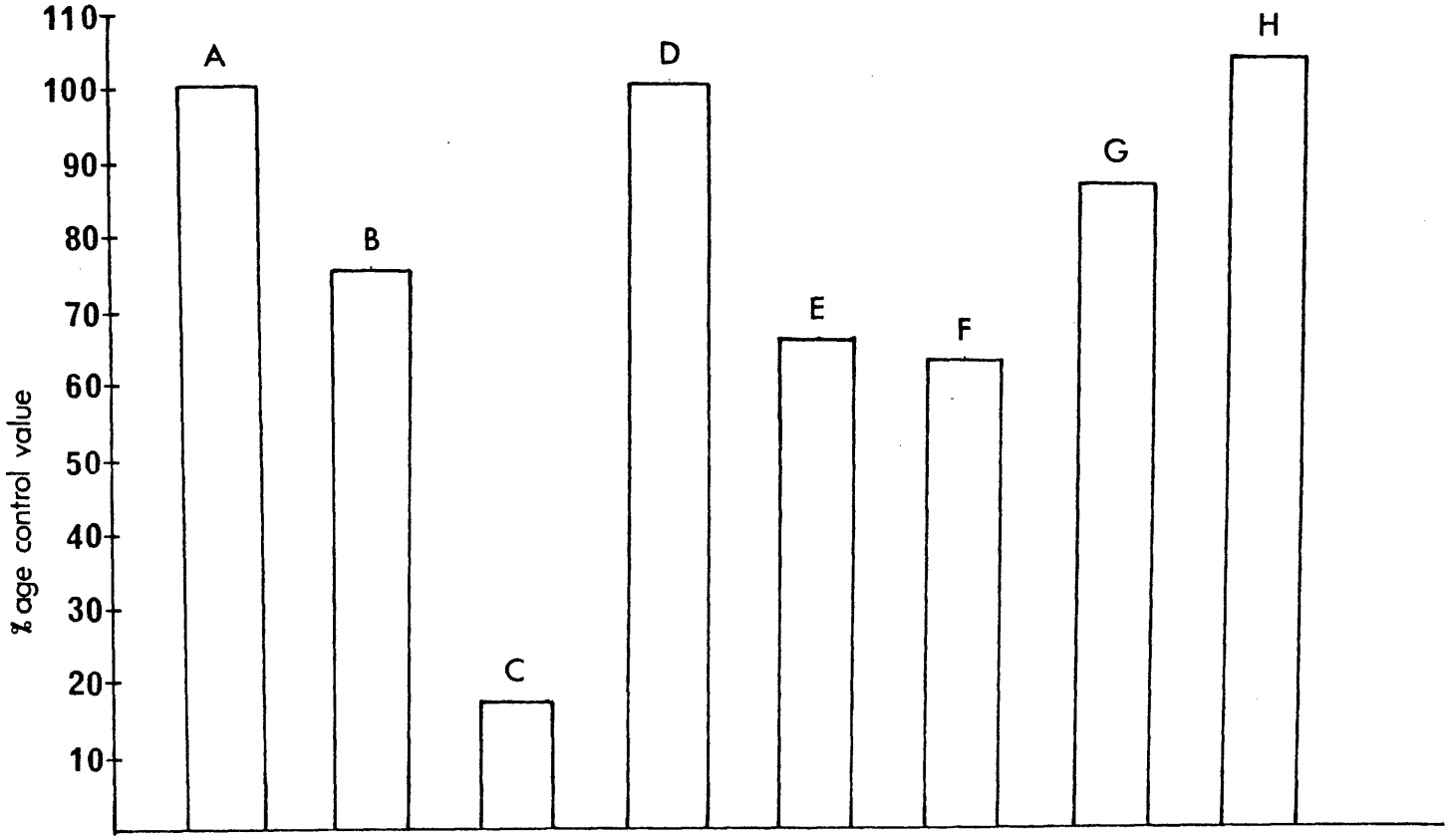


FIGURE R39: Competition with ^{125}I-Ac-LDL by PE-LDL, Human LDL and Control Rabbit LDL for Degradation by the Acetyl LDL receptor on Cultured Macrophages.

A:	control (no addition)	E:	500 µg/ml control rabbit LDL
B:	500 µg/ml PE-LDL	F:	1000 µg/ml control rabbit LDL
C:	1000 µg/ml PE-LDL	G:	1000 µg/ml human LDL
D:	control (no addition)	H:	250 µg/ml EDTA

250 ug/ml EDTA was 10 times the concentration used in the other assays.

(each was the mean of duplicate experiments)

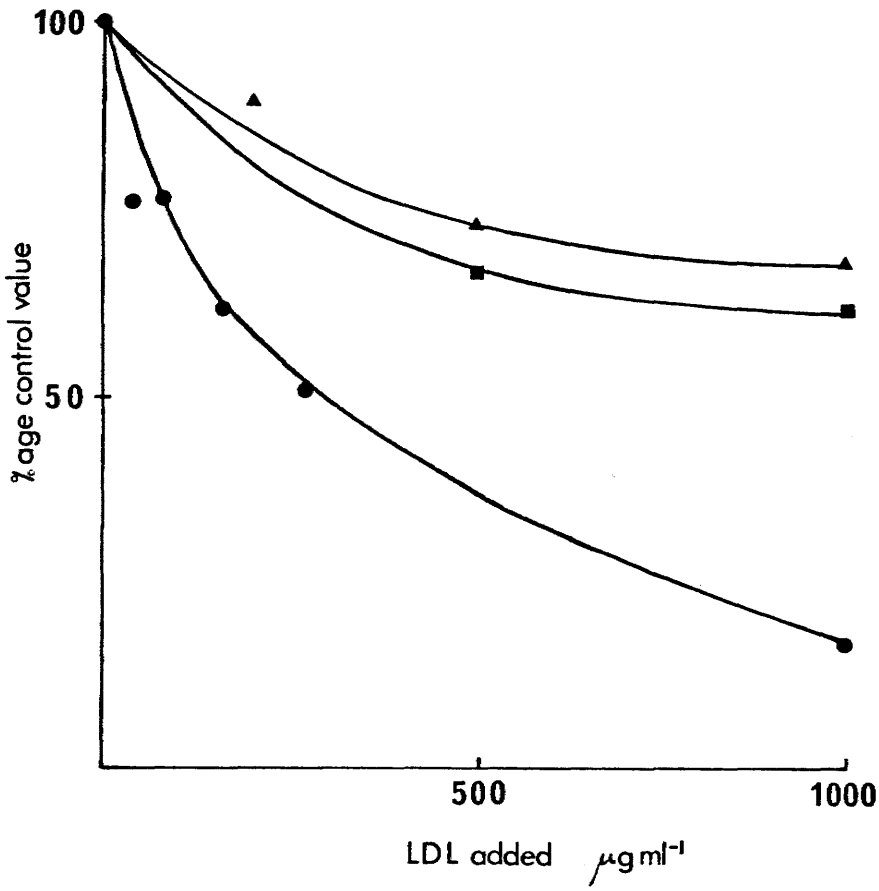


FIGURE R40: Competition with ^{125}I-Ac-LDL by Control Rabbit LDL, Human LDL and PE-LDL for the Acetyl LDL Receptor on Cultured Macrophages.

● : PE-LDL

▲ : Human LDL

■ : Control Rabbit LDL

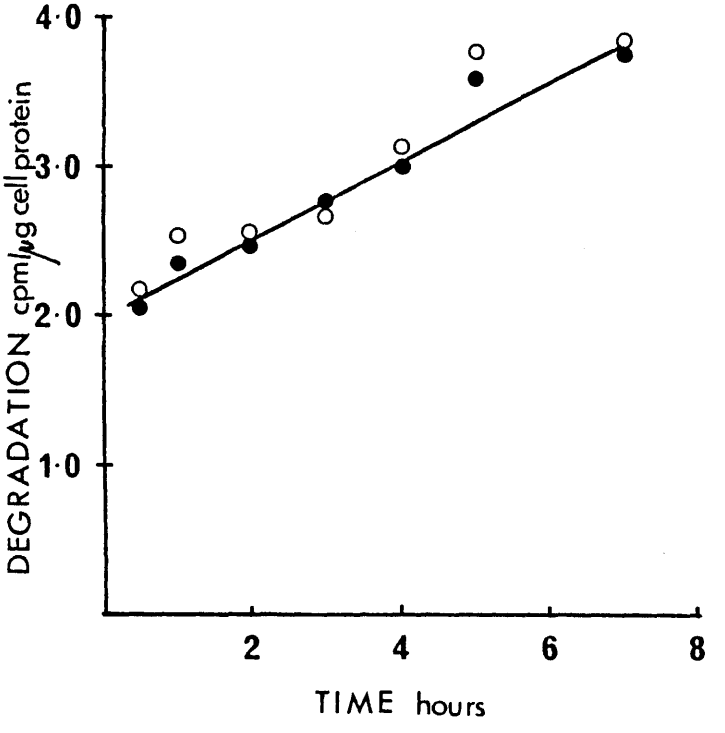


FIGURE R41: Degradation of Control Rabbit LDL and Ethyl Oleate Treated Rabbit LDL (PE-LDL) by Cultured Fibroblasts.

o : PE-LDL

● : Control LDL

(Each point was the mean of duplicate experiments).

6.12: Clearance of Control LDL, after Reisolation from Control Rabbits and from Rabbits Treated with Ethyl Oleate, from the Plasma of Control Rabbits.

LDL from the plasma of control rabbits was divided into two aliquots and labelled with ^{125}I and ^{131}I. The ^{125}I-LDL was injected into control animals and the ^{131}I-LDL into ethyl oleate treated animals. After 48 hours the radiolabelled LDLs were reisolated. Agarose gel electrophoresis showed that the LDL reisolated from ethyl oleate treated rabbits had a higher electrophoretic mobility (towards the anode) than the LDL from the control rabbits (Fig. R42). Both tracers were injected simultaneously into control animals and plasma clearance rates measured. Plasma clearance of the LDL incubated *in vivo* in ethyl oleate treated rabbits was greater than that incubated in control animals (Fig. R43). Screening in treated rabbits appears to confer the properties of PE-LDL onto injected control LDL.

6.13: Clearance of PE-LDL and Control LDL, after Reisolation from Control Rabbits, from the Plasma of Control Animals.

^{125}I-PE-LDL was injected into the plasma of control rabbits and the radioactivity in the LDL density range reisolated 24 hours later. ^{131}I-LDL was treated in the same way in separate animals. Both LDLs were then injected, together, into control rabbits. Agarose gel

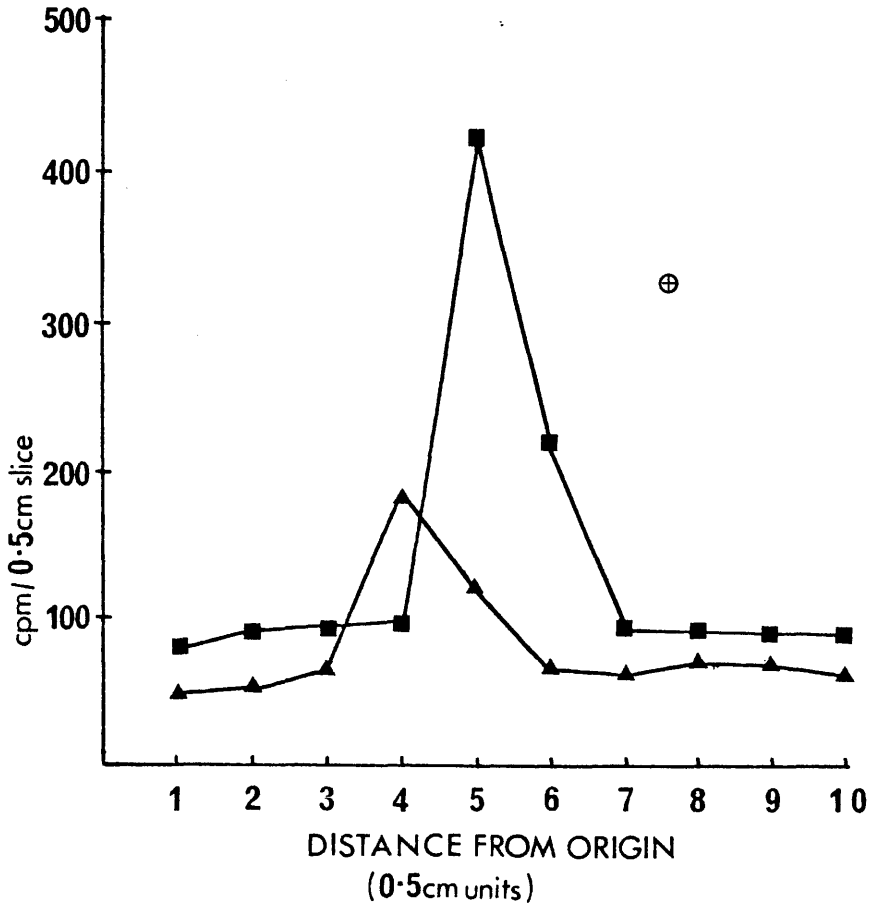


FIGURE R42: Agarose Gel Electrophoresis of Control LDL Screened in Control and Ethyl Oleate Treated Rabbits.

■: Control LDL screened in vivo in an Ethyl Oleate Treated Rabbit.

▲: Control LDL screened in vivo in a Control Rabbit.

Run Time - 30 minutes.

⊕ indicates direction of anode.

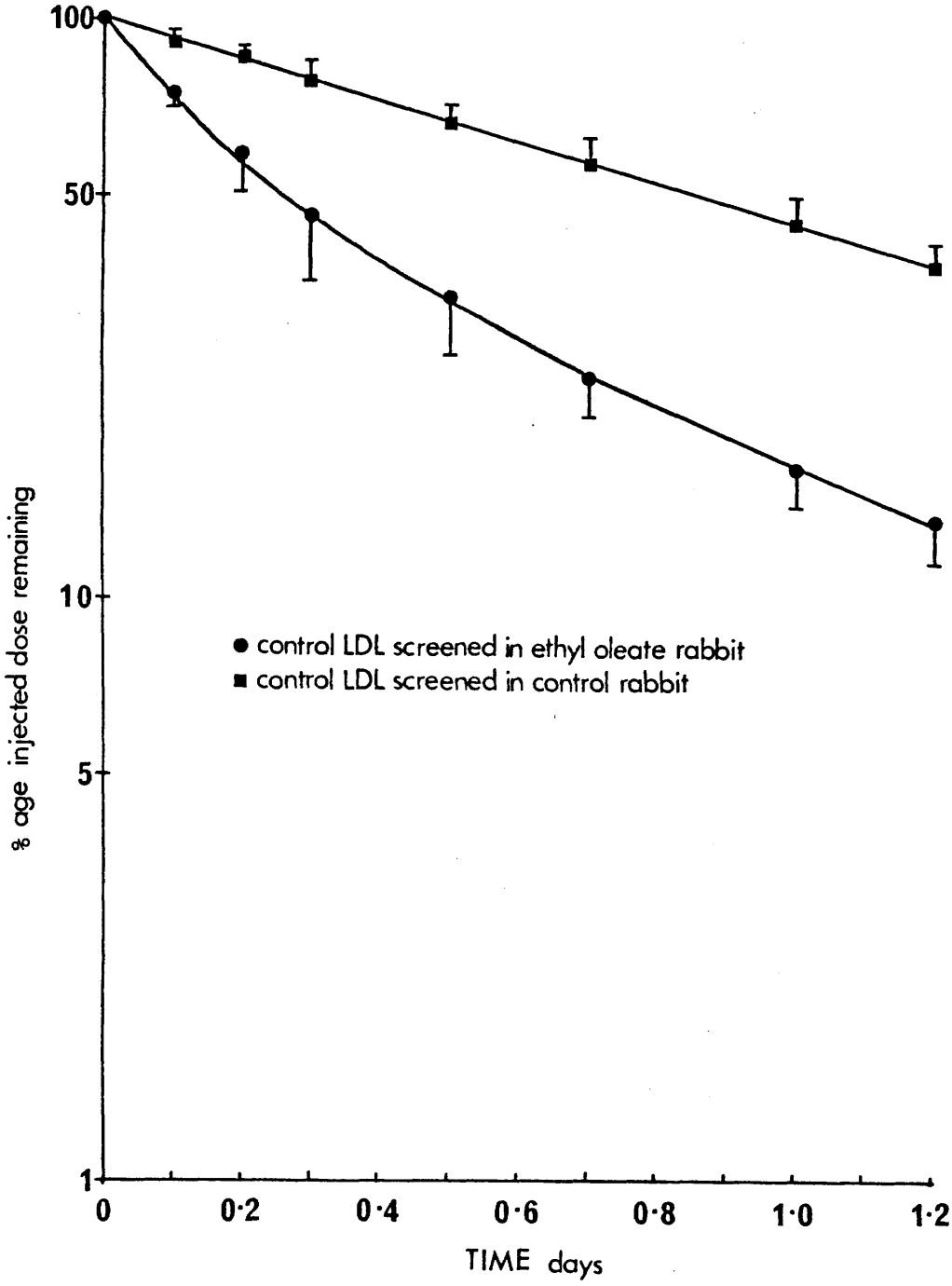


FIGURE R43: Plasma Clearance of Control LDL Biologically Screened in Control and Ethyl Oleate Treated Rabbits.

^{125}I-LDL was injected into control rabbits and reisolated 48 hours later, ^{131}I-LDL was injected into ethyl oleate treated rabbits and reisolated 48 hours later. The two tracers were then injected simultaneously into control animals and the plasma clearance rates followed for 30 hours.

(n = 5 in both cases)

electrophoresis failed to show any difference in electrophoretic mobility of the two LDLs (Fig. R44) and, further, there was no difference between these LDLs and the electrophoretic mobility of control LDL reisolated from control rabbits (section 6.12; Fig. R42).

Plasma clearance from control rabbits occurred at the same rate for both LDLs (Fig. R45) and it was noted, at the same rate as the LDL incubated in control rabbits (Fig. R43).

6.14: Effects of Cholesterol Feeding and Ethyl Oleate on the Plasma Clearance of Native and Met-LDL in Rabbits.

Plasma clearance rates were determined for native and met-LDL in two groups of rabbits. Group 1 received a diet supplemented with 1% cholesterol for 40 days and were injected with ethyl oleate emulsion twice per week for the duration of the experiment. The second group were fed the same diet for the same time but received no ethyl oleate.

In group 2, there appeared to be a slight difference between the clearance rates for the tracers, native-LDL being removed faster (Fig. R46). However, on calculation of fractional clearance rates this difference was shown to be not significant ($p>0.5$; Table R6). In group 1, receiving ethyl oleate, there was no difference in the rate of removal of the tracers (Fig. R47) as confirmed by calculation of their FCRs (Table R7). There

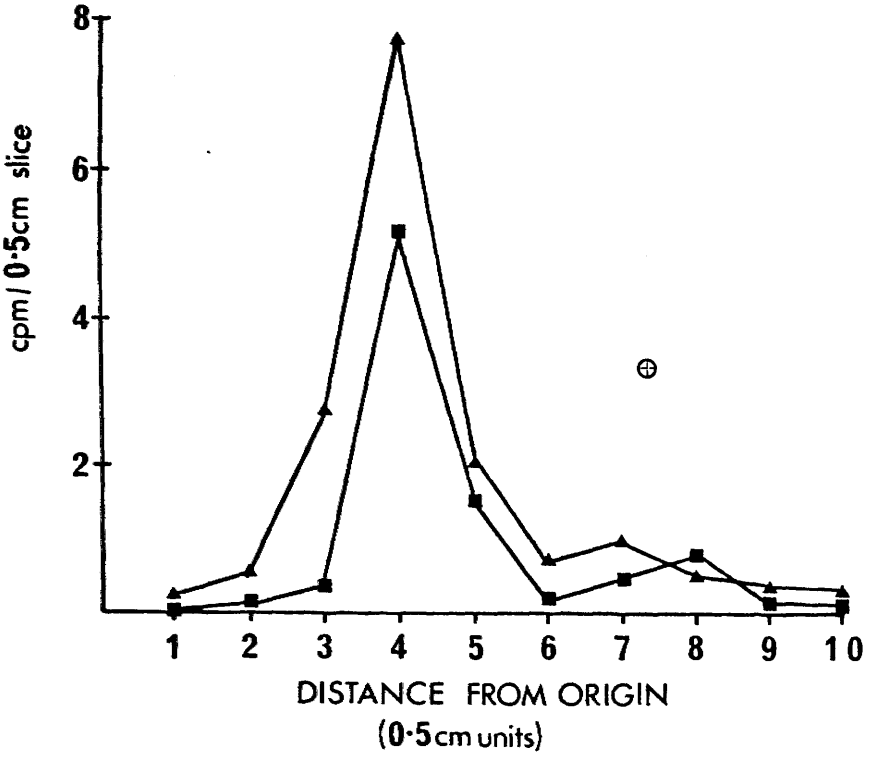


FIGURE R44: Agarose Gel Electrophoresis of Control LDL and PE-LDL Biologically Screened in Control Rabbits.

▲: Control LDL screened in vivo in a control animal.

■: PE-LDL screened in vivo in a control animal.

Run Time = 30 minutes.

(⊕ indicates direction of anode)

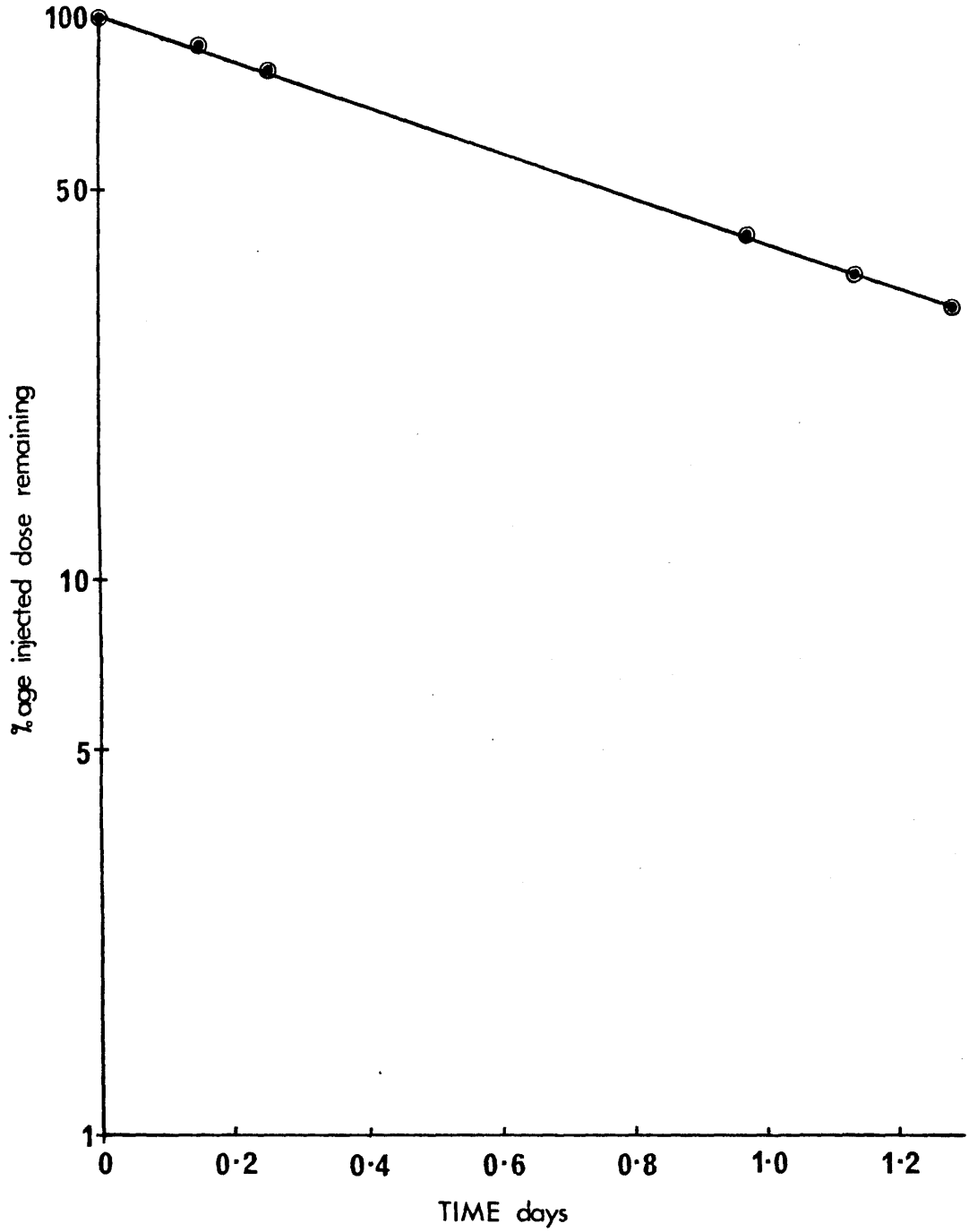


FIGURE R45: Plasma Clearance of Control and Ethyl Oleate Treated Rabbit LDL after Biological Screening in Control Rabbits.

^{131}I-LDL was injected into control NZW rabbits and reisolated after 24 hours.

^{125}I-PE-LDL was injected into control NZW rabbits and reisolated after 24 hours. The tracers were then injected simultaneously into control animals and their plasma clearance rates followed for 30 hours.

●: ^{125}I-PE-LDL reisolated from control animals

o: ^{131}I-LDL reisolated from control animals.

(n = 3)

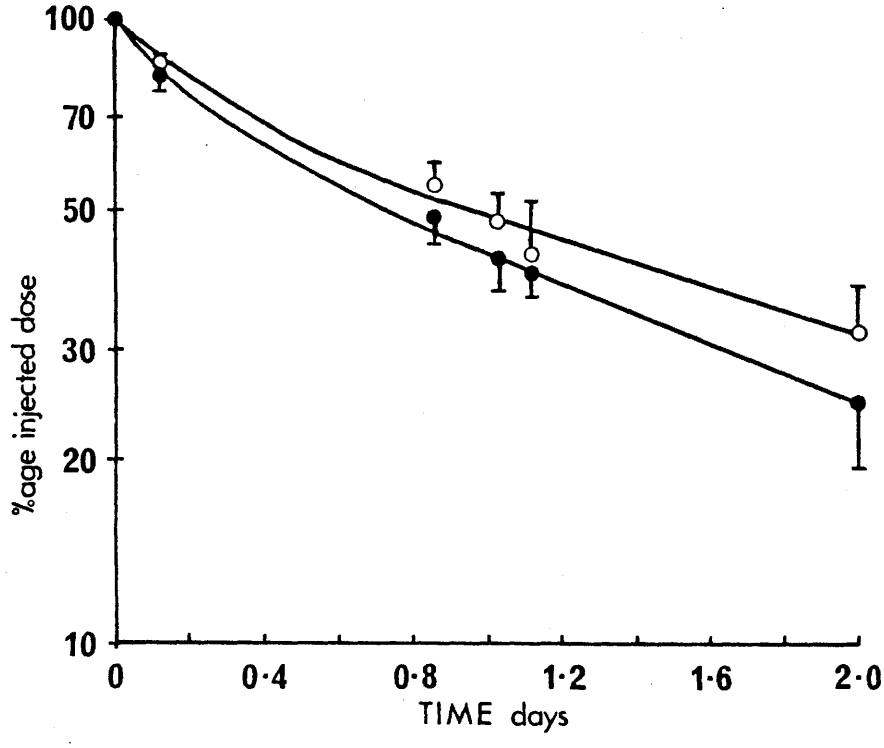


FIGURE R46: Plasma Clearance of ^{125}I-LDL and ^{131}I-Met LDL in NZW Rabbits Fed a Cholesterol Rich Diet.

● : Met-LDL (n = 5)
o : Native-LDL (n = 5)

see Table R6.

RABBIT NO.	FCR NAT-LDL	FCR MET-LDL
6	0.87	-
7	0.77	0.58
8	0.50	0.51
9	0.84	0.64
10	0.62	0.58
MEAN ± 1 S.D.	0.72 ± 0.15	0.58 ± 0.05

TABLE R6:

unpaired t-test Met LDL vs Nat LDL $p > 0.05$

Fractional catabolic rate (FCR) for native LDL (NAT-LDL) and reductively methylated-LDL (MET-LDL) in cholesterol fed animals.

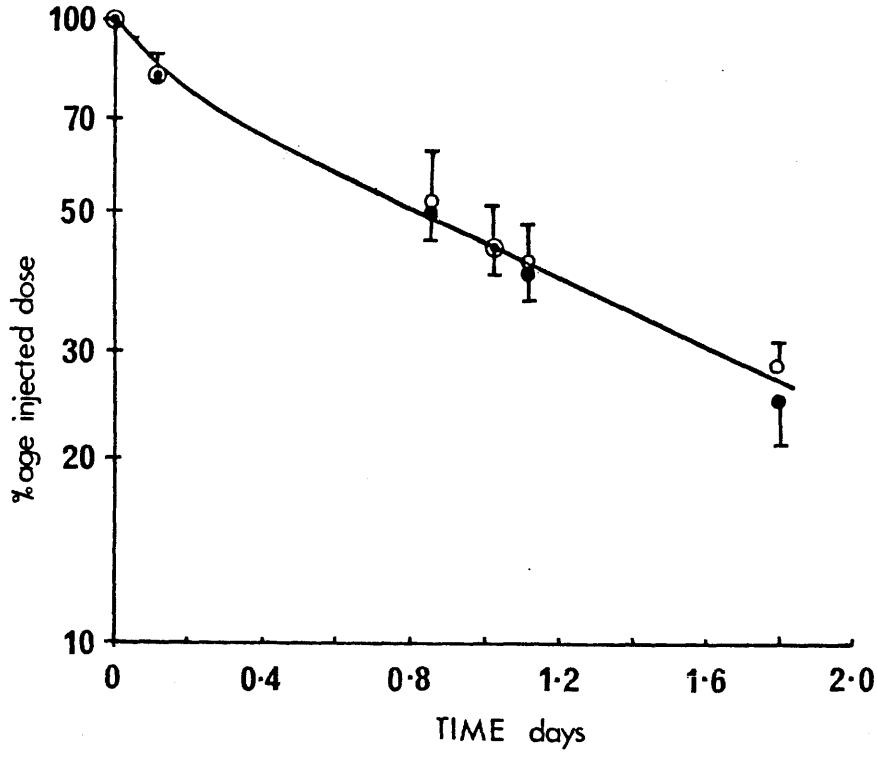


FIGURE R47: Plasma Clearance of ^{125}I-native LDL and ^{131}I-Met LDL in NZW Rabbits Fed Cholesterol Rich Diet and Treated with Ethyl Oleate.

● : Met-LDL (n = 5)
o : Native-LDL (n = 5)

see Table R7.

RABBIT NO:	FCR NAT-LDL	FCR MET-LDL
1	0.81	0.79
2	0.63	0.61
3	1.04	1.22
4	0.69	0.58
5	0.76	0.92
MEAN ± 1 S.D.	0.78 ± 0.15	0.79 ± 0.27

TABLE R7: unpaired t-test nat-LDL vs met-LDL $p >> 0.5$

Fractional catabolic rate (FCR) for native LDL (NAT-LDL) and reductively methylated-LDL (MET-LDL) in rabbits fed a cholesterol rich diet and receiving twice-weekly injections of ethyl oleate emulsion.

was no significant difference between the rate of removal of tracers from group 1 and group 2 ($p>0.1$).

6.15: Effect of Cholesterol Feeding and Ethyl Oleate on the Development of Atherosclerosis in Rabbits.

The percentage of atherosclerotic aortae ((weight of affected areas/weight of aorta) x 100) was compared between two groups of 5 rabbits. Group 1 received a diet supplemented with 1% cholesterol for 40 days and were injected with ethyl oleate twice per week for that time. Group 2 received the same diet as group 1 but were not treated with ethyl oleate. At the end of the 40 day period the rabbits were anaesthetised, exsanguinated and their aortae removed. The percentage affected areas were calculated for both groups (Table R8). Group 1 had a significantly higher percentage of affected surface area than group 2 ($p < 0.001$; two of the worst affected aortae from each group are schematically represented in Figure R48), despite the fact that there was no difference in total cholesterol levels between the groups (Fig. R12). Agarose gel electrophoresis of LDL from two animals of each group revealed that LDL from ethyl oleate treated group 1 had a higher anodic mobility than that from group 2 (Fig. R49).

TREATMENT	ETHYL OLEATE PLUS CHOLESTEROL FEEDING	CHOLESTEROL FEEDING ONLY
1	24.03	3.47
2	44.71	5.43
3	47.97	7.79
4	77.92	14.61
5	89.03	27.77
MEAN $\pm$ 1 S.D.	[a] 56.73 $\pm$ 26.38	[a] 11.81 $\pm$ 9.86

TABLE R8: Affected Areas of the Aortae of NZW Rabbits Fed a Cholesterol Rich Diet and Treated with Ethyl Oleate.

a: unpaired t-test cholesterol diet vs cholesterol diet + ethyl oleate $p < 0.001$

Percent of the surface area of the aortae which were affected by fatty infiltration and raised plaques after feeding with a cholesterol rich diet or treatment with ethyl oleate and a cholesterol rich diet.

See Fig. R48.

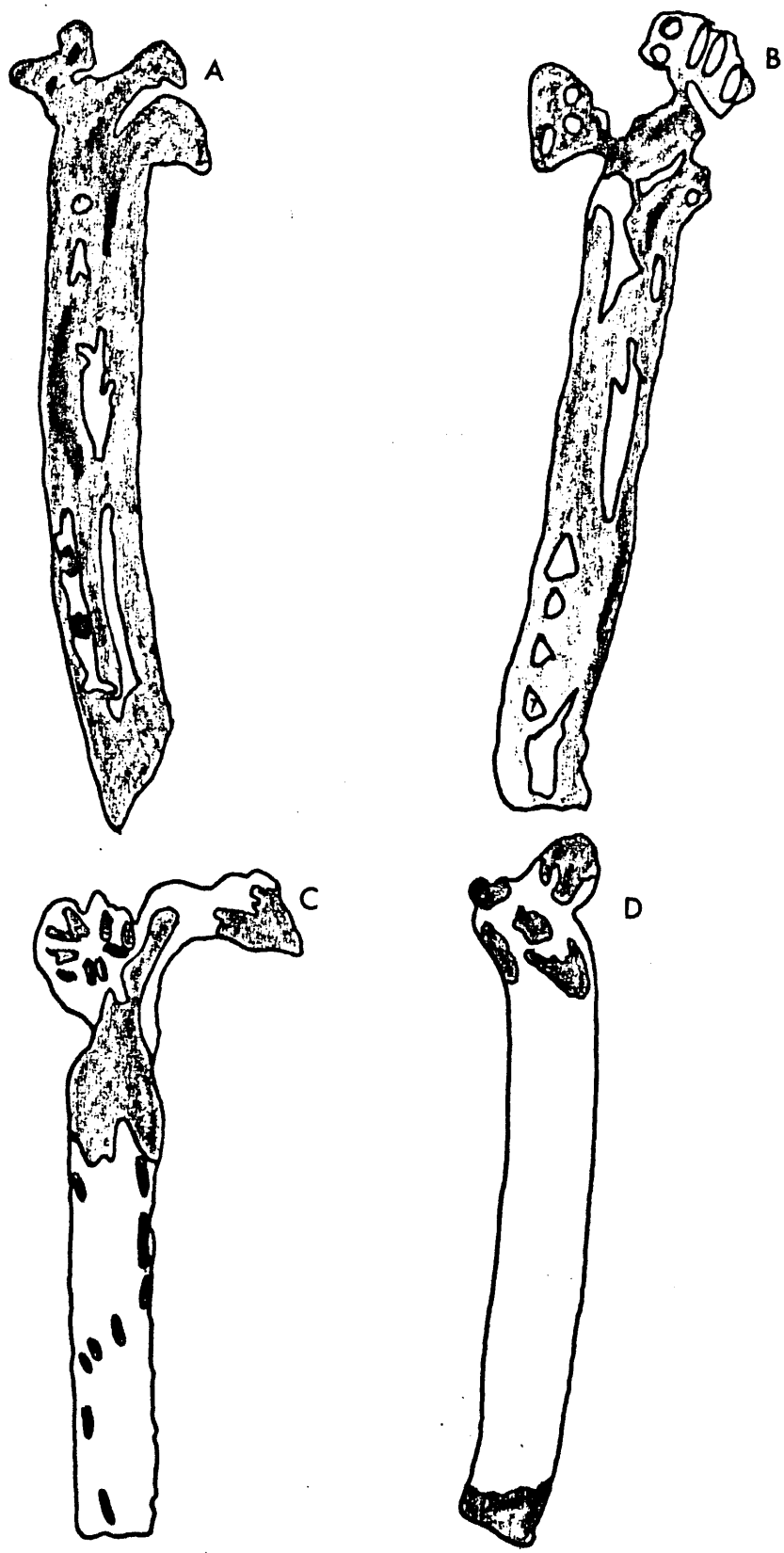


FIGURE R48: Aortae from Group 1 and Group 2 NZW rabbits.

Representations of the aortae of 2 animals from group 1 (cholesterol feeding and ethyl oleate). The shaded areas represent affected intima (A:89%, B:78% affected). C and D are aortae from rabbits which received only the cholesterol rich diet and were 28% and 15% affected, respectively.

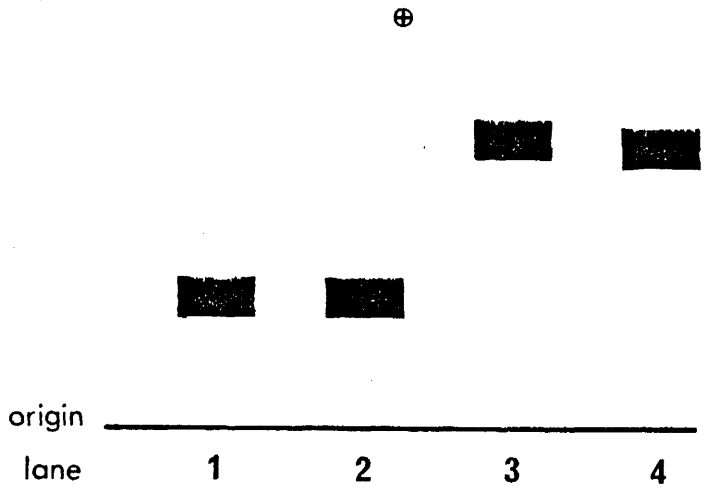


FIGURE R49: Agarose Gel Electrophoresis

Schematic Representation of:

Lane 1. LDL from Cholesterol fed Rabbit.

Lane 2. LDL from Cholesterol fed Rabbit.

Lane 3.) LDL from Cholesterol fed
) Rabbits treated with ethyl
Lane 4.) oleate.

Run Time 50 minutes.

7: DISCUSSION

7.1: General Discussion

Cells of the reticuloendothelial system (RE-system) include tissue macrophages (or histiocytes), alveolar and peritoneal macrophages and the endothelial macrophages of blood sinusoids. These cells are able to phagocytose particulate matter of macromolecular size, such as colloidal suspensions of dye stuffs (158). They are also thought to play an important role in atherosclerosis. There is evidence to show that they are the precursors of foam cells found in atherosclerotic plaques (7,8,11).

When endothelial cells lining the aorta are damaged by mechanical (13,14), chemical (15) or immunological (16) insult, insudation of blood components into the intima occurs (5). Monocytes migrate into the artery wall from the lumen, becoming histiocytes (7,11). These cells may be present, originally, to phagocytose and degrade blood components (such as lipoproteins) and to remove damaged cells and matrix components in an attempt to prevent necrosis of the lesion. However, it is postulated that macrophages along with platelets, which aggregate at the site of endothelial damage, release highly active growth factors (17,19). These growth factors are chemotactic to smooth muscle cells and promote their migration from the media and proliferation in the intima. In addition there is evidence that growth

factors increase the number of LDL receptors present on the surface of smooth muscle cells (17), promoting uptake of that lipoprotein by these cells.

The concentration of LDL in the intima is dependent on the plasma level of the lipoprotein (27). In cases where this is high macrophages catabolise large amounts of LDL. These cells catabolise little or no LDL by the LDL receptor pathway (139) but alternative routes are available for the assimilation of the lipoprotein. They will be discussed in detail below. The macrophages become engorged with lipid, especially cholesteryl ester, to the extent that they may form foam cells (7,11).

Macrophages, endothelial cells and vascular smooth muscle cells appear to release oxidising free radicals (172,173). These highly reactive chemical agents may cause further damage to cells and matrix components in the aorta. Previously published results demonstrate that free radicals can react with LDL (171,172) causing changes which increase uptake by macrophages.

Thus monocyte derived histiocytes which are present at the sites of damage to the aorta to aid in the repair process become contributing factors in atherosclerosis.

Receptor dependent catabolism of LDL is a reasonably well understood process (61,63,87,90,91). LDL and certain other lipoproteins which contain apolipoprotein B (apoB) or apoE are ligands for a specific

receptor associated with the cell membrane. Many types of cell have been shown to express this receptor (61,62). After binding of the ligand lipoprotein to the receptor it is internalised in a receptosome and degraded by hydrolysis in lysosomes (66). Cholesteryl ester is hydrolysed and free cholesterol released into the cytoplasm. This cholesterol has various effects on cellular processes. There is a stimulation of cholesterol ester formation, a decrease in cellular cholesterol synthesis and a down regulation of receptor uptake of LDL (90,91). In normal individuals, one third to one half of the plasma LDL pool is catabolised via the LDL receptor, depending on the LDL pool size of the subject (92,93,94).

Individuals with the disease familial hypercholesterolaemia have a genetically determined lack of functional LDL receptors (90). In heterozygous sufferers receptor dependent LDL degradation is reduced by about 50% (94). The fully affected homozygous form is manifested by a complete lack of functional LDL receptors (92). This results in grossly elevated plasma LDL levels. Despite the lack of LDL-receptors total catabolism of the lipoprotein is increased in these individuals (92). Clearly another mechanism, independent of the receptor system, is operational. It also seems to be responsible for degradation of at least one half of the LDL pool in normal subjects. This catabolic mechanism has been termed the receptor independent or

scavenger pathway but is still poorly understood (155). The reticuloendothelial system has been implicated as part of the scavenger mechanism (102,155). Macrophages are an important part of the RE system (158). They have a greater capacity for non-specific uptake and catabolism of LDL. Pittman has, however, demonstrated that, although this process contributes to the receptor independent pathway, the contribution is limited even at high plasma levels of LDL (157).

Macrophages express few LDL receptors in culture (139) and thus do not degrade LDL by the LDL receptor pathway. In familial hypercholesterolaemia the receptor does not function but macrophages from these subjects still degrade large amounts of LDL (102). In patients with myeloproliferative disease the RE-system is hyperactive (174). Studies have shown (99,100) that such individuals catabolise LDL and chemically modified LDL (which does not interact with the LDL receptor) at a significantly increased rate. Receptor dependent catabolism is unaffected.

It is evident that some mechanism for the degradation of LDL by macrophages is functioning independently of the LDL receptor.

Cells of the RE-system have been shown to degrade LDL chemically modified in such a way as to increase its net negative charge. These modifications include citraconylation (103), maleylation (139), malondialdehyde treatment (140) and acetylation

(103,139). The process was found to be mediated by a receptor on macrophages which was specific for negatively charged proteins (139). It was denoted the acetyl-LDL receptor (Ac-LDL receptor). In contrast to the LDL receptor, the Ac-LDL receptor is not down regulated by influx of cholesterol into the cell (139). Consequently large amounts of LDL can be catabolised by this route, leading to massive deposition of sterol in the cell.

There is some evidence for the formation of chemically modified LDL, which serves as a ligand for the Ac-LDL receptor, *in vivo* (145). Substances such as malondialdehyde, released during the oxidation of arachidonic acid by aggregating platelets, have been shown to modify LDL so that the product interacts with the macrophage receptor. Although the concentration of this substance in the plasma is low it may reach appreciable levels in the microenvironment of platelet thrombi. This may be of some consequence in the formation of foam cells since one of the sequelae associated with atherosclerosis is platelet aggregation.

Oxidation of LDL by free radicals has been shown to occur *in vitro*. The primary action appears to be on the lipids of the lipoprotein but may produce a secondary effect on the apolipoprotein moiety (171,172). Such secondary effects include breakage of peptide bonds, loss of amino acids and crosslinking (171). There is evidence that oxidation converts LDL to a form recognised by the Ac-LDL receptor (172). Macrophages and endo-

thelial cells release superoxide ions (172,173) which are active alone or can generate highly reactive free radicals. It is postulated that this oxidation may have some role in LDL degradation by macrophages in atherosclerotic plaques.

Hendriksen (142,147) has reported that incubation with endothelial cells and aortic smooth muscle cells in vitro converts LDL into a form recognised by the macrophage receptor. This EC-modified LDL was found to have altered physical and chemical characteristics. The density of the particle increased as did its electrophoretic mobility (overall negative charge). The cholesterol/protein ratio of EC-modified LDL was lower than that for control LDL. This lower ratio may explain the increase in density of the altered lipoprotein; increased protein or, more likely, decreased cholesterol content is consistent with higher density in lipoproteins. These changes did not occur if LDL was incubated with endothelial cell conditioned medium. It was shown that the increase in average density was not due to preferential degradation of a lower density fraction of LDL by endothelial cells. The cells catabolised less than 10% of the total amount of lipoprotein present (147).

Several mechanisms were considered to explain the changes observed in LDL:

a) chemical modification (103,139,140) would produce an increase in net negative charge of the

lipoprotein (125,126,127). However, this has been shown not to alter the density of the particle (93). Therefore it seems unlikely that chemical modification, alone, was responsible for changes observed.

b) passive or enzymatic alteration of LDL at the cell surface cannot be ruled out (147) but there is strong evidence that LDL is internalised by the cells. Conversion does not occur in the presence of inhibitors of endocytosis or at 4°C (147,178).

c) retroendocytosis is a process in which LDL that has been internalised via the LDL receptors is excreted from the cell without being degraded in lysosomes (142,147). It has been suggested that retro-endocytosis may be a widely occuring cellular process but the mechanism is not well understood. Removal of material from, or addition to, LDL may occur within the cells (147) possibly inducing conformational changes in the particle or complexing with sulphated polysaccarides or proteoglycans.

d) most convincing, however, is the finding by Morel (172) that inclusion of antioxidants or free radical scavengers in the medium prevent the physical and chemical changes observed on incubation of LDL with endothelial cells. These cells are capable of producing superoxide ions (173) which may react with the internal-ised lipoprotein converting it to the form recognised by the Ac-LDL receptor.

It has been shown that retroendocytosis *in vitro* occurs in many cell types and is quantitatively significant (175). Despite this the evidence for such a mechanism occurring *in vivo* is scarce. No EC-modified LDL has been found in the plasma but this observation is not unexpected. If altered LDL were generated by, for example, endothelial cells of the blood vessels, it would be rapidly catabolised by macrophages of the RE-system through the Ac-LDL receptor (142,147). This rapid removal from the plasma would mean that concentrations were normally very low.

There is some evidence for similarly altered LDL in the interstitial fluid of rabbits (176). These particles have increased density and electrophoretic mobility. However, there are some differences between EC-modified LDL and LDL of interstitial fluid. The latter is larger and triglyceride enriched. These are properties usually associated with less dense lipoproteins (81). It is not known if interstitial fluid LDL is degraded rapidly by macrophages.

If LDL altered by the mechanisms described were present in the plasma its concentration would be very low. In hypercholesterolaemics, where the LDL receptor is defective, the Ac-LDL receptor still functions (139). In these individuals large amounts of the lipoprotein are degraded by the RE-system. The most important cells in this catabolism would be the endothelial macrophages of the liver and spleen (158).

These cells have been shown to catabolise over 95% of markers of macrophage function *in vivo* (158). It is possible that larger amounts of altered LDL are produced in familial hypercholesterolaemics but, again, it would be catabolised rapidly by the still functioning RE-system macrophage receptors.

7.2: Suppression of the Reticuloendothelial System

7.2.1: Agents Used to Achieve Suppression

According to the above argument, suppression of the RE-system might be expected to reduce catabolism of any lipoprotein normally degraded by that route and cause an increase in plasma concentration of that lipoprotein.

Two agents were found to be particularly effective as suppressants of RE function:

1) Ethyl Oleate.

The monoesters of long chain fatty acids are well documented as inhibitors of the RE-system (131). Emulsions of lipid, including ethyl oleate, are cleared rapidly from the plasma by the RE-system (158). The liver and spleen are responsible for the clearance of most of the emulsion due to their content of macrophages (158). This properly was exploited to deliver the inhibitory ethyl oleate, as an emulsion, specifically to these cells.

2) Muramyl Peptides.

Bacterial cell walls contain peptide conjugates of N-acetyl muramic acid. These peptidoglycans are responsible for the endotoxic activity of the organisms (161). The minimum configuration which can mimic this effect is muramyl dipeptide (MDP). The adjuvant properties of MDP have been known for some time (160,161,162). More recently, however, the immunosuppressive properties of peptidoglycans have been recognised (165) and confirmation that MDP can also inhibit the RE-system reported (166,167).

These endotoxins have powerful effects on plasma lipid levels (163,164). Plasma triglyceride was shown to increase dramatically and resolve to base-line values within 24 hours of a single injection. Cholesterol levels did not increase as rapidly but remained elevated for longer periods (163,164). The mechanism of action of the endotoxins on lipid levels is little understood.

Two methods were successfully used to target MDP to the RE-system. Firstly, MDP can be attached to a phospholipid moiety through a third amino acid: this was denoted muramyl tripeptide phospholipid (MTP). Through the hydrophobic properties of the phospholipid the endotoxin was incorporated into an emulsion formed from glyceryl trioleate (triolein). Triolein emulsion was removed rapidly from the plasma of experimental animals (95% in 15 minutes) mainly by the liver and spleen.

Secondly, the Ac-LDL receptor can recognise other acetylated proteins such as bovine serum albumin (Ac-BSA). Ac-BSA was also cleared from the plasma by the liver and spleen. The spleen, however, was less active in catabolism of acetylated protein than it was with triolein emulsion. This difference may be accounted for by liver endothelial cells. These are not endothelial macrophages (Kupffer cells) but have high affinity binding sites for acetylated proteins (159). It was shown (159) that these cells, although contributing less than 4% of total liver protein mass, contained approximately 50% of the Ac-LDL catabolised by the liver as a whole. There is, however, some evidence that these cells represented a subpopulation of actual endothelial cells present in the liver, rendering this estimate high (159).

Total liver uptake of radioactive tracer was 40% of the injected dose after 30 minutes. It is important to note that this is not a cumulative estimate and does not include catabolites of the tracer (ie iodotyrosine) which were excreted back into the plasma.

7.2.2: Effects of RE-System Suppression In-Vivo

The effect of ethyl oleate and MTP-triolein on the activity of the RE-system was measured in vivo using the rate of clearance of colloidal carbon from the plasma of experimental animals before and after treatment. Kupffer cells take up approximately 90% of injected carbon

particles and the splenic macrophages account for most of the remainder (131). Clearance of colloidal carbon is an established method for the determination of the level of RE activity (131). Phagocytic index (K) is calculated from the gradient of the graph produced by plotting the logarithm of plasma carbon concentration (determined by measurement of the optical density of the plasma at 700 nm) against time.

Treatment with ethyl oleate or MTP-triolein reduced K to approximately 50% or 20% of control values, respectively (Fig.R18). This demonstrates a significant reduction in activity of the RE-system. Redetermination of phagocytic index two weeks after treatment with MTP-triolein showed that, after that time interval, previously phagocytosed carbon particles had no suppressive effect on macrophages.

In previous studies (163,164) investigators found that administration of bacterial endotoxins produced a rise in plasma cholesterol and triglyceride levels. In this study similar effects were observed in the plasma concentrations of both lipids after treatment with ethyl oleate, MTP-triolein and MDP-Ac-BSA (Fig.R6-R9).

The effects observed on treatment with MDP-Ac-BSA demonstrated that the lipid targeting systems used were not responsible for the rise in plasma lipids. This was supported by analysis of total plasma fatty acids. On administration of ethyl oleate no increase in

plasma oleic acid (c18:1) levels were noted despite the injection of a total of 6 grams of ethyl oleate over four days. Repeated injections of ethyl oleate produced a new steady level of plasma cholesterol. Analysis of lipoprotein concentrations showed this to be almost entirely accounted for by an increase in the LDL level.

Seifter (163,164) found that the response of plasma cholesterol on administration of bacterial endotoxin did not follow the same pattern as that for triglyceride. It was suggested that the increase in cholesterol was due to either an increased mobilisation or a decreased utilisation of that lipid. In light of the results discussed here the latter of these possibilities seems more likely. The increase in plasma cholesterol was due to a decrease in catabolism of cholesterol rich lipoproteins (ie LDL) by the RE-system.

LDL which has been reductively methylated (met-LDL) does not interact with LDL-receptors (125,126,127) and so is a marker of alternative (scavenger) degradative mechanisms. Lipoproteins decay from the plasma in a biexponential manner. The terminal phase, known as the monoexponential phase, was used to examine the effects of RE-system suppressants on the catabolism of met-LDL and native LDL. The rate of monoexponential decay was reduced by approximately 50% on administration of MDP-Ac-BSA or MTP-triolein. It has been demonstrated that ethyl oleate has a similar effect

(102), and that triolein emulsion alone elicited no suppressive effects on the plasma clearance of LDL.

The reduction in catabolism of both tracers suggests that treatment with suppressants mainly affects the scavenger pathway. Examination of a number of biochemical and haematological parameters after administration of RE-system suppressants revealed no significant changes in hepatic, thyroid or renal function. It did, however, show a small reduction in the number of circulatory white blood corpuscles but haemoglobin and red cell morphology were unaffected.

Cholesterol feeding appeared to mask the effect of RE suppressants on plasma lipid levels. There was no difference between cholesterol levels in cholesterol fed animals and those also receiving ethyl oleate emulsion. This may result from the massive rise in plasma cholesterol which occurs on feeding of the sterol (reaching 50-60 mmolar in 40 days: Fig.R12, ref.21). Triglyceride levels in the plasma of the two groups were not significantly different.

Starvation of control rabbits produced a slight rise in plasma cholesterol levels which was possibly due to a decreased utilisation of LDL by the liver for the production of bile. Simultaneous ethyl oleate treatment produced a rapid rise in cholesterol level which appeared to reach a new steady state by the fourth day. Starvation produced a mixed but small response in plasma triglyceride and treatment of these

animals with ethyl oleate produced a rapid rise in triglyceride which resolved to control levels by the fourth day despite continued treatment. The reason for the transient response of triglyceride is unclear but may not be a product of RE-system suppression.

7.2.3: Effects of Re-System Suppression *In Vitro*

Formaldehyde denatured albumin has been known for some time to be an *in vitro* marker of macrophage function (168). It is rapidly degraded by cultured macrophages.

MDP or MTP dissolved in culture medium reduced the degradation of denatured albumin to less than 13% and 4% of control values, respectively. This demonstrates the potency of these substances in affecting the catabolic processes of macrophages. Macrophages treated with MTP-triolein degraded less than 4% as much denatured albumin as control cells. This shows that the effects seen *in vivo* on the administration of MTP-triolein were a manifestation of the suppression of macrophages. Triolein vesicles alone had no significant effect on the degradation of denatured albumin but there was a short lag phase in macrophage function immediately after treatment. The reason for this is not understood.

Macrophages were also treated with ethyl oleate but cell death occurred even at low concentrations (15 μg lipid/ml culture medium). This observation was inconsistent with earlier work done *in vivo* (169) which suggested that treatment of animals with ethyl oleate

resulted in suppression of macrophages without cell death.

Catabolism of lipoproteins by macrophages in culture was also reduced by preincubation of the cells with RE-system suppressants. MDP-Ac-BSA reduced LDL and met-LDL degradation to 61% and 53% of control rate respectively. It has been shown that J-774 mouse macrophages express very low numbers of LDL receptors (140,143), thus only small amounts of LDL are degraded via this route. This, along with the quantitative effects on the degradation of native and met-LDL, suggests that the LDL-receptor independent or scavenger pathway was affected by MDP-Ac-BSA.

Cell protein was shown to decrease as the concentration of MDP-Ac-BSA in the incubation medium increased. This probably indicates a reduction in cell numbers in each culture at the end of the incubation period. It is likely that this is due to an inhibition of cell replication. Cell division would proceed at normal rates in control cultures but be suppressed in treated cultures. This would reduce the amount of cell protein present in treated flasks when compared to controls. Cell death could be ruled out because the cells remained attached to the wall of the flask and there was no increase in the number of cells floating free in the medium (a sign of cell mortality) in cultures treated with MDP-Ac-BSA. Degradation of markers was calculated per microgram of cell protein so this factor

was not responsible for the effects observed. Ac-BSA, at concentrations used to deliver MDP, had no significant effect on either LDL catabolism or cellular protein when compared to control values.

β-VLDL degradation was also greatly reduced by suppression of cultured macrophages with MDP-Ac-BSA. This lipoprotein is bound by a specific receptor on macrophages (73) which does not bind control LDL, Ac-LDL or control VLDL (73,74). MDP-Ac-BSA reduced catabolism of β-VLDL by up to 55% compared with 57% for LDL measured concomitantly. β-VLDL are present at low concentrations in the plasma of normal animals but accumulate when the diet is rich in cholesterol (74). The suppression of β-VLDL degradation by macrophages indicates a general inhibition of catabolic activity. The significance of this effect in normal rabbit does not appear to be great, indeed, as has been stated, β-VLDL concentrations are very low in normal animals. In cholesterol fed animals, where the plasma concentration of β-VLDL is much higher, this reduced degradation might be expected to be more pertinent. However, treatment of cholesterol fed animals with ethyl oleate had no significant effect on the already grossly elevated plasma cholesterol levels. There is some evidence that β-VLDL receptors are to some extent down regulated by the influx of cholesterol into the cell (73) but whether this is sufficient to explain the similarity in cholesterol levels between RE suppressed and normal cholesterol fed animals is unknown. The

effects of MDP-Ac-BSA on lipoprotein degradation by macrophages in culture are summarised in Figure R28.

It appears that macrophages *in vitro* were affected by RE-system suppressants. Their ability to degrade denatured albumin (a marker of the activity of cultured macrophages), LDL, met-LDL and β-VLDL was inhibited. These findings support the *in vivo* results discussed in section 7.2.2.

7.3: The Effects of Reticuloendothelial Suppression on Low Density Lipoproteins

As has been discussed, LDL incubated with endothelial cells undergoes an increase in density, an increase in electrophoretic mobility and a decrease in cholesterol to protein ratio (142,147,148). Various mechanisms were proposed to explain this. The most likely of these appeared to be retroendocytosis following oxidation within the cell (171,172,173).

Treatment of rabbits with RE suppressants produces an increase in plasma LDL cholesterol levels. This LDL, which is normally degraded by the RE-system, may be the equivalent *in vivo* of the EC-modified LDL produced in culture.

Anion exchange resin binds negatively charged proteins, which include LDL. The affinity of binding depends on the magnitude of the negative charge on the protein and the concentration of counter ions in the surrounding buffer.

Anion exchange column chromatography was employed to determine if there was a species of LDL, which had a higher negative charge than control LDL, in the plasma of RE suppressed animals.

The technique demonstrated that this was indeed the case. LDL from animals treated with MDP-Ac-BSA contained two separate fractions. One had the same net negative charge as control LDL and the second bound more strongly to the column, indicating a greater net negative charge.

PE-LDL eluted as a single peak but this also bound more strongly to the column than control LDL. There was, however, no distinct peak at the point where control LDL normally eluted.

So, LDL from animals treated with RE-system suppressants contains a species which has an increased net negative charge compared to control LDL. The presence of two peaks in MDP-treated rabbit LDL may be explained by the length of time the animal's RE-system was suppressed. Rabbits received a single dose of MDP-Ac-BSA and the LDL prepared from their plasma 24 hours later. The process of conversion of LDL operated in these animals for only that length of time. In animals treated with ethyl oleate the RE-system was suppressed for a period of 5 days in all. The alterations occurring in LDL may have proceeded to a much greater extent in that case.

Another factor may contribute to the difference between the LDL produced by ethyl oleate and by MDP-Ac-BSA suppression. As was stated above (section 7.2.1), the profiles of tissue uptake of emulsions and acetylated proteins are different. This may be due to uptake of the latter by liver endothelial cells (159) and may have an effect on the alteration of LDL.

LDL from ethyl oleate treated animals was separated into two fractions by density gradient ultracentrifugation. The first of these had a near normal hydrated density but the second was significantly denser than control LDL (Fig.R34). Individual peaks were not analysed for electrophoretic mobility or cholesterol/protein ratio. It is unclear why PE-LDL could be separated into two species by density but not by charge.

Analysis of total PE-LDL (containing both density fractions) showed a slight but significant increase in cholesterol/protein ratio which is inconsistent with the decrease observed in endothelial cell modified LDL. Attempts to detect oxidation products and free radicals in PE-LDL were inconclusive.

Nevertheless it appeared that RE-system suppression produced at least one new species of LDL which had increased density and increased net negative charge compared to control LDL. Interpretation of these results, at the simplest level, would seem to indicate that this species of LDL was normally catabolised rapidly by macrophages. This would mean that it was present in

normal plasma and, indeed, in plasma from LDL-receptor deficient subjects at low concentrations. The conversion and rapid uptake of LDL by macrophages may be a naturally occurring "ageing" process which plays a significant role in the scavenger catabolism of LDL.

7.4: Metabolic Fate of LDL Produced During RE-System Suppression

Fractional catabolic rates (FCRs) were calculated (120) for the clearance of LDL and PE-LDL from the plasma of normal rabbits. The FCR of PE-LDL was significantly greater than that for control LDL ($p<0.001$; Table R5). As discussed in section 7.1, EC-modified LDL is rapidly catabolised via the Ac-LDL receptor on macrophages (142). In the present study it was shown that PE-LDL was catabolised by tissues rich in RE-system macrophages, namely the liver, the spleen and the adrenal glands. The liver appears to be the most active tissue as far as the RE-system is concerned. It is said to catabolise the majority of blood borne substrates of the RE-system (131). This is consistent with the tissue uptake of PE-LDL: if PE-LDL is catabolised via the Ac-LDL receptor then the tissue which is richest in macrophage activity would degrade a major portion of that LDL. It is probable therefore, that the species of LDL produced on treatment with ethyl oleate is catabolised by macrophages.

Further studies indicated that the altered species of LDL was formed in the circulation rather than being synthesised de novo. Control LDL reisolated from ethyl oleate treated rabbits had increased electrophoretic mobility compared to control LDL reisolated from control rabbits. It was also cleared from the plasma at a greater rate when injected into normal animals. When control LDL and PE-LDL were reisolated from normal rabbits both had the same electrophoretic mobility and plasma clearance rates. This suggests that PE-LDL contains at least two species, one of which decays rapidly from the plasma where the other is removed at the same rate as control LDL. Presumably the rapidly cleared LDL has the larger overall negative charge. The two species model is further supported by examination of the terminal monoexponential portion of the decay curve for LDL preincubated in an ethyl oleate treated rabbit. This monoexponential decay closely resembles the decay of control LDL. According to Figure R35 the curve becomes monoexponential approximately 12 hours after injection at which time 80% of the tracer has been removed. It is interesting to note that more than 75% of total PE-LDL is present as the higher density form (Fig.R34). These data are consistent with earlier findings, and the simple model proposed above (section 7.3).

In vitro PE-LDL competes effectively for the acetyl-LDL receptor on macrophages, reducing degradation of Ac-LDL to less than 20% of that in the absence of

competitor. This was not achieved with control rabbit LDL or human LDL at the same concentrations (Fig.R40). It has been shown that EC-modified LDL also competes effectively for the Ac-LDL receptor (142). It seems likely that PE-LDL is catabolised via this route *in vivo*. This evidence lends more weight to the two species model, and the proposal that PE-LDL is produced naturally *in vivo* then degraded rapidly by the RE-system.

7.5: LDL from RE-System Suppressed Animals and its Role in Atherosclerosis

As has been discussed, the foam cells present in atherosclerotic plaques are partly derived from macrophages (11,17). Macrophages do not catabolise large enough quantities of normal lipoproteins to explain the gross amounts of lipid found in foam cells (139). Various explanations for the accumulation of lipid in macrophages have been given (section 7.1).

The ability of lipoproteins from RE suppressed animals to form foam cells and promote atherosclerosis was tested *in vivo*.

Under normal conditions rabbits have low levels of plasma lipoproteins (40) and do not develop atherosclerosis. However, if the animals are fed a diet containing cholesterol (1% w/w) their plasma cholesterol concentration increases rapidly (21,24). In 30-40 days it may reach 55-60 mmol/l. After 12 weeks on this

regimen the animals develop significant numbers of atheromatous deposits in their aortae (24).

During the first 35-40 days, the influx of cholesterol into the aortae is steady but fairly slow (21). Thereafter an increasingly rapid deposition of cholesterol occurs leading to severe lesion formation over most of the intimal surface (21).

The effect of ethyl oleate treatment on the formation of atherosclerosis in rabbits was determined using cholesterol feeding to enhance atherogenesis. The animals were fed a cholesterol supplemented diet and half were injected with ethyl oleate twice weekly. There was no significant difference between the plasma cholesterol levels in treated and untreated rabbits at any time during the 40 days. The same was true for plasma triglyceride concentration. Forty days was chosen because, for the reasons given above, lesion formation is moderate in rabbit aortae after this period and would not obscure the possible effects had by ethyl oleate on the atherosclerotic process.

Fractional catabolic rates (FCRs) were calculated for LDL and met-LDL (as a marker of RE-system degradation) in both groups. There was no significant difference in the FCR of LDL and met-LDL in either of the groups. The tracers were also cleared at the same rate in both groups. Thus cholesterol feeding appeared to mask any effect ethyl oleate had on either cholesterol

and triglyceride concentrations or on the clearance of LDL and met-LDL from the plasma.

Examination of the aortae of the animals revealed that treatment with ethyl oleate significantly increased the preponderance of atherosclerosis (Table R8, Fig.R48). Agarose gel electrophoresis showed that LDL from treated rabbits had a higher electrophoretic mobility than that from untreated animals.

It would appear, therefore, that suppression of the RE-system and hence macrophages caused a build up of more negatively charged LDL which had a greater capacity for atherogenesis. This would seem to be in contradiction to the mechanism discussed previously (sections 7.1 and 7.2).

The rabbits were injected with a macrophage inhibiting agent which reduces degradation of altered LDL. This would, at first, be expected to apply to histiocytes, preventing them from becoming foam cells. However, the liver and spleen are responsible for the uptake of more than 95% of the targeted drugs. The percentage taken up by the aorta was too small to be measured in these terms. It is possible that ethyl oleate vesicles were removed by the liver and spleen at a rate which did not allow for suppression of tissue macrophages.

Although there is a slight reduction in the number of blood borne monocytes, there is no evidence that these cells are otherwise affected by RE-system

suppressants. Injections of ethyl oleate were given at 3-4 day intervals and cleared from the plasma in a matter of minutes. It is, therefore, possible that monocytes, and hence histiocytes, which are affected by the suppressants, are few in number. This would mean that normally functioning macrophages were present in the intima with acetyl-LDL receptors available for the uptake of altered LDL (which would be present in the plasma in increased amounts under these circumstances).

7.6: Conclusions

Suppression of the RE-system is associated with an increase in the plasma concentration of an altered form of LDL. This altered LDL has an increased density, an increased electrophoretic mobility and an increased cholesterol to protein ratio. It is removed rapidly from the plasma of control animals probably via the Ac-LDL receptor, since it competes for that receptor *in vitro*.

Various mechanisms have been proposed for the formation of altered LDL *in vivo*. Most realistic of these, in light of the amount and characteristics of LDL produced, would seem to be retroendocytosis. However, other mechanisms may play a role in atherosclerosis. These include chemical modification by agents released on the aggregation of platelets, complexes formed with polyanionic macromolecules present in the intima, oxidation of LDL by superoxide ions or free radicals and

insudation of altered LDL into the intima from the plasma (Fig.D1).

It would seem that because circulating altered LDL is catabolised very rapidly it would have little relevance to the atherosclerotic process and only the conversions taking place in and around a plaque could contribute to foam cell formation. However, in individuals with high plasma levels of LDL, the amount of altered LDL being produced in the circulation may increase. There is no evidence available to support this in familial hypercholesterolaemics but little work has been done in these individuals to determine whether altered LDL is present and, if so, in what quantities.

The main findings arising out of this study are:-

a) Suppressive drugs can be successfully targeted to the Reticuloendothelial system, specifically to the macrophages thereof.

b) Suppressive agents (such as ethyl oleate and MDP) elicit an increase in plasma cholesterol and triglyceride concentrations, though not necessarily by the same mechanism.

c) The rise in plasma cholesterol is caused mainly by an increase in LDL cholesterol, suggesting that a mechanism for the catabolism of that lipoprotein is inhibited. This mechanism appears to be the LDL-receptor independent pathway.

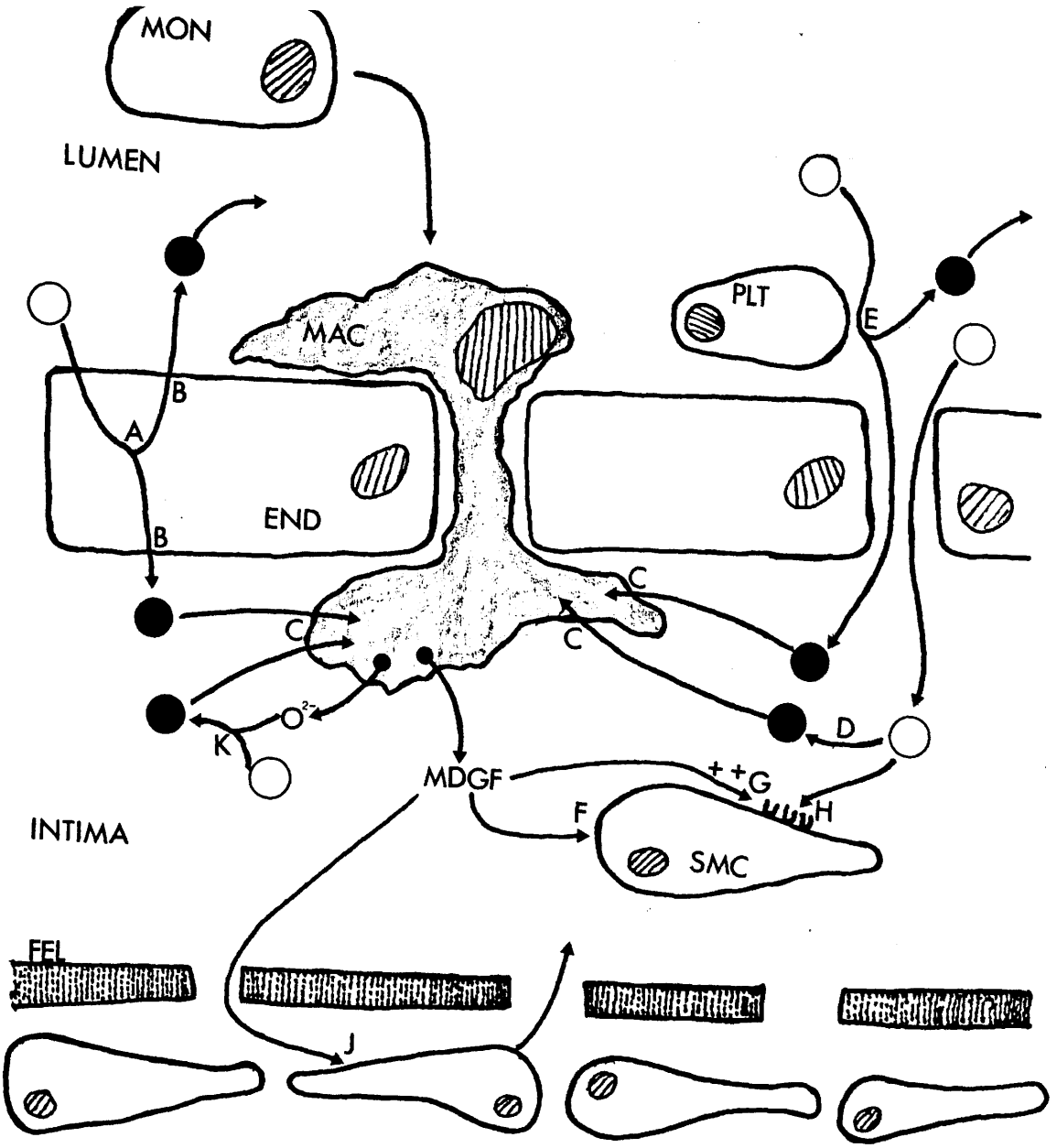


FIGURE D.1:

MON - monocyte
MAC - macrophage (histiocyte)
PLT - platelet (aggregating)
END - endothelial cell
SMC - smooth muscle cell
FEL - fenestrated elastic lamina
○ - normal LDL
● - converted LDL capable of interaction with the ac-LDL receptor
A - conversion of LDL within endothelial cells
B - retroendocytosis
C - uptake of altered LDL via the ac-LDL receptor
D - interaction of LDL with polyanionic substances in the intima
E - reaction of LDL with chemical agents released on the aggregation of platelets
F - stimulation of smooth muscle cell proliferation by macrophage derived growth factor
G - macrophage derived growth factor increases the number of LDL receptors (U) on smooth muscle cells
H - increased uptake of LDL by smooth muscle cells
J - macrophage derived growth factor stimulated the migration of smooth muscle cells from the media into the intima
K - oxidation of LDL by superoxide ion or free radicals

d) The LDL which accumulated in vivo had an increased density and an increased net negative charge. It was an effective competitor for macrophage degradation of acetyl-LDL.

e) Altered LDL was cleared from the plasma of normal rabbits with an FCR almost 2 fold greater than that for control LDL. This species was formed in the circulation rather than being synthesised de novo.

f) Treatment with ethyl oleate significantly increased the amount of atherosclerosis found in the aortae of rabbits fed a diet supplemented with cholesterol for 40 days.

The formation of altered LDL in the plasma may be a naturally occuring "ageing" process which aids in the overall catabolism of the lipoprotein. However, it does not account for all the LDL degraded by the RE-system. Individuals with myeloproliferative disorders have significantly higher fractional clearance rates for LDL (99,100,174). There is no physiological or biochemical reason why these subjects should produce more altered LDL, therefore, it must be surmised that the RE-system degrades LDL by more than one mechanism.

Significant amounts of LDL do, however, accumulate in the plasma of RE-system suppressed animals so it would appear that this mechanism does play an important role in the LDL-receptor independent pathway.

Some questions remain unanswered here, and more have been raised. Perhaps foremost among these concerns the mechanism by which LDL is altered *in vivo*. Some proposals have been made, mainly involving the phenomenon of retroendocytosis. There are some similarities between the accumulated LDL and that obtained on incubation with cultured endothelial cells (142,147) but they are not identical.

Treatment of cholesterol fed animals with ethyl oleate produces a greater preponderance of atherosclerosis but it is not known if RE suppression alone can induce atherosclerosis.

The physical and chemical properties of altered LDL are not fully documented. Effects on the lipids and apoproteins of LDL require further investigation.

In this study low density lipoproteins have been investigated but, apart from plasma concentrations, the effect of RE suppression on other lipoprotein classes has not been considered.

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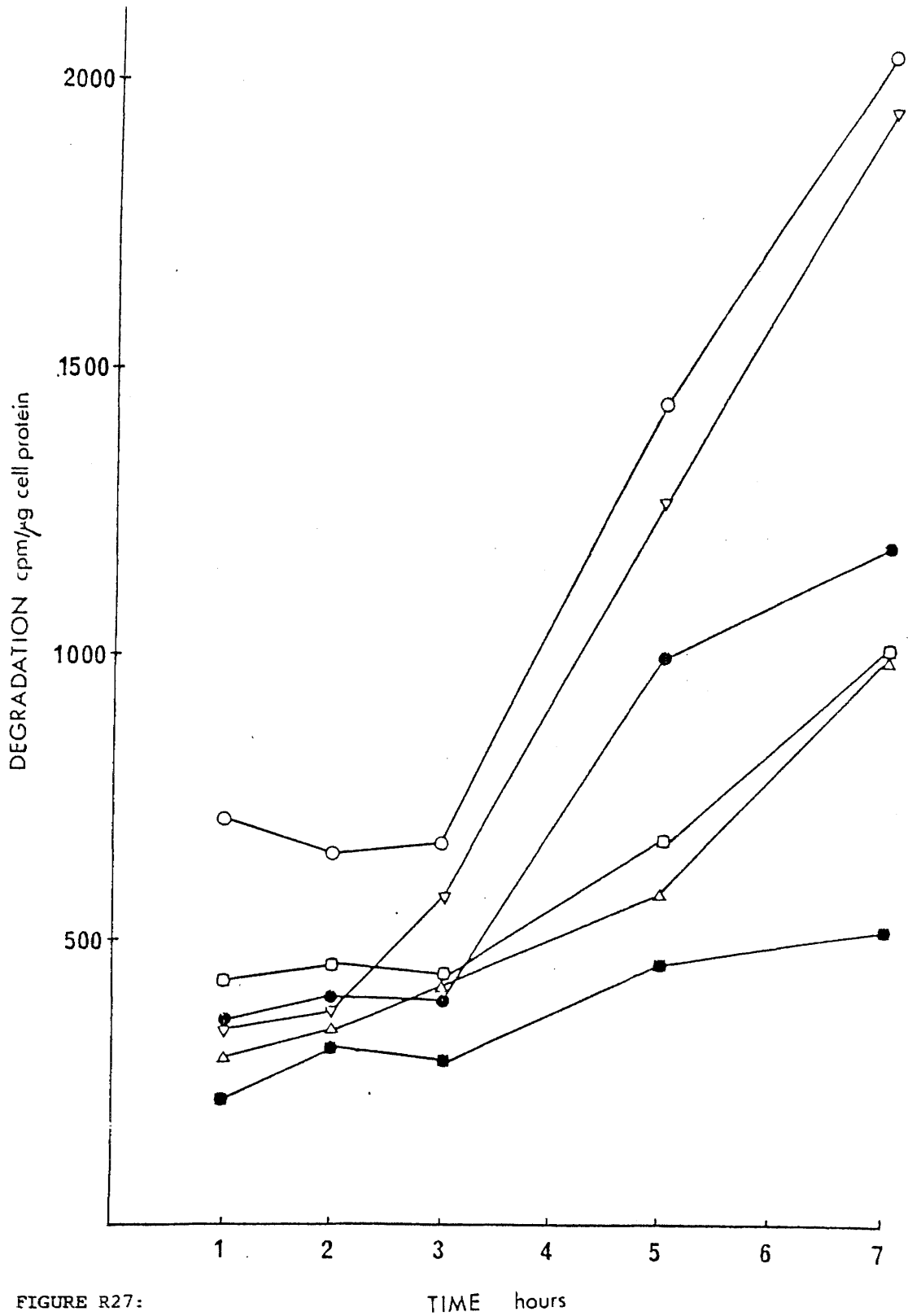

FIGURE R27:

Degradation of LDL and β-VLDL by Cultured Macrophages in the Control State and After Treatment with MDP-Ac-BSA.

- o β-VLDL control
- □ LDL control
- ▽ β-VLDL + 500 µg/ml Ac-BSA
- △ LDL + 500 µg/ml Ac-BSA
- ● β-VLDL + 500 µg/ml MDP-Ac-BSA
- ■ LDL + 500 µg/ml MDP-Ac-BSA

(each point was the mean of duplicate experiments)

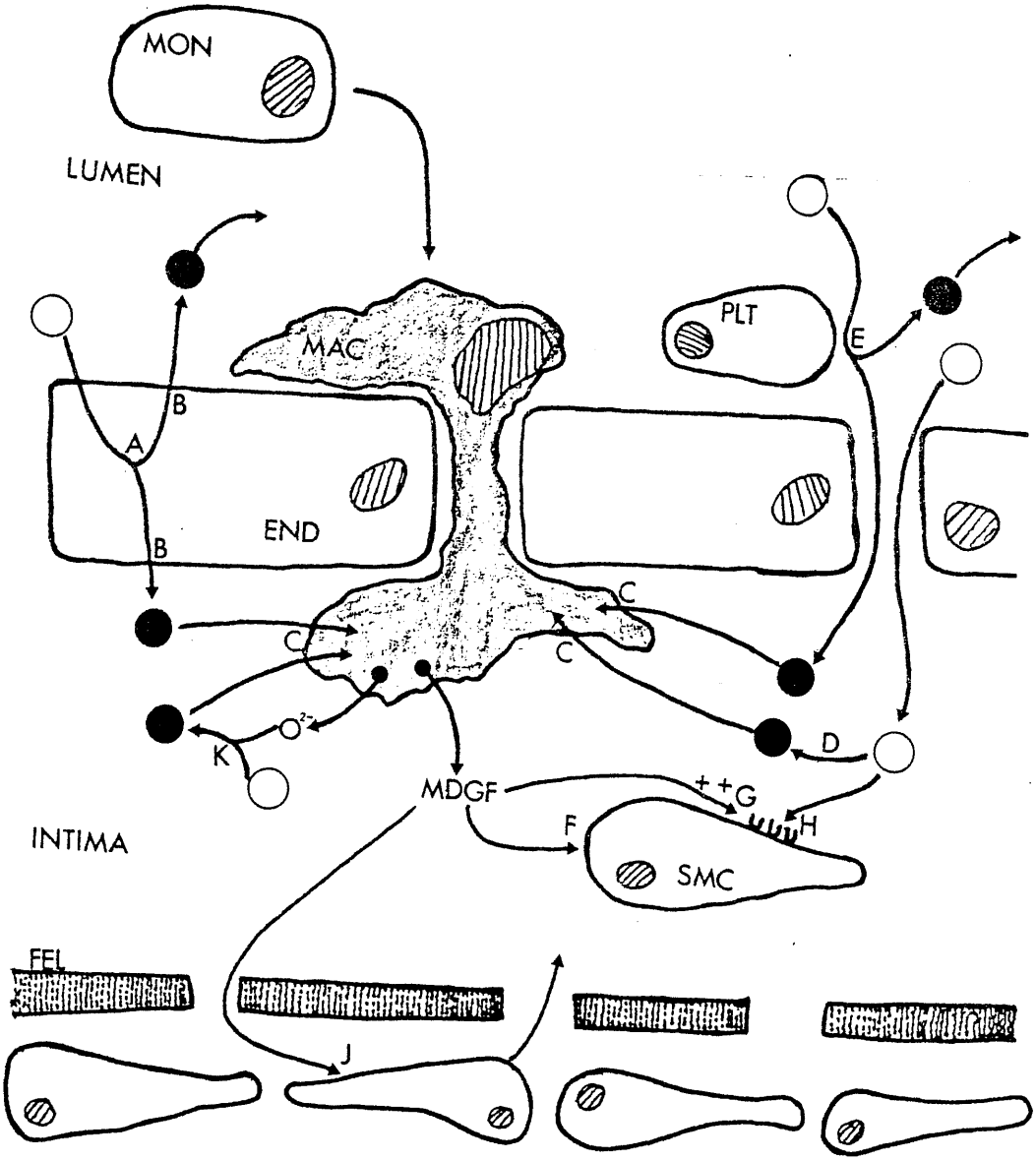

FIGURE D.1:

MON - monocyte
MAC - macrophage (histiocyte)
PLT - platelet (aggregating)
END - endothelial cell
SMC - smooth muscle cell
FEL - fenestrated elastic lamina
○ - normal LDL
● - converted LDL capable of interaction with the ac-LDL receptor
A - conversion of LDL within endothelial cells
B - retroendocytosis
C - uptake of altered LDL via the ac-LDL receptor
D - interaction of LDL with polyanionic substances in the intima
E - reaction of LDL with chemical agents released on the aggregation of platelets
F - stimulation of smooth muscle cell proliferation by macrophage derived growth factor
G - macrophage derived growth factor increases the number of LDL receptors (U) on smooth muscle cells
H - increased uptake of LDL by smooth muscle cells
J - macrophage derived growth factor stimulated the migration of smooth muscle cells from the media into the intima
K - oxidation of LDL by superoxide ion or free radicals

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