LIPOPROTEIN RECEPTORS IN CULTURED BOVINE ENDOTHELIAL CELLS

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ABSTRACT

Endothelial cells take up and degrade both low density lipoproteins (28) and low density liproteins which have been modified by acetylation (AcLDL) (84). In this study, receptors that may be involved in the uptake of these lipoproteins were characterized.

The cells used were aortic endothelial cells obtained from a bovine foetus, with subsequent cloning (A_3Cl_2). A cell culture system which closely resembled the *in vivo* monolayer was established, by growing the cells on gelatin-coated Petri dishes. Endothelial cell and lipoprotein interactions were examined by incubating the cells with ^{125}I-labelled lipoproteins under various conditions. The main findings were the following:

The receptor affinity of bovine aortic endothelial cells was higher for AcLDL than that for LDL. The half-maximal rates of degradation, obtained from degradation saturation curves which were linearized using the Scatchard method (136), were about 20 µg protein/ml for LDL and about 2 µg protein/ml for AcLDL. Analyses of binding data were not accurate due to the large amount of non-saturable material bound. However, the bulk of the lipoproteins was taken up and degraded via the saturable process.

Competition studies demonstrated that there were two distinct receptors for LDL and AcLDL on the endothelial cells. AcLDL

did not compete with LDL for the LDL receptor, and conversely LDL did not compete with AcLDL for the AcLDL receptor.

The receptor activities for LDL and AcLDL were examined as a function of culture age. Sparse cultures incubated at low lipoprotein concentrations (10-20 µg protein/ml) had a higher receptor activity for LDL than for AcLDL. In contrast, confluent cultures, catabolized more AcLDL than LDL. In comparing sparse to confluent cell cultures, the rate of ^{125}I-labelled LDL degradation decreased about twice, while the degradation rates of ^{125}I-labelled AcLDL increased about three times.

Whereas the LDL receptor could be regulated, the AcLDL receptor was not as susceptible to regulation. Up-regulation was measured by pre-incubation of the cells with lipoprotein-deficient serum medium (LPDS-medium) for 48 h. Using degradation data, the LDL receptor was up-regulated about 4-fold, whereas the AcLDL receptor was not up-regulated under these circumstances. Down-regulation by incubating the cells with 25-hydroxycholesterol for 24 h resulted in a 96% decrease in the LDL receptor activity and only a 30% decrease in the AcLDL receptor activity. Furthermore, both LDL and AcLDL could down-regulate the LDL receptor, but neither could down-regulate the AcLDL receptor.

Upon exposing endothelial cells for 72 h to either LDL or AcLDL, it was found that the total amount of cellular cholesterol increased (by about 50%). However, the increase of

total cholesterol was largely in the form of free cholesterol. This is in contrast to macrophages, where the increase in total cholesterol upon exposure to AcLDL is largely in the form of cholesteryl esters.

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ABBREVIATIONS AND SYMBOLS

AcLDL	: acetylated LDL
ACAT	: acyl-coenzyme A : cholesterol acyltransferase
BSA	: bovine serum albumin
^{o}C	: degree Celcius
Ci	: Curie (3.7×10^{10} disintegrations per second)
cpm	: counts per minute
δ	: density
DMSO	: dimethyl sulphoxide
dpm	: disintegrations per minute
EDTA	: ethylenediaminetetraacetic acid
Expt.	: experiment
FITC	: fluorescein isothiocyanate
g	: acceleration due to gravity
Hepes	: N-2-hydroxyethylpiperazine-N'-2'-ethanesulphonic acid
HDL	: high density lipoprotein
k_{δ}	: Equilibrium dissociation constant
LDL	: low density lipoprotein
LPDS	: lipoprotein deficient serum
M	: molar
MDA-LDL	: malondialdehyde-treated LDL
MW	: molecular weight
No.	: number
PAGE	: polyacrylamide gel electrophoresis
PBS	: phosphate buffered saline
S	: concentration of free lipoprotein
SDS	: sodium dodecyl sulphate

S.E.M. : standard error of the mean

$$\left(= \frac{\{\Sigma(\bar{x}-x)^2 \;/\; (n-1)\}^{\frac{1}{2}}}{n^{\frac{1}{2}}} \right)$$

Tris : tris-hydroxymethyl amino methane

TCA : trichloroacetic acid

V : concentration of lipoprotein bound to receptor

VLDL : very low density lipoprotein

CONTENTS

1. INTRODUCTION

Atherosclerosis, an important factor in most cardiovascular abnormalities, can be accelerated by various factors which influence the elevation of blood cholesterol, such as genetic disorders (1,2), high cholesterol diet (3,4), hypertension (5) and smoking (5) (for a review see reference 6). Most of the circulating cholesterol is synthesized in the liver and is secreted as very low density lipoproteins (VLDL) which are converted to low density lipoproteins (LDL) upon the loss of mainly triglycerides (7). Low density lipoproteins carry about 70% of the blood cholesterol which is delivered to extrahepatic cells mainly for membrane synthesis (for reviews see references 8,9). A massive accumulation of cholesteryl ester deposits in the vascular lumen leads to the formation of atherosclerotic plaques. Various cells are involved in the complex mechanism of plaque formation, among which smooth muscle cells, macrophages and endothelial cells play a prominent role. It has recently been shown that these cells in culture are able to accumulate cholesteryl esters upon exposure to modified LDL (for a review see reference 10). The endothelium, consisting of endothelial cells, lines the lumen of the vascular system, forming an interface between the circulatory system and the underlying vascular tissue. Injury of the endothelium and functional abnormalities are thus prominent in the pathology of atherosclerosis (11,12). In this introductory chapter, the morphology and functions of the endothelium will be discussed,

with particular reference to its response to injury (for reviews see references 12,13,14,15) and metabolism of low density lipoproteins (for reviews see references 7,8,9,16, 17).

1.1. MORPHOLOGY OF THE VASCULAR ENDOTHELIUM

Vascular endothelial cells grow in an ordered monolayer of highly flattened, polygonal cells resembling a cobblestone pavement. Their long axis, which is about 20-50μ in length is orientated in the direction of blood flow. The width of these cells is about 10-15μ (for review see reference 12). The endothelial cells have large nuclei and the cytoplasm contains many mitochondria, rough and smooth endoplasmic reticulum, well-developed Golgi apparatus, lysosomes and microtubules (Plate 1.1). All arterial endothelial cells, with the exception of bovine aortic endothelial cells, are further characterized by the presence of Wiebel-Palade bodies (18,19,20,21). These are rod-shaped organelles with as yet undetermined functions.

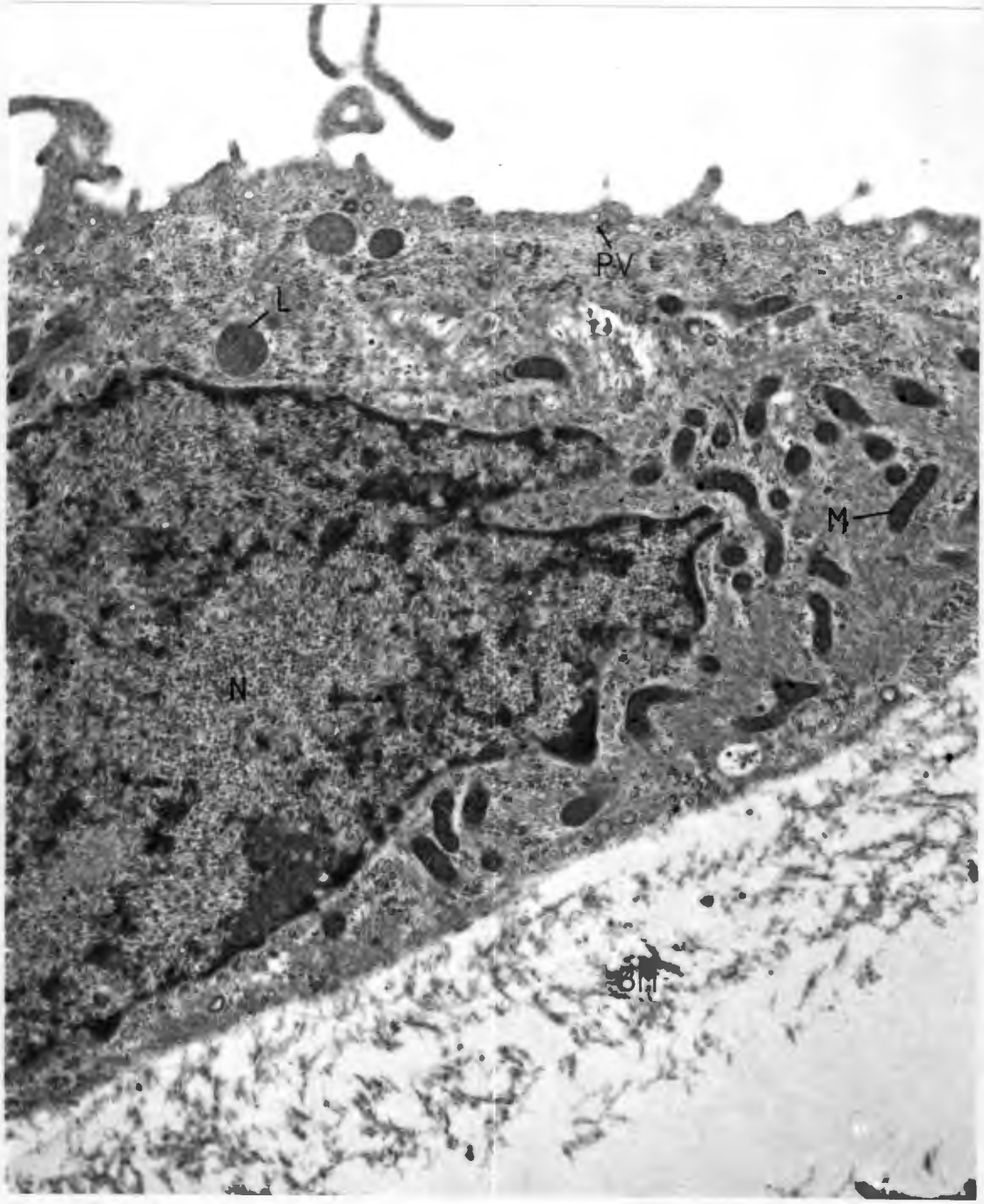
The endothelium exhibits both a morphological and functional polarity. The side exposed to the bloodstream is non-thrombogenic, whereas the abluminal surface is thrombogenic (21,22). This is of great importance, as platelet adhesion to the endothelium is normally prevented, as well as the possibility of thrombus formation which could initiate the atherosclerotic plaque (13). In addition, the luminal

PLATE 1.1: ELECTRON MICROSCOPY OF ENDOTHELIAL CELLS IN CULTURE

Transmission electron micrograph of cell strain A_3Cl_2 in culture (transverse section).

A portion of an endothelial cell (A_3Cl_2) in culture. The cell can be seen to have a large nucleus (N). The cytoplasm consists of numerous mitochondria (M) and plasmalemmal vesicles (PV) as well as lysosomes (L). The basement membrane (BM) is found on the abluminal surface.

Magnification = 25000 x


PV
L
M
N
OM

surface synthesizes and secretes various metabolites such as prostacyclin (PGI_2), adenosine, plasminogen activator and the angiotensin-converting enzyme which aid in the maintenance of a thrombo-resistant surface (20,23,24). Furthermore, the luminal surface of the endothelium is characterized by the presence of the antihaemophilic factor antigen (19,25,26). This is thus a useful means for identifying endothelial cells. The basement membrane is synthesized by the thrombogenic surface. Although the exact nature and composition of the basement membrane is still to be elucidated, it is composed of multiple layers of different collagenous material, as well as glycosaminoglycans, proteoglycans and glycoproteins (21,22,27). Studies *in vitro* have shown that fibronectin, which coats the membrane, is responsible for cell adhesion (21,28), and another major cell surface protein with a molecular weight of 60 000 (CSP-60) is responsible for the confluent, contact-inhibited monolayer (21,28,29).

Furthermore, the endothelium contains numerous plasmalemmal vesicles (30) and a variety of intercellular junctions (31). The plasmalemmal vesicles have a mean diameter of 600 Å. Ultrastructural studies have shown that some plasmalemmal vesicles are situated entirely within the endothelial cytoplasm, while others open into the luminal or subendothelial space, by fusion with the plasma membrane (30). Not only are the plasmalemmal vesicles far more abundant on the abluminal surface, but their relative number also varies

between different segments of the vascular system (12). For example, Simionescu et al (32) observed that the arteriolar endothelium had a much smaller population of plasmalemmal vesicles than did the capillaries. Interendothelial junctions with a mean diameter of 30-40 Å, are also found to differ morphologically and functionally, depending on the type of endothelium (12). The endothelium can be divided into two main categories: the continuous endothelium, which is found in the arteries, veins and capillaries; and the fenestrated endothelium of various forms of visceral capillaries (33,34). Simionescu et al (32,35) observed that arterioles have the most elaborate junctional system. This system is composed of tight junctions surrounding large gap junctions. The arteries have a similar network with considerably less complex tight and communicating junctions (35). Capillaries, by contrast, have no communicating junctions and are less organized. Venules have the least organized junctions, which are mostly devoid of junctional particles. The complexity of the junctional arrangement in the endothelium is further emphasized by the work of Schwartz and Benditt (36). In the aortic intima of rats alone, they identified four types of inter-endothelial junctions. The most common junction was a mortice junction, followed by the complexly-folded junction, the simple-overlap junction, with the least common being the single end-to-end junction. It has been shown in vitro that both the plasmalemmal vesicles and the intercellular junctions of endothelial cells are involved in a transport mechanism (see Introduction 1.5).

1.2. GROWTH AND PROLIFERATION OF VASCULAR ENDOTHELIAL CELLS IN VITRO

The ease with which vascular endothelial cells are obtained and grown *in vitro*, is largely due to the pioneering work carried out by Lewis since the 1920s (37). Vascular endothelial cells are isolated either by mechanical scraping (38,39) or by enzymatic release (19,20,40,41,42). The growth of endothelial cells in culture varies according to the substrates used. When bovine aortic endothelial cells are exposed to plastic, their orientation is such that the basal layer adheres. Once these cells form a confluent monolayer with newly synthesized basal materials, such as collagen, proteoglycans and glycoproteins, a second layer is formed beneath. The latter attaches in an inverted manner to the original culture, with the apex of the cells in contact with the plastic (43). When this second layer is restricted to focal areas, where the cells do not proliferate beyond a small number, then this phenomenon has been referred to as "sprouting" (19), in contrast to cells which take over the whole culture and form multilayers. McAuslan *et al* (44), who observed a similar morphology, prefer to refer to the "sprouting" cells as variant endothelial cells, which are found to grow both over and under the monolayer of endothelial cells. Since correlations have been drawn between endothelial cell behaviour and function, and biosynthetic or artificial extracellular substratum, the growth of endothelial cells on extracellular matrix derived from corneal endothelial cells (21,22,45), collagen (42,46,47) and gelatin

(48) have been studied. The extracellular matrix from corneal endothelial cells was able to sustain the growth rate of corneal and vascular endothelial cells (45). Bovine aortic endothelial cells and capillary endothelial cells were grown on collagen gels in order to study tumour invasion of the in vitro endothelial monolayer (47,49). Human umbilical endothelial cells grown on fibrillar type I collagen were used as a model to study platelet adhesion (42). Gelatin was used as a parameter together with tumour-conditioned medium for maintenance of capillary endothelial cells for a long period of time in culture (48). A substratum for endothelial cell growth in vitro is not essential, but it may allow for enhanced growth, as a substitute for growth factors (27).

As mentioned, the proliferation of vascular endothelial cells which has been extensively studied (for reviews see 21,22,50), is found to be rather varied. Population doubling times in bovine endothelial cells vary in different media and sera (41) having a range from 29 h (51) to 3-4 days (19). This wide range is also dependent on the proliferative agents used to maintain growth of these cultures.

A potent mitogen for endothelial cells is fibroblast growth factor (22,38,52,53). Gospodarowicz and coworkers (38) isolated and maintained bovine endothelial cells in the presence of fibroblast growth factor (FGF, 100 ng/ml). In the absence of FGF, cells proliferated at a very much slower

rate and lost the ability to form a confluent contact-inhibited monolayer as found *in vivo* (22,54). However, if cells were grown in the presence of FGF for some time (12 days), and thereafter FGF was removed, the confluent, contact-inhibited monlayer was maintained for at least 7 days (38). Controversies regarding FGF have arisen, with Duthu *et al* (41) reporting that cells grown in the presence of FGF do in fact multilayer. Schwartz *et al* (50) also contradict Gospodarowicz *et al* (22) in that these workers were unable to show an increased mitogenic effect of FGF.

Platelets have been shown to produce growth factors which enhance the proliferation of smooth muscle cells (22,21). The effect of these factors on endothelial cells was looked at by Gotlieb and Wong (55) and Davies and Ross (56). Although incubation of endothelial cells in plasma-derived serum (PDS) slowed down cell spreading if compared to incubation of endothelial cells in whole blood serum (WBS) (55), the cell growth rate did not differ between cells exposed to 20% PDS, 20% WBS or 20% PDS containing the platelet-derived growth factor (56). It was assumed therefore that the presence of platelets and their growth factors did not enhance endothelial cell proliferation. In a report by Gospodarowicz and Charles (57), bovine aortic endothelial cells plated on plastic had a slower growth rate when exposed to plasma than to serum. In contrast, when the cells were plated on an extracellular matrix, they proliferated equally well, whether they were exposed to plasma or serum. When the effect of

whole blood serum (WBS) alone was looked at, it was shown that endothelial cell growth relied on the correct concentration for optimal growth. This was found to be a concentration of 20% serum (50). At the low concentration of 1% serum endothelial cells grew very slowly, even upon the addition of mitogens such as FGF, EGF (epidermal growth factor) and insulin (50).

Recently, Gospodarowicz et al (27) have shown that if endothelial cells are grown on an extracellular matrix (ECM) in the presence of plasma, FGF is no longer required to attain a confluent monolayer. Endothelial cells grown on an ECM no longer proliferate if they are exposed to lipoprotein-deficient serum (LPDS). It thus appears that the presence of lipoproteins is necessary for endothelial cell proliferation. In adding either low density lipoproteins (LDL) or high density lipoproteins (HDL) to the LPDS-medium, it was found that although low concentrations of LDL (150 µg protein/ml) expressed full mitogenic capabilities, higher concentrations became toxic for the cells (58). The toxicity of high concentrations of LDL was a function of cell density. When the cells were confluent, LDL even at a physiological level (600-800 µg protein/ml) was no longer toxic (59). HDL, on the other hand, proved to be mitogenic over a wide range of concentrations, even at the physiologic concentration of 1000-1500 µg protein/ml (59), and was thought to be the preferred lipoprotein for enhanced proliferation. In addition, endothelial cells maintained on

ECM could be grown in completely serum-free medium if supplemented with HDL and transferrin (58). The requirement of transferrin could reflect either its role as an iron-carrying protein capable of delivering this metal to the cells or as a detoxifying protein removing trace amounts of toxic metals that would otherwise have been removed by serum proteins (60). Furthermore, Tauber and Gospodarowicz (61) investigated the effect of LDL and HDL on cell migration and proliferation on the repair process of a wounded confluent monolayer. Whereas HDL added to LPDS-medium leads to a rapid repair process, LDL at high concentrations adversely affects the repair process. Further studies will be indispensable in elucidating the role of lipoproteins in wound repair.

Wound-derived macrophages and wound fluids have also been shown to stimulate vascular endothelial proliferation (62). A 2% wound fluid concentration causes proliferation of endothelial cells comparable to 100 ng/ml FGF (62). The substance in wound fluids responsible for the mitogenic behaviour is not yet known. However, it has been shown that macrophages produce a growth factor (MDGF) independently of plasma- or platelet-serum growth factors (63,64,65), and it is possible that this factor, which has also been shown to be mitogenic for endothelial cells (63,52) may be released by the macrophages into the wound fluid.

Although there are many different and often conflicting reports on the growth and proliferation of vascular endothelial cells, it has been possible to maintain them in

culture. As far as growth *in vitro* is related to LDL metabolism, it has been reported that when cells are grown to a confluent, contact-inhibited monolayer (21,28,66), the LDL particle is bound, but no longer internalized or degraded. This has been found to be the case only with endothelial cells grown according to the method of Gospodarowicz (22,38, 54). However, Coetzee and coworkers (67) did not find that the amount internalized and degraded by bovine endothelial cells in confluent cultures was negligible, although they did observe a decrease in LDL metabolism with increasing cell confluence.

1.3. THROMBOGENESIS

When the endothelial layer of cells is removed to expose the underlying surface, platelets can adhere and thrombus formation occurs. This can be further aggravated if macrophages and platelets release growth factors which cause smooth muscle cells to proliferate into the intima and eventually lead to atherosclerotic plaque formation (13). As a preventative measure, the endothelium actively metabolizes and secretes various vasoactive substances, which participate in maintaining a non-thrombogenic luminal surface. The endothelium and blood platelets produce metabolites from arachidonic acid which are involved in thrombogenesis.

In both endothelial cells and platelets, arachidonic acid is converted to prostaglandin endoperoxides (PGE_2 and PGH_2) by

the action of cyclo-oxygenase (68,69). In the case of the endothelial cells, the prostaglandin endoperoxides are converted to prostacyclin (PGI_2) by prostaglandin synthase, while in platelets they are converted to thromboxane A_2 (TxA_2) by thromboxane synthetase. PGI_2 is a vasodilator and inhibitor of platelet aggregation. TxA_2, on the other hand, is a vasoconstrictor and inducer of platelet aggregation. The protection by endothelial cells against thrombogenesis is also seen in the ability by these cells to catabolize exogenous adenosine triphosphate (ATP) to adenosine (24), which is an inhibitor of platelet aggregation (70). Promotors of platelet aggregation such as serotinin (23), adenine nucleotides (24,70), brad bardykinin (20) and angiotensin I (20) are removed by endothelial cells. For example, angiotensin I is converted to angiotensin II, which indirectly stimulates the release of PGI_2(20).

When thromboxane A_2 formation is increased, or prostacyclin formation inhibited, increased platelet adhesion to the endothelium occurs (71). Thrombin, an activated coagulation factor, which is known to bind to cultured human and bovine endothelial cells (72,73) is a strong inducer of platelet aggregation. There is a large production of malondialdehyde (MDA) by platelets *in vitro* in response to thrombin (74). Aspirin, which inhibits platelet aggregation (75), also inhibits production of malondialdehyde by platelets (74). Hammerström *et al* (68) have shown that malondialdehyde is a by-product of prostaglandin endoperoxide conversion to thromboxane A_2 in platelets.

The production of platelet substances *in vitro* have been shown to modify LDL, and cause cholesteryl ester accumulation in macrophages (76). Macrophages had been shown to have receptors for negatively-charged LDL which can be involved in cholesteryl ester accumulation under certain conditions (77). Brown *et al* (78) used acetylated-LDL (AcLDL) as a source of negatively-charged LDL, while Fogelman used malondialdehyde-treated LDL (MDA-LDL) (76,77,79). Both these modified lipoproteins bound to the same receptor on macrophages (78). When the LDL, which had been modified upon exposure to platelet substances, was compared to MDA-LDL, it was found to have the same elution profile on Sepharose 4B. Upon hydrolysis of the LDL particle, which had been exposed to platelet substances, it was spectrophotometrically determined to consist of malondialdehyde (76). Not only was malondialdehyde the first molecule to be found *in vivo* at the appropriate site where the initiation of atherosclerosis could occur, but it was also produced in large enough quantities to modify LDL in such a way that the lipoprotein was able to cause subsequent cholesteryl ester accumulation (76) (See Introduction 1.5).

1.4 ENDOTHELIAL RESPONSE TO INJURY

As early as 1856, Virchow (80) suggested that injury to the artery wall somehow led to a series of tissue responses, that culminated in the lipid-filled lesions of atherosclerosis. In recent years, the studies of injury to the artery wall

have focussed on the endothelium (for reviews see references 13,14). When the intact endothelium is injured in experimental procedures by means such as exposure to mechanical forces, various toxins, immunological injury, viruses and chemical agents such as low density lipoproteins (13,14,81), the endothelial monolayer is disrupted, exposing the underlying connective tissue. The presence of basement membrane collagens, type IV and V (42) and the interstitial collagens, type I and III (82), greatly enhance platelet adhesion. Disruption of the endothelial monolayer may lead to changes such as separations between endothelial cells, altered endothelial permeability or desquamation of the endothelial cells, in which both adherence of platelets and of monocyte-macrophages may occur. Substances derived from platelets and monocyte-macrophages, as well as from the plasma, such as lipoproteins and hormones may stimulate the migration of smooth muscle cells from the media into the intima, where proliferation of these cells may be induced (13) (Figure 1.1).

Habernicht et al (83) reported that the platelet-derived growth factor (PDGF), in addition to enhancing quiescent smooth muscle cell division, also increased cholesterol biosynthesis in cultured smooth muscle cells. Furthermore, as mentioned earlier, the release of malondialdehyde into the bloodstream by platelets upon aggregation can cause modification of the LDL particle. At the site of injury of the endothelial monolayer, where platelet aggregation occurs, the modification of the LDL particle can have far-

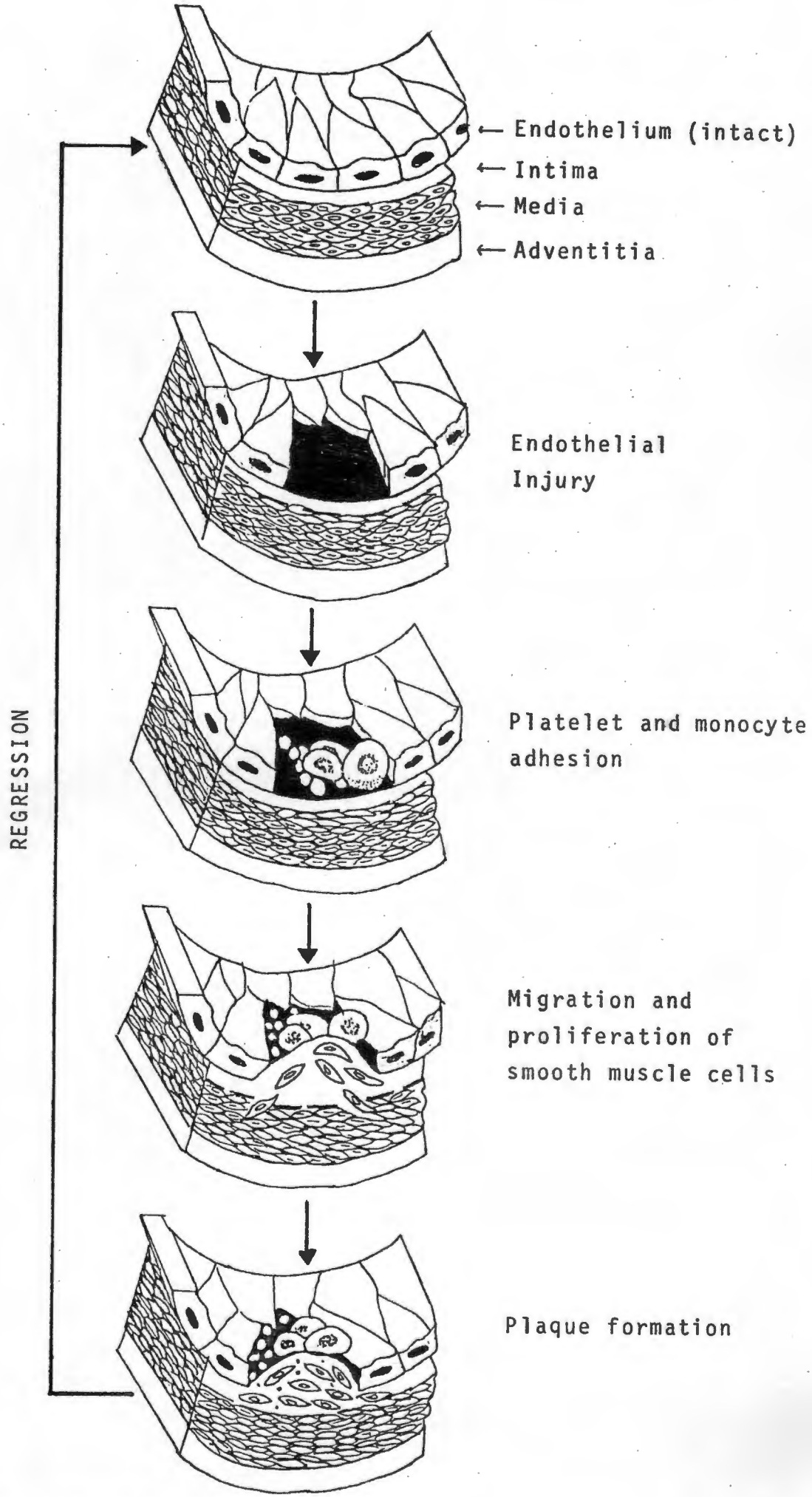


FIGURE 1.1. ARTERIAL WALL: CONSEQUENCES OF ENDOTHELIAL INJURY

reaching implications, since this modified LDL (MDA-LDL) has been shown to be taken up by monocyte-macrophages with the result of a cholesteryl ester accumulation (76). It has been reported that the ability of macrophages to metabolize negatively-modified LDL (MDA-LDL or AcLDL) *in vitro* is shared by endothelial cells (84), but not by smooth muscle cells or fibroblasts (10). However, when smooth muscle cells are exposed to the medium of either macrophages or endothelial cells incubated with negatively-modified LDL, in the form of AcLDL, these cells accumulate cholesteryl esters (85,86). Although it is rather appealing to link various *in vitro* studies to explain the *in vivo* situation of lipid-filled macrophages and smooth muscle cells at the site of atherosclerosis, it should be pointed out that the *in vitro* systems used are rather specialized and have yet to be confirmed *in vivo*.

It is further postulated that if injury to the endothelial monolayer is limited, the process of plaque formation is reversible and regression of the lesion is obtained. Regression of the lesion occurs by the two separate mechanisms of migration followed by replication (15). Upon wounding a confluent endothelial cell layer in culture, the cells at the wound edge first appear to spread themselves over a larger area to close the wound, while remaining attached to the adjacent cells (13,15). It is thought that only if the wound is too large do the cells respond by replication. The migration of endothelial cells is possible due to the presence of actin fibres. Cell movement usually

begins with the protrusion of a broad flattened portion of the cytoplasm (the lamellipodium) away from the main cell body in the direction of cell movement (87). After attachment, translocation occurs by retraction of the actin fibres associated with the cell membrane. Cytochalasin B has been shown to inhibit cell motility by disrupting the submembraneous web of actin fibres (21,88). Apart from preventing migration, cytochalasin B also prevents replication at wound edges (89), strongly suggesting the possibility that replication is dependent on the movement away from the intact sheet.

1.5. TRANSPORT ACROSS THE AORTIC ENDOTHELIUM

In the 1950's, the classical pore theory was developed to clarify the mode of transport through the continuous layer of endothelial cells which line the vascular lumen of blood vessels. This theory postulated the presence of two-pore systems: A large-pore of 250-700 Å and a small-pore of 50-90 Å in diameter (91). Since the exact location of these pores on the endothelium had been disputed for many years, various tracer molecules of known size have been used to resolve this uncertainty. This section concentrates mainly on investigations in search of lipoprotein transport using tracer molecules. (For a more general review of endothelial transport see reference 12).

Stein and Stein (31) used the tracers horseradish peroxidase (MW 40 000) and bovine milk lactoperoxidase (MW 82 000) to

follow the macromolecular transport through the endothelium of mouse aorta. They observed that horseradish peroxidase transport was concentration-dependent. When 0.25 - 0.5 mg of horseradish peroxidase was injected, transport was via the plasmalemmal vesicles, while injection of 5 mg of enzyme resulted in transport via both plasmalemmal vesicles and intercellular junctions (31). On the other hand, lactoperoxidase was transported only via the plasmalemmal vesicles. These early investigations suggested that the intercellular junctions were the location for the small-pore system. This was further investigated with markers of smaller molecular size, such as myoglobin (MW $\sim$ 17 800) and heme peptides (MW $\sim$ 1 900). Neither of these tracers was observed on the intercellular clefts, although they had already appeared in the subendothelial space (92). It was thus suggested that any sign of marker in the intercellular junctions was probably due to diffusion with time, and that these were not the location of the small-pore system. The discovery of transendothelial channels which are formed by transient fusion of plasmalemmal vesicles, led to the acceptance of these channels as the small-pore system (92). The channels fit the requirement of the pore theory in that they are water-filled passages connecting the lumen with the tissue spaces, having size limiting structures of about 100 Å.

Agreement has been reached as to the location of the large-pore system in the plasmalemmal vesicles by tracers such as

ferritin and lactoperoxidase. It is thought that lipoproteins use the large-pore system of transport, after various studies in different species (90,93). The studies carried out can be divided into two main groups: those that made use of labelling the cholesteryl ester moiety and those that labelled the protein component of lipoproteins. The method of radioautography using protein-labelled lipoproteins was employed to study the transport of ^{125}I-lipoproteins, such as human VLDL, LDL, HDL, delipidated HDL apolipoprotein and rat HDL in a perfusion of rat aorta and heart (93). The outcome of this investigation was that plasmalemmal vesicles participated in the transport and that the whole lipoprotein particle was taken up. Apart from this non-receptor mediated transport of lipoproteins, it is known that endothelial cells possess saturable high affinity receptors for LDL, which allow for a regulated uptake of LDL (see Introduction 1.7).

1.6. THE ORIGIN AND COMPOSITION OF LDL

The cholesteryl esters found in atherosclerotic plaques have been suggested to be mostly derived from the cholesterol which is delivered to the cells by the LDL particle (10,94). There are two lipid sources for the circulating plasma lipoproteins: exogenous dietary fat and endogenous synthesis by the liver (7). Chylomicrons, rich in triglycerides, are secreted from the intestinal mucosa following the absorption of digested fats. The other triglyceride-rich lipo-

protein, VLDL, is synthesized predominantly in the liver from non-lipid precursors such as glucose (7)(Figure 1.2). The catabolism of VLDL and chylomicrons is initiated by hydrolysis of their triglyceride moiety by lipoprotein lipase. The enzyme is bound at the luminal surface of capillary endothelium. The released fatty acids are taken up largely by the muscle cells and adipocytes. The structural peptide of VLDL, apoprotein B, is retained as the lipoprotein particle, and is catabolized through a range of products, including smaller VLDL, then intermediate-density lipoprotein (IDL) and finally low density lipoprotein (LDL). Not only the apo B, but also cholesteryl esters are stable components of this series of lipoproteins. Although some studies indicate that most of the apo B of VLDL is normally converted to the apo B of LDL, other observations suggest that this conversion is only partial (95). In addition, the cholesteryl ester component of LDL is increased, presumably through the action of the plasma enzyme, lecithin: cholesterol acyl transferase (LCAT) (9). The studies of Glomset (96) suggest that HDL is the source of free cholesterol that becomes the esterified cholesterol of LDL.

The low density lipoprotein particles of human plasma have a density range of 1.019 to 1.063 g/ml and a molecular weight of about 2 - 3.5 x 10^6 daltons with a diameter of 200 - 250 Å (9). The variation in size and molecular weight has been shown to be due to the varying amount of lipid in the core of the lipoprotein particle, rather than

Figure 1.2: Schematic representation of endogenous LDL synthesis

Code:
VLDL = very low density lipoproteins
IDL = intermediate density lipoproteins
LDL = low density lipoproteins
HDL = high density lipoproteins
R = receptor site
NR = non-receptor site

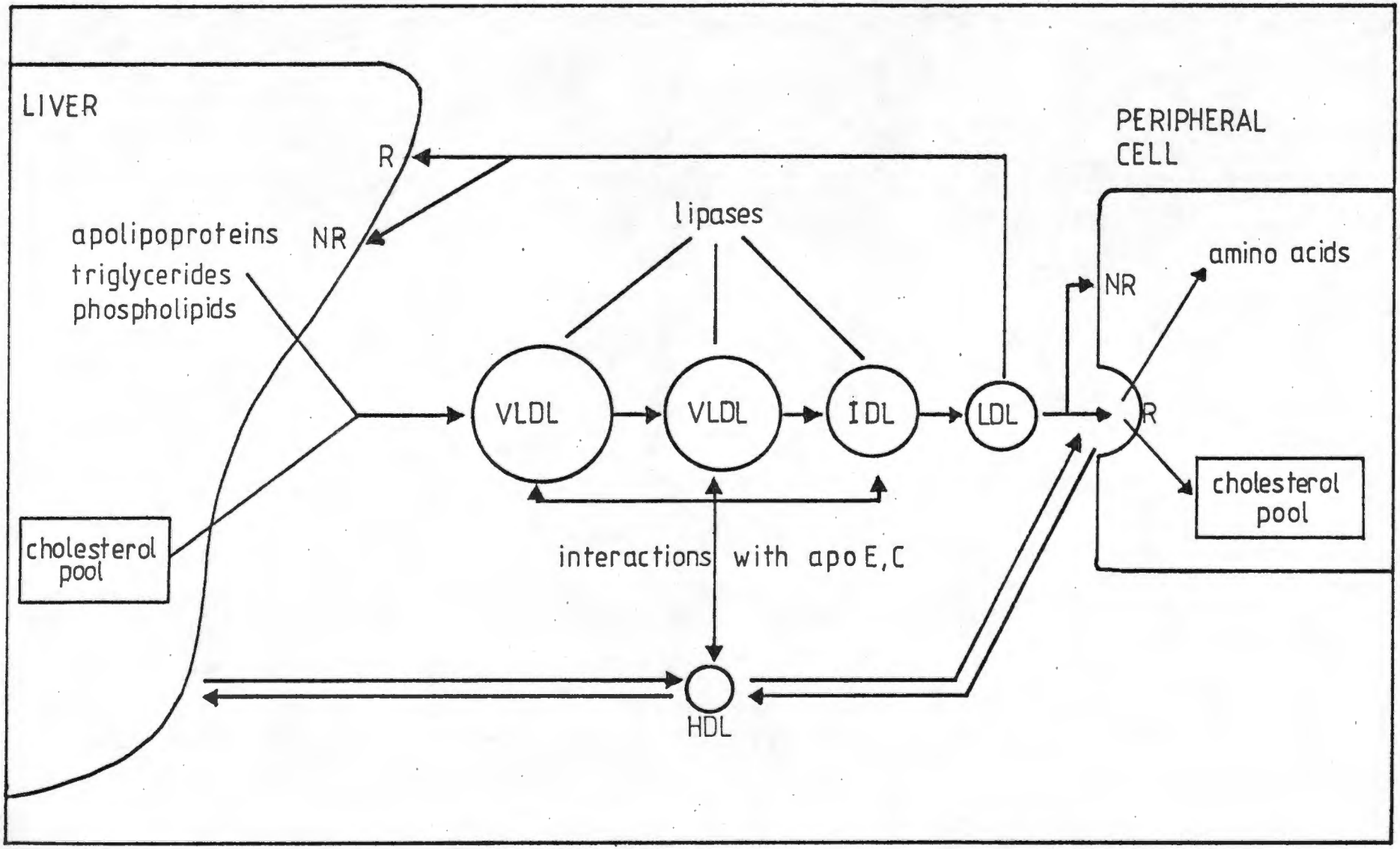


FIGURE 1.2: SCHEMATIC REPRESENTATION OF ENDOGENOUS LDL SYNTHESIS
(Modified from reference 171)

to the variation in the protein composition (97). Lipids comprise 75% of the mass of the LDL particle and the protein makes up 25%. The lipid component of LDL consists primarily of an apolar core of neutral lipids, mostly esterified cholesterol (60%) with phospholipids (30%) and free cholesterol (10%) surrounding it. Apolipoprotein B (apo B), the polypeptide moiety of LDL, directs the particle to its site of catabolism. This protein has remained poorly characterized, primarily due to its marked insolubility in aqueous solutions in the absence of amphiphiles or denaturants (104). Many of its properties continue to be disputed with widely varying estimates of its molecular weight. These conflicting results could be attributed to the different methods employed to characterize the protein, such as delipidation (98), proteolytic activity (99,100), the use of various denaturing agents (101,102) and detergents (103,102). In addition, there is a high tendency for the subunits of the apoprotein B to aggregate (105) and the possibility of reaction between protein and auto-oxidizing lipids (106) which further complicate the research. Some data in the literature indicate that apo B consists of high molecular weight proteins (107, 109), while others indicate that apo B consists of subunits (98,105). The estimates of the molecular weight of apo B have ranged from 8 000 to 450 000 (101,98,108,106). Socorro and Camejo (108) drew some interesting conclusions, that when apo B is prepared by side chain modifications and organic solvent extraction, intermediate molecular weight subunits (26 000 - 80 000) are detectable in the presence of ionic

detergents and/or urea; when only organic solvents are used, apo B is associated and has molecular weights ranging from 250 000 to 318 000; and when detergents are used in the absence of organic solvents, then small subunits of molecular weight 8 000 to 10 000 are present.

In general, there appears to be some agreement that the monomeric state of apo B consists of a molecular weight of 250 000 to 270 000 (102,107,112). Additionally, it has been shown that delipidated LDL can be reconstituted with yolk lecithin yielding a 500 000 g/mol complex, which is the dimeric form of apo B, forming a quaternary structure (110). Zampighi et al (111) complexed the apo B to the detergent, n-dodecyloctaethyleneglycolmonoether. This protein-detergent complex was investigated using electron microscopy, and a rod-shaped particle composed of 2 copies of apo B and 5-6 equivalent micelles of detergent was observed. This asymmetrical particle had a high degree of flexibility with a strong tendency to self-associate, the latter being pH-dependent. Although the width of the complex remained fairly constant (4-5 nm), the length was variable ($\sim$80 nm).

Since apoprotein B directs the LDL particle to its site of catabolism, it is of immense value to obtain increasing knowledge of the structure of the protein, in order to gain a deeper insight of its functional aspect. In a later section (Introduction 1.8), modification of amino acids of

the apoprotein B will be discussed, with special reference to the effect of LDL uptake by a variety of cells.

1.7. THE LOW DENSITY LIPOPROTEIN PATHWAY

High affinity receptors, specific for LDL, have been shown to be situated on the surface of extrahepatic cells such as human skin fibroblasts (113,114), endothelial cells (21,54, 66,67), smooth muscle cells (115,116) and adreno-cortical cells (117). These glycoprotein cell surface receptors are also known as the apo B-E receptors as they can bind lipoproteins containing either apoprotein B or apoprotein E (166,167) (see Figure 1.3).

LDL particles bind to the apo B-E receptors, which are situated in coated pits on the cell surface. These coated pits, which represent 2% of the cell surface, contain about 70% of the apo B-E receptors (168). The major constituent of the coated pits is a polypeptide, clathrin (118). Subsequent to binding, the LDL particle is internalized via endocytotic vesicles, which eventually fuse with the lysosomes (169). It has been speculated that receptor-ligand dissociation occurs before the endocytotic vesicle has reached and fused with the lysosome (165), with the subsequent event of receptor recycling. Within the lysosomes, the apoprotein moiety is degraded by lysosomal proteases to amino-acids. The cholesteryl esters are hydrolysed to free cholesterol by a lysosomal acid lipase. The unesterified cholesterol crosses the lysosomal membrane and enters the cyto-

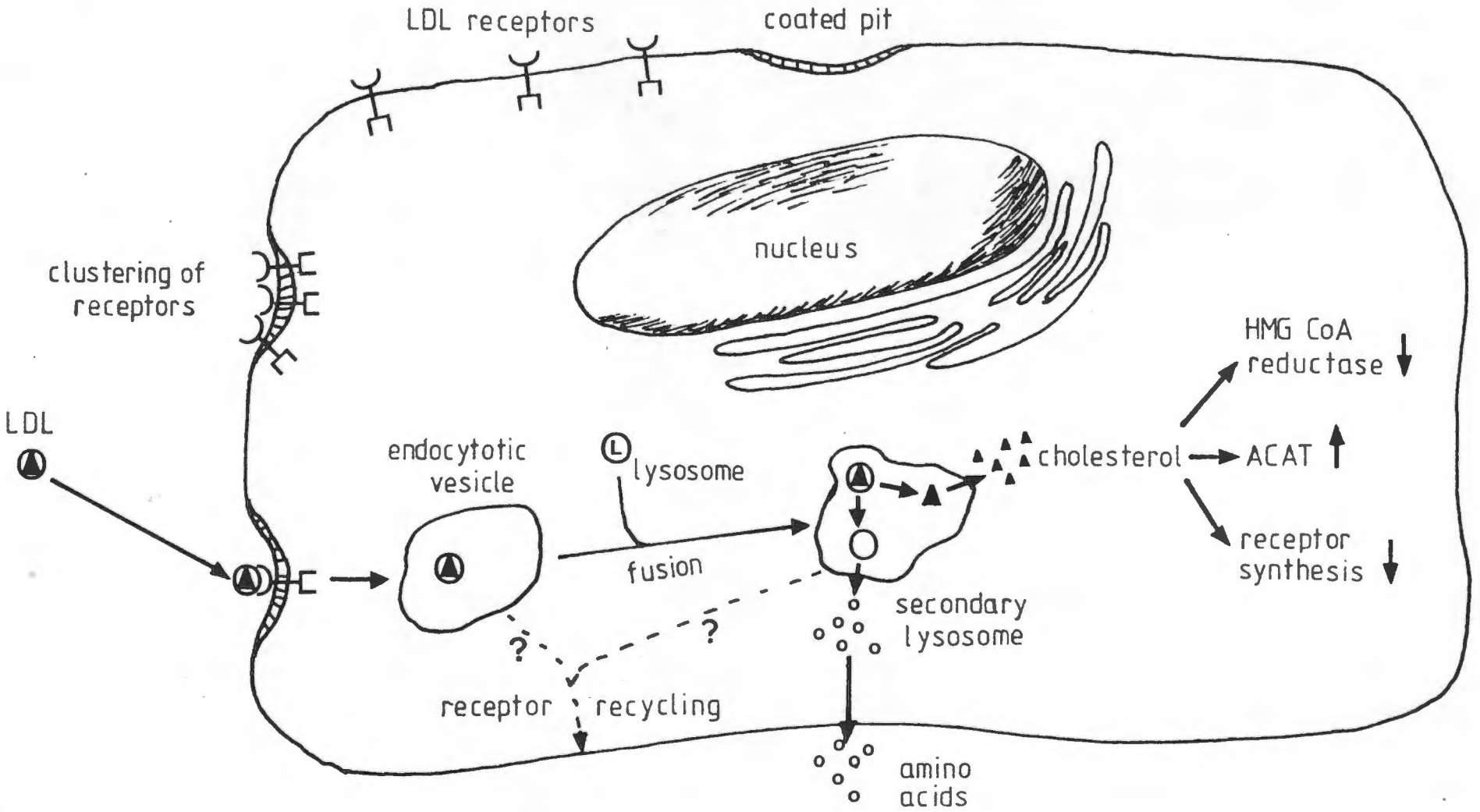


FIGURE 1.3: SCHEMATIC REPRESENTATION OF THE EXTRAHEPATIC LDL PATHWAY

plasmic compartment, where it triggers three regulatory responses:

(1) the suppression of the activity of 3-hydroxy-3-methyl-glutaryl CoA reductase. This is the rate-limiting step in cholesterol biosynthesis within the cell;
(2) the suppression of the synthesis of the LDL receptors; and
(3) the activation of acyl CoA: cholesterol acyl transferase (ACAT). This enzyme converts excess cholesterol to cholesteryl esters for storage as lipid droplets (119).

The continual functioning of these regulatory mechanisms is of utmost importance in the regulation of cellular cholesterol levels.

1.8. MODIFICATIONS OF LDL RELATED TO CHOLESTERYL ESTER ACCUMULATION IN CELLS

It is difficult to define the mechanism responsible for the lipid accumulation characteristic of atherosclerosis, since LDL, the major carrier of cholesterol into the arterial wall is taken up by cells via a well-regulated pathway (for reviews see 8,9). An excess of cholesterol triggers the three feedback control mechanisms (described in Introduction 1.7), preventing abnormal cellular accumulation of cholesteryl esters in cells under normal conditions.

When the apoprotein B moiety of LDL is chemically modified,

an over-accumulation of esterified cholesterol in various cell types *in vitro* such as human monocyte-derived macrophages (76,120), mouse peritoneal macrophages (78), smooth muscle cells (83,86), endothelial cells (84) and fibroblasts (121), has been observed. When LDL is chemically modified to a cationized form, which is more electropositive than normal LDL, it is taken up by fibroblasts (121) and smooth muscle cells (122) by a low affinity, non-saturable mechanism. An enhanced electropositive charge on LDL was also obtained by desialyzation of the LDL particle by neuraminidase treatment (123,124,125). However, although cells incubated with cationized LDL resulted in the appearance of numerous cholesteryl ester droplets, those with desialyzed-LDL did not (10). Desialyzed-LDL is taken up via adsorptive endocytosis, and interestingly, if exposed to receptor-deficient cultured human skin fibroblasts, was shown to have the ability to regulate cholesterol metabolism (125). Although cationized-LDL caused a cholesteryl ester increase in some cells, the problem remained that the LDL isolated and purified from plaques had a more electronegative charge relative to plasma LDL (126). This led to various modifications of LDL to obtain an electronegative particle that would be taken up by cells and result in a cholesteryl ester accumulation.

Recent studies by Goldstein *et al* (127) showed that mouse peritoneal macrophages bind and internalize LDL by a high affinity process if the LDL has been acetylated, thus increasing its net negative charge. The uptake of acetylated-LDL (AcLDL) is not via the apo B-E receptor. The feedback

control found with apo B-E receptors is absent and thus cholesteryl ester accumulation in these macrophages occurs. Since mouse peritoneal macrophages do not catabolize normal LDL (78,127), lipid accumulation in these cells is largely due to the uptake of modified LDL. Mahley *et al* (128) have also shown that canine peritoneal macrophages take up and degrade acetoacetylated-LDL at a much higher rate than native LDL. Furthermore, *in vivo* studies of the clearance of acetoacetylated-LDL from the plasma of rats, resulted in the modified lipoprotein being found in the liver, where the Kupffer cells were responsible for its rapid removal (129, 130).

Although it has not yet been demonstrated that chemically modified LDL, such as cationized LDL or AcLDL are produced *in vivo*, the production of malondialdehyde upon platelet aggregation (50,74,131) can result in a negatively modified LDL which causes cholesteryl ester accumulation in monocyte-macrophages (76). Another source of malondialdehyde is at the site of lipid peroxidation (132). Incubation of macrophages *in vitro* with malondialdehyde-treated LDL (MDA-LDL) has resulted in massive cholesteryl ester accumulation (76, 77,133). Neither native LDL nor MDA-LDL can regulate the MDA-LDL receptor, yet both can downregulate the LDL receptor (133). This inability to regulate the receptor for MDA-LDL could perhaps account for the large cholesteryl ester accumulation which occurs in these monocyte-macrophages. An interesting factor of this MDA-LDL receptor in monocyte-macrophages is that AcLDL is also recognized (77). Conversely,

the receptor in mouse peritoneal macrophages which bound AcLDL, also recognizes MDA-LDL, and thus it appears that there is one receptor which recognizes and binds both forms of negatively-modified LDL (77,78). This receptor found in macrophages is not expressed in fibroblasts, smooth muscle cells, lymphocytes or adrenal cells (78), but it is found in endothelial cells (84). Although this receptor is not found in smooth muscle cells, when these cells are incubated with medium containing AcLDL pre-incubated with either macrophages or endothelial cells, they become lipid-laden (85,86).

During the same period, Henricksen et al (94) showed that if medium containing LDL was exposed to endothelial cells, the LDL particle is modified in such a way that when post-incubated with macrophages, it is taken up via the same receptor as for AcLDL. This effect was specific for endothelial cells, and as yet has not been used to look at possible cholesteryl ester loading. The ability of endothelial cells in vitro to modify LDL and allow for its uptake by a receptor-mediated pathway in macrophages is a rather interesting observation, especially if it was to be found to cause lipid loading in macrophages as well. Together with the various other ways in which LDL is modified, it leads to an attractive proposition of how atherosclerosis may occur.

1.9. CONCLUSION

Numerous *in vitro* studies on endothelial cells have revealed their various mechanisms for maintaining an intact monolayer of contact-inhibited cells. Among these are their ability to produce or respond to metabolites to overcome endothelial damage and to prevent thrombogenesis. An interesting feature of endothelial cells is that they possess a receptor for negatively-modified LDL which has also been found in macrophages, but not in fibroblasts, smooth muscle cells, lymphocytes or adrenal cells (78). In macrophages, the receptor for negatively-modified LDL has been studied with respect to its activity, regulation and cholesteryl ester accumulation, but as yet this receptor in endothelial cells has not been thoroughly investigated. An attempt has been made to answer the following questions regarding the interaction of endothelial cells and low density lipoproteins: How do the receptor affinities for LDL and AcLDL differ? What effect does the cell density of endothelial cells have on the receptor activities? Are there two separate, distinct receptors? If so, how do they differ? How and to what extent are these receptors regulated? Are they able to regulate the cholesterol content within the cells, or do they allow intracellular cholesteryl ester accumulation?

OUTLINE OF PROPOSED RESEARCH

The aim of the present study was to investigate the uptake and catabolism by endothelial cells of LDL which had been acetylated (AcLDL). In the acetylation reaction, acetyl groups are attached to the lysine residues of LDL; this neutralizes the positive charges and increases the net negative charge of the protein. A comparative study of the handling of LDL and AcLDL by endothelial cells was envisaged.

The experimental model system chosen was cultured bovine aortic endothelial cells. These cells were obtained by mechanically scraping the lumen of the aorta of a 2-3 month old bovine foetus. Although cultured cells need not reflect the *in vivo* situation, there are far more possibilities for experimental manipulations over the whole animal system.

To establish the receptor behaviour in endothelial cells suitable conditions for assaying lipoprotein binding and catabolism were investigated. These conditions would allow for the characterization of receptor and non-receptor pathways and the distinction between LDL and AcLDL receptors.

Since there had been a controversy about the LDL receptor activity with cell density (21,66,67,163), it was useful to establish the behaviour of the LDL receptor in these cells. The possible regulation of receptor activity in cultured

cells by factors such as cell density and cholesterol were therefore examined.

The effect of inhibitors of lysosomal function was measured to establish whether AcLDL was delivered to the lysosomes for degradation. Furthermore, the release of cholesterol from AcLDL was investigated for the possibility of causing cholesteryl ester accumulation in endothelial cells.

2. EXPERIMENTAL PROCEDURES

2.1. METHODS

2.1.1. Cells

(a) Bovine aortic endothelial cells: A_3Cl_2 was a clonal cell strain derived from the thoracic aorta of a 2-3 month old bovine embryo, prepared according to the method of Ross (137). The foetus, obtained from the abattoirs, was kept on ice for about 30 min until the aorta was removed. All procedures were carried out under sterile conditions. The foetus was washed with betadine and the heart was removed with the aorta intact. The aorta was then dissected free, cleaned of adherent fat, rinsed and placed into a sterile 100 mm Petri dish containing phosphate buffered saline, pH 7.4 (PBS). With minimal handling, the aorta was cut open longitudinally. A sterile scalpel blade was used to lightly scrape free the endothelial layer. The blade was rinsed into a 35 mm Petri dish containing 2 ml of FCS-medium (Eagle's minimal essential medium buffered with Earle's salts, and containing 10% tryptose phosphate broth, 10% heat-inactivated foetal calf serum (FCS), 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate). The FCS-medium is the normal growth medium. The cells were maintained at 37°C in a humidified atmosphere (95% air : 5% CO_2) until they attached to the dish.

Cloning: When the cultures reached confluence, the cells

were trypsinized (0.05% trypsin - 0.02% EDTA) and plated into 60 mm Petri dishes at a density of 100 - 400 cells/4 ml FCS-medium. The cells were left for 1-2 weeks to allow colony formation from single cells. The colonies that appeared to be derived from a single cell were marked and later harvested using cloning rings. When cell growth appeared to be confluent, these cells were subdivided for storage under liquid nitrogen.

Storage and Passaging: Cells were frozen in 1 ml aliquots of FCS-medium containing 10% dimethylsulphoxide (DMSO). The cell concentration was 10^6 cells/ml. When required, the frozen cells were thawed in a 37°C water-bath, with subsequent addition of 5 ml of FCS-medium, followed by a low speed centrifugation to remove the DMSO. The cells were then resuspended in FCS-medium and seeded into a 75 cm^2 flask in a total of 12 ml of FCS-medium. After 1-2 weeks when the cells had reached confluence, they were dissociated by trypsinization and seeded at a plating density of 10^5 cells/4 ml in 60 mm Petri dishes. Cells were counted in a Coulter Counter - Model Z_f (Coulter Electronics Inc., Hialeah, Florida). These endothelial cells were always used below the 12th passage.

Factor VIII antigen detection: One of the distinguishing characteristics of endothelial cells is the presence of the glycoprotein, Factor VIII antigen, on the cell surface

(26). This was detected using a fluorescent antibody technique as described by Schwartz (19) (Plate 2.1). Cells were grown to confluence on glass coverslips in 60 mm Petri dishes. These were rinsed with phosphate buffered saline containing 2 mM $MgCl_2$ (PBS-$MgCl_2$) and fixed in acetone for 10 min at 0^oC. The coverslips were then overlaid with rabbit antibody globulin to human factor VIII (AHG) associated protein. The coverslips were placed in a humidified incubator (95% air: 5% CO_2) at 37^oC for 30 min. Next they were rinsed in PBS-$MgCl_2$ for 45 min, changing the PBS-$MgCl_2$ every 15 min. The coverslips were dried, and fluorescein-conjugated goat antibody globulin to rabbit immunoglobulins (diluted 1:3) was added to the cells on the coverslips. The cells were again incubated at 37^oC for 30 min, followed by a 45 min rinse with PBS-$MgCl_2$. Coverslips were mounted on microscope slides with the cells face down onto a drop of glycerol-PBS, pH 7.6. The edges of the coverslips were sealed to the slides with clear nail varnish. Controls were carried out in duplicate, using either normal rabbit serum or PBS-$MgCl_2$ in place of rabbit antibody globulin to human factor VIII (AHG) associated protein. All preparations were examined by epifluorescent microscopy, using a Leitz Laborlux 12 microscope. In the blue light fluorescence system, the exciting frequency was 495 nm and the emitted frequency was 510 nm. A real suppression barrier filter (cut off at 530 nm) was used. Images were

PLATE 2.1: FACTOR VIII ANTIGEN DETECTION

Fluorescent antibody technique performed on bovine aortic endothelial cells (A_3Cl_2):

A. Cells treated with rabbit antibody globulin to human factor VIII (AHG) associated protein and exposed to FITC-conjugated goat antibody globulin to rabbit immunoglobulins.

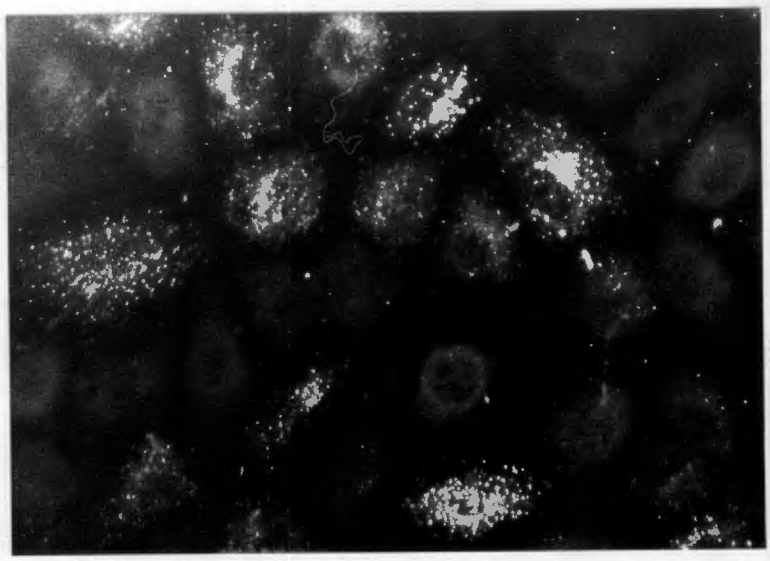
B. Cells treated with normal rabbit serum and exposed to FITC-conjugated goat antibody globulin to rabbit immunoglobulins.

C. Cells treated with PBS-$MgCl_2$ and exposed to FITC-conjugated goat antibody globulin to rabbit immunoglobulins.

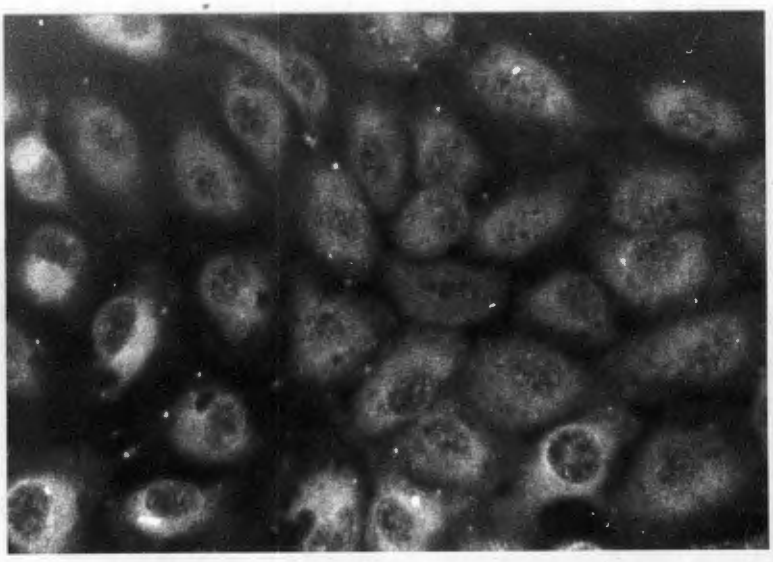
Magnification = x480.

PLATE 2.1: FACTOR VIII ANTIGEN DETECTION

A



B



C

recorded on Kodak Tri-X film by a 20 sec exposure under standard illumination conditions.

Gelatin-coated Petri-dishes: Endothelial cells were grown on gelatin-coated Petri dishes to obtain a culture which exhibited and maintained a confluent monolayer. A 1% gelatin solution was made by allowing the gelatin to swell at $4^{o}C$ in distilled water for 30 min. This solution was centrifuged and washed 7 times with ice-cold distilled water at low speed centrifugation (1000 x g) for a duration of 15 min each. Finally the gelatin pellet was resuspended in the correct volume of distilled water and sterilized by boiling for 20 min. Each Petri dish was covered with 4 ml of the sterile 1% gelatin. The excess was removed by aspiration and the dishes were allowed to dry overnight in a laminar flow hood.

(b) Human skin fibroblasts: Two receptor normal fibroblast cell strains were used, namely GM0203 and SB. GM0203 was obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey. SB was obtained from a skin biopsy from the medial part of the forearm of a male normo-lipaedimic adult and maintained in culture by the same method as described for the bovine endothelial cells. These cells were always used between the 10th to 16th passage.

(c) Macrophages: A continuous cell line (J774) originating from cultured murine lymphoblastoid cells having several characteristics of macrophages (138) was kindly donated by Dr Z. Werb, San Francisco. These cells were stored and seeded in the same way as the endothelial cells and fibroblasts, but passage number was not recorded.

2.1.2. Preparation of lipoproteins and lipoprotein-deficient serum

Human LDL (δ = 1.019 - 1.063 g/ml) and human lipoprotein-deficient serum (δ > 1.25 g/ml) were isolated from the plasma of normolipidaemic donors by ultracentrifugation, using a Beckman L8-70 ultracentrifuge (Beckman Instruments Inc., Fulleston, California). Fractions were isolated by discontinuous gradient centrifugation using the method of Chung et al (139).

A discontinuous NaCl/KBr density gradient was formed by adjusting the density of the plasma (containing 0.01% EDTA) to 1.3 g/ml with solid KBr. The 25 x 89 mm polyallomer ultracentrifuge tubes were filled with 13 ml aliquots of serum (δ = 1.3 g/ml which were layered carefully under 27 ml of 0.9% NaCl - 0.01% EDTA, pH 7.0 (δ = 1.006 g/ml). Specimens were centrifuged at 50 000 rpm for 2.5 h at 10^{o}C in a VTi 50 rotor. The various lipoproteins layered in well-defined bands corresponding to their specific density ranges (Figure 2.1).

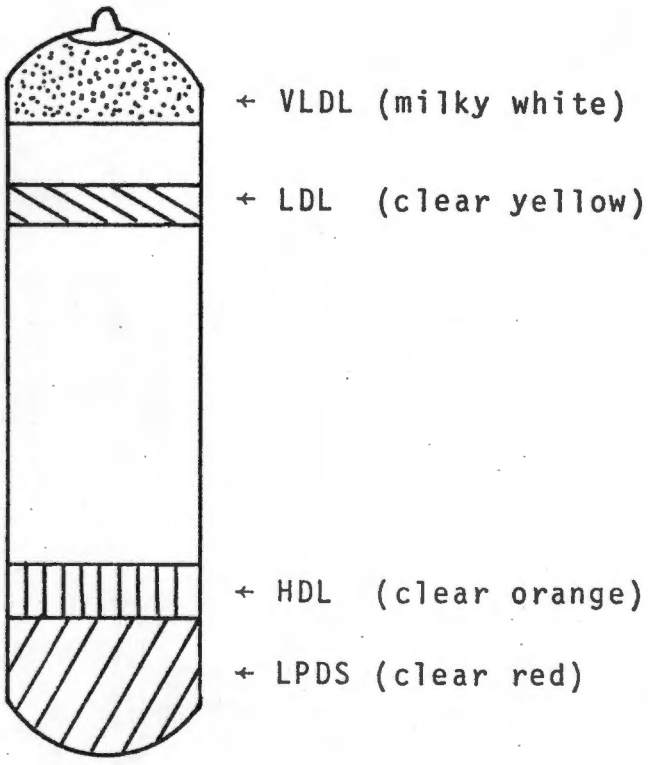


FIGURE 2.1: Separation of lipoproteins.

The centrifuge tube was sliced with a tube-cutter (Beckman Instruments Inc., Fulleston, California) to recover the LDL and the bottom fraction. The LDL fraction was washed at a density of 1.063 g/ml by an overnight centrifugation at 40 000 rpm in a Ti 60 rotor. The yellow layer at the top of the tube which contained LDL was collected.

The bottom fraction obtained after the initial 2.5 hour centrifugation was adjusted to 1.25 g/ml with solid KBr and washed by recentrifugation at 50 000 rpm for 6-8 h in a VTi 50 rotor. The residual HDL at the top of the tube was removed

and the lipoprotein-deficient serum fraction (LPDS) was retained.

Both the LDL and LPDS fractions were dialysed extensively against 0.9% NaCl - 0.01% EDTA, pH 7.4 at 4°C. They were subsequently sterilized by filtration through a Millipore filter of 0.45 µm pore diameter. The protein content was determined by the method of Lowry et al (140), using bovine serum albumin (BSA) as standard. The LPDS was heat-inactivated (56°C, 30 min) and stored at 0°C until required. LDL was stored at 4°C and used within a month.

Preparation of acetylated-LDL: Acetylated-LDL (AcLDL) was prepared according to the method of Fraenkel-Conrat (141). An ice-cold solution of saturated sodium acetate was added to LDL in a 1:1 ratio (v/v), and gently mixed. This was then allowed to stand on ice for 5 min, before the addition of acetic anhydride. The amount of acetic anhydride used was 1 µl acetic anhydride/1 mg LDL. The acetic anhydride was added over a period of an hour with mixing on every addition. The solution was subsequently extensively dialysed at 4°C against 0.9% NaCl - 0.01% EDTA, pH 7.4, followed by a 1 min centrifugation in a Microfuge B (Beckman Instruments, Inc., Spinco Division, Palo Alto, California) to remove any precipitated protein. The protein content, as measured via the Lowry method (140), resulted in a 10-20% loss of lipoprotein. AcLDL was stored at 4°C and used within 3-4 weeks.

Radioactive labelling of lipoproteins: LDL and AcLDL were iodinated using the iodine monochloride method of McFarlane as modified by Bilheimer et al (142). The lipoprotein (10 mg) to be iodinated was placed in a test-tube containing glycine buffer, pH 10 (0.3 - 0.5 M), and 50 µl of ^{125}I-sodium iodide (100 mCi/ml; 13-17 mCi/µg). A 4.2 mg/ml solution of iodine monochloride was diluted 1:5 with 2 M NaCl. Of this solution, 0.4 ml was syringed with a quick and forceful action into the mixture contained in the test-tube. The resulting solution was then extensively dialysed at 4^{o}C against 0.9% NaCl - 0.01% EDTA, pH 7.4, to remove any unbound ^{125}I. The label in both LDL and AcLDL was 98% precipitable with 5% trichloroacetic acid (TCA), and the lipid radioactivity as determined by the method of Folch et al (143) was approximately 3% in both cases. Using the above procedure to label the lipoproteins, the specific activities of the labelled lipoproteins ranged from 160 - 250 cpm/ng protein.

2.1.3. Electrophoretic characterisation of the lipoproteins

Agarose gels: Lipoproteins were separated on preformed agarose gels (Beckman Paragon Lipoprotein Electrophoretic kit) at pH 8.6. The slab gel was run at 100 mA for 20 min, after which it was fixed for 10 min. It was subsequently dried at 90^{o}C (10 min) and stained for 5 min in Lipo stain. The gel was destained in a solution of ethanol diluted 3:1 (Plate 2.2).

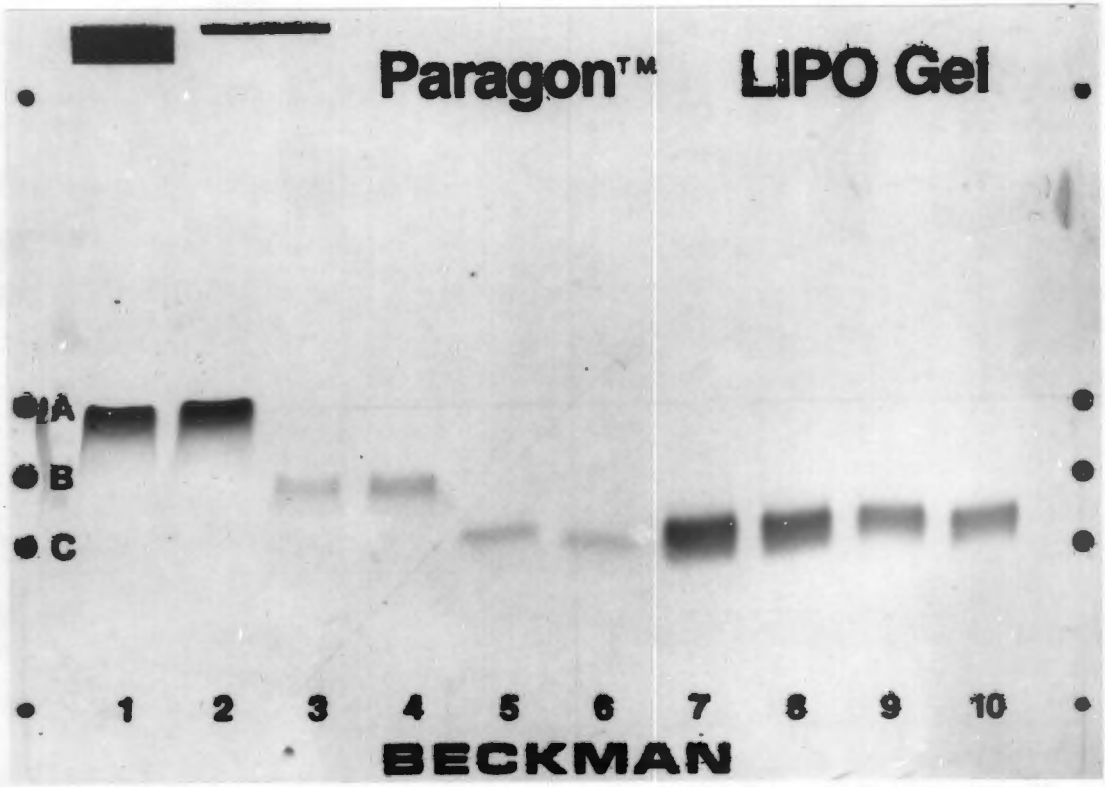


PLATE 2.2: AGAROSE GEL SEPARATION OF LIPOPROTEINS

1,2 = LDL

3,4 = VLDL

5,6 = HDL

7,8 = AcLDL

9,10 = ^{125}I-AcLDL

SDS-PAGE: Lipoprotein proteins were separated according to molecular weight on SDS-polyacrylamide slab gels according to the method of Laemmli (144) (Plate 2.3). A linear 6-12% polyacrylamide gradient containing glycerol (7.5 - 20%; v/v) was used, with a 5% acrylamide stacking gel. Samples of lipoproteins were prepared in solubilizing buffer (0.062 M Tris-HCl (pH 6.8), 2% SDS and 10% glycerol) containing 5% β-mercaptoethanol to give a final concentration of about 1 mg protein/ml. High and low molecular weight standards used were those from Electrophoresis calibration kits (Bio-rad Laboratories, Richmond, California). The electrode buffer contained 0.025 M Tris-HCl, pH 8.3, 0.1% SDS, 0,02 M glycine and the gel was run at 25 mA for the first hour, then at 30 mA until the tracking dye (bromophenol blue) reached the bottom of the gel (about 4 h). The gel was stained in 0,025% Coomassie blue stain in 25% isopropyl alcohol and 10% acetic acid. The destaining solution used was 10% acetic acid.

2.1.4. Determination of bound, internalized and degraded ^{125}I-lipoproteins.

Unless otherwise stated in the legends, lipoprotein receptor activity was up-regulated (145,146) by exposing the endothelial cells to 2 ml of LPDS-medium (Eagle's minimal essential medium buffered with Earle's salts, and containing human LPDS (5 mg protein/ml, i.e. ~10%), which had been heat-inactivated, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate). The LPDS-medium was changed every 24 h

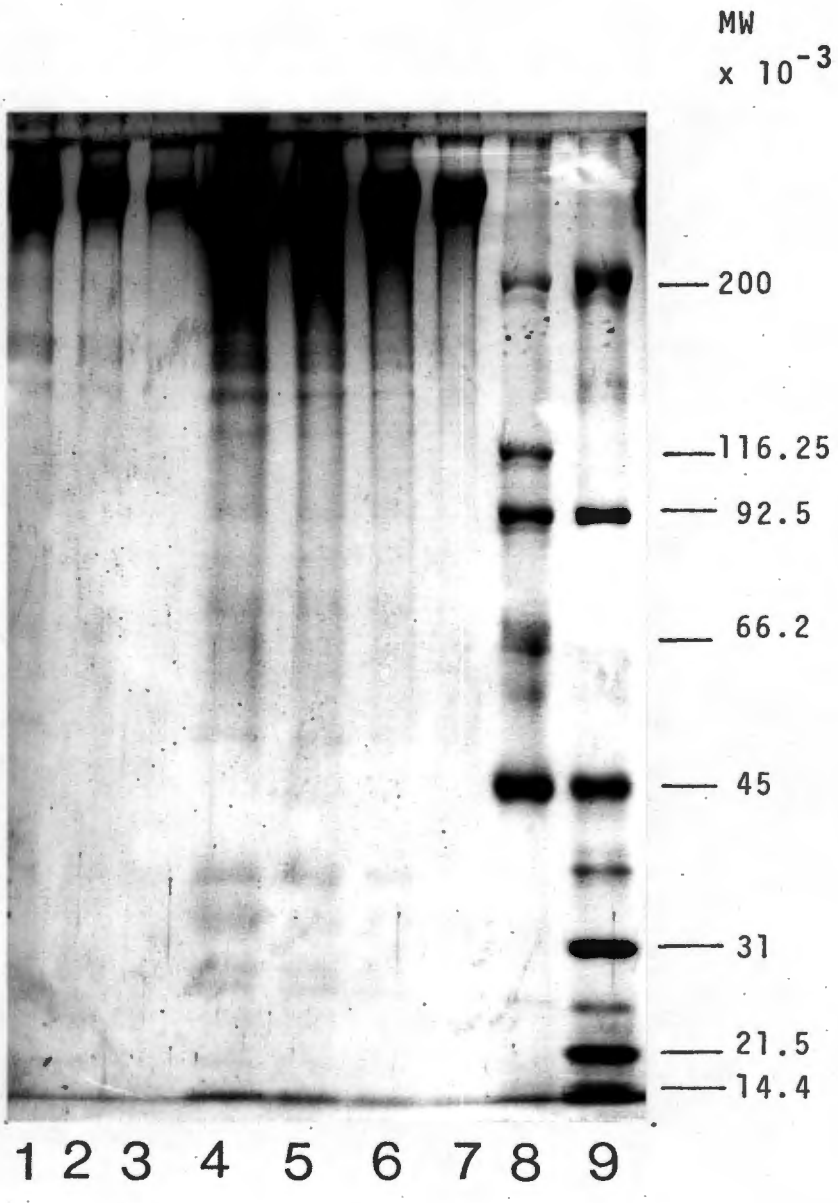


PLATE 2.3: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

1,2,3 = 2.5, 5, 10 µl of AcLDL (1 mg/ml) respectively

4,5,6,7 = 2.5, 5, 10, 20 µl of LDL (1 mg/ml) respectively

8 = high molecular weight standards

9 = low molecular weight standards

when longer incubations were used.

After upregulation, the cells received 2 ml of fresh LPDS-medium containing the required amounts of ^{125}I-labelled lipoproteins as specified in Results. (Note: Throughout the text ^{125}I-labelled lipoproteins are referred to as ^{125}I-lipoproteins). The cells were incubated at 37^{o}C under humidified conditions (95% air : 5% CO_2). After 4 h, the cells were cooled to 4^{o}C and the medium collected for the determination of ^{125}I-lipoprotein products (115).

Determination of degraded lipoproteins: The medium was treated with 0.6 ml of 50% trichloroacetic acid (TCA) to obtain a final concentration of 12.5% TCA. Samples were placed at 0^{o}C for 30 min to ensure complete precipitation, after which they were centrifuged at 1000 x g for 20 min. Any free iodide in the TCA-soluble fraction was converted to I_2 by the addition of hydrogen peroxide. The standard procedure involved treatment of 1 ml of the TCA-soluble fraction with 20 µl of 40% KI and 50 µl of hydrogen peroxide. This was followed by the addition of 2 ml of chloroform to extract the I_2, and centrifugation at 1000 x g for 15 min. An aliquot (750 µl) of the resulting aqueous phase was counted (Packard PGP autogamma (Prias) counter). The counts were used to calculate the degradation amounts, which were expressed as ng ^{125}I-lipoprotein protein degraded/mg cell protein. Routinely, lipoproteins in LPDS-medium were incubated in cell-free dishes. Following incubation, the

medium was processed as described above. The radioactivity obtained was subtracted from that obtained in the presence of cells to correct for any extracellular lipoprotein degradation and/or any acid-soluble material present in the ^{125}I-lipoprotein preparations.

Determination of bound lipoproteins: Following removal of the incubation medium, the cells were washed 4 times with 3 ml of phosphate-buffered saline containing 0,2% bovine serum albumin (PBS-BSA) and 4 times with 3 ml of PBS to remove all unbound radioactivity. All washing was carried out in a cold-room at 4°C and all solutions were kept at 0-4°C. The standard method employed to release surface-bound ^{125}I-lipoproteins was by the addition of 1 ml of 0.05% trypsin - 0.02% EDTA for 20 min at 4°C. During this time all the cells were released from the dish. Trypsin was inactivated by the addition of 1 ml of LPDS-medium and the suspended cells were centrifuged at 1000 x g for 5-10 min. The supernatant was decanted and taken to represent the amount of receptor-bound ^{125}I-lipoprotein (116).

Alternatively, the release of surface-bound material was executed using 10 mg/ml of dextran sulphate (or 10 mg/ml of sodium heparin) for 60 min at 4°C. The resulting solution was analysed for ^{125}I-radioactivity and taken to represent the amount of receptor-bound material.

Determination of internalized lipoproteins: The cell pellet was extracted with a 60% : 40% methanol : chloroform mixture

and centrifuged at 1000 xg (5-10 min). The supernatant was discarded and the pellet dried at 60°C for 15 min. The cell pellet was then dissolved in 1 ml of 1 M NaOH by incubation at 37°C overnight. This solution was counted for the amount of ^{125}I-radioactivity to determine the intracellular content of ^{125}I-lipoprotein. The amount of cell protein was determined using the method of Lowry (140).

4°C Binding of ^{125}I-lipoproteins: Cells were up-regulated for 48 h in LPDS-medium at 37°C in a humidified incubator (95% air : 5% CO_2). Subsequently cells were pre-chilled for 10 min at 4°C before the addition of ^{125}I-lipoproteins in 2 ml of Hepes-medium (Eagle's minimal essential medium without bicarbonate, containing 10 mM Hepes buffer, pH 7.4, 60 µg/ml penicillin G, 100 µg/ml streptomycin sulphate and 5 mg/ml heat-inactivated human LPDS). All procedures at 4°C were carried out by placing the cell dishes on steel trays in a 4°C cold room. All solutions were cooled to 4°C prior to use. After incubation for 2 h at 4°C, the cells were washed extensively at 4°C as previously described. At 4°C no internalization or degradation of ^{125}I-lipoproteins occurs; therefore the 4°C binding assay measures only surface bound lipoproteins (114,147).

Inhibition of lipoprotein degradation: In certain experiments, known inhibitors of lysosomal activities were used to prevent ^{125}I-lipoprotein degradation. Cells were incubated at 37°C with LPDS-medium containing ^{125}I-lipoprotein in the presence

of 70 μM chloroquine (148) or 100 μg/ml leupeptin (149). The various procedures outlined above to determine the amount of bound, internalized and degraded lipoproteins were executed in each case.

2.1.5. Affinity constants and maximum capacity determinations

Affinity constants and maximum capacity binding of lipoproteins to the cell surface can be obtained using the analogy of hormone-receptor interactions (150). Assuming that the lipoprotein-receptor interaction is a simple bimolecular reaction, the following equilibrium equation holds:

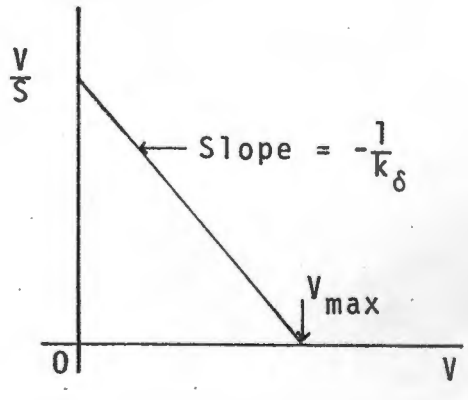
$$|L| + |R| \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} |L - R| \quad ;$$

where $|L|$, $|R|$ and $|L\text{-}R|$ represent the concentration of lipoprotein, receptor and receptor-bound lipoprotein respectively. The equilibrium dissociation constant (k_δ) is the ratio of the dissociation (k_{-1}) to the association (k_1) rate constants, i.e. ($k_{-1}.k_1^{-1}$).

Two linearization techniques were employed to calculate the apparent equilibrium dissociation constant (k_δ) and the maximum capacity (V_{max}) of lipoprotein receptor activity.

Scatchard Analysis (136)

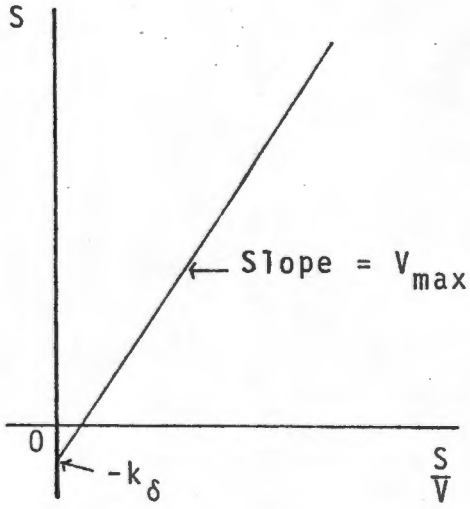
The ratio of receptor-bound (V) to free lipoprotein(s) was plotted against receptor-bound lipoprotein. The slope of the



resultant straight line is equal to $-\frac{1}{k_\delta}$, and the intercept on the x-axis corresponded to the V_{max}. This plot is weighted for k_δ analysis.

Riggs Analysis (151)

Free lipoprotein (S) was plotted against the ratio of free to receptor-bound lipoprotein (V). The slope of the resultant straight line is the maximum lipoprotein bound (V_{max}) and the point of intersection on the y-axis is equal to $-k_\delta$.



This technique is weighted for V_{max} analysis.

Receptor number per cell: The numbers of receptors per cell were determined using the following equations and assumptions:

no. of receptors per dish = LDL particles bound per cell at saturating concentrations of LDL

$$= \frac{\text{bound LDL protein (g/dish)}}{(\text{MW lipoprotein})(\%\text{ protein in LDL})} \times (\text{Avogardo's no.})$$

where MW lipoprotein = 2.6×10^6 daltons

% protein of LDL = 20%

$$\therefore \text{ no. of receptors per cell} = \frac{\text{(no. of receptors per dish)}}{\text{(cells per dish)}}$$

2.1.6. Determination of DNA synthesis

The synthesis of DNA was determined by measuring the amount of thymidine |methyl-^{3}H| taken up by the cells. The cells were incubated with 2 µCi ^{3}H-thymidine in 2 ml of FCS-medium at 37°C in a humidified incubator (95% air : 5% CO_2). After one hour, the cells were trypsinized (0.05% trypsin - 0.02% EDTA) and well aspirated to disperse the cells. An aliquot of the cell suspension was counted in a Coulter counter. The remaining suspension was precipitated by 50% TCA to give a final concentration of 10% TCA. After mixing, the solution was kept at 4°C for 30 min. The TCA-precipitated material was filtered under suction onto GF-C millipore filters (Whatman 2.5 glass filters), washed five times with 5% TCA and once with 96% ethanol. The filter was dried at 37°C overnight, and subsequently prepared for counting by the addition of scintillation fluid (Beckman Ready-Solv™ EP).

2.1.7. Determination of ACAT activity

Preparation of ^{14}C-oleic acid - BSA complex: Chloroform (2 ml) was added to 5.6 mg of oleic acid (corresponding to 20 µmoles of fatty acid) and 125 µCi of ^{14}C-oleic acid in a glass test-tube. After evaporation of the chloroform under nitrogen, 60 µmoles of sodium hydroxide in 1.9 ml of 0.15 M NaCl were added. The test-tube was heated to boiling over a flame with extreme care, after which it was placed in a waterbath at 40°C. Bovine serum albumin (340 mg) in 3 ml of 0.9% NaCl - 0.01% EDTA, pH 7.4, was heated to 40°C in a waterbath. The oleic acid solution was added dropwise with

a heated pipette to the continually stirred bovine serum albumin (BSA) solution. Following the addition of all the oleic acid, the solution was then made up to 10 ml with pre-warmed (40°C) 0.9% NaCl - 0.01% EDTA, pH 7.4.

ACAT assay: Cells were up-regulated for 48 h in LPDS-medium and were then incubated with 200 µg protein/ml of lipoprotein in LPDS-medium at 37°C in a humidified incubator (95% air : 5% CO_2). Controls using 10 µg/ml of 25-hydroxycholesterol or LPDS-medium alone, were also carried out for each assay. After 5 h, 0.1 mM ^{14}C-oleic acid - BSA (± 15 000 dpm/nmol) was added to each of the dishes, which were returned to the incubator. After 2 h the cells were thoroughly washed three times with 3 ml of phosphate buffered saline, followed by a 60% : 40% methanol : chloroform extraction. The suspension was then centrifuged at 1000 x g for 20 min. The supernatant was poured off into glass test-tubes and the pellet was dried at 60°C for 15 min. Subsequently, 1 ml of 1 M NaOH was added to the pellet, and was left at 37°C overnight. This solution was then used for protein determination via the Lowry method (140). Chloroform (2.5 ml) and 0.04% $CaCl_2$ (1.5 ml) was added to the supernatant and the solution was mixed and then centrifuged at 1000 x g. The upper aqueous layer was removed by aspiration and discarded. Methanol was added dropwise to the organic layer until clear, and this solution was then dried under nitrogen. Each sample was resuspended in 50 µl of cholesterol ester (triolein) (1 mg/ml) and 50 µl of tri-glyceride (1 mg/ml), and spotted onto thin layer chromatography

plates. The sample runs with the solvent front. The solvent is a mixture of 170 ml hexane, 30 ml diethyl ether and 2 ml acetic acid. The plates are cut up and transferred to scintillation vials containing scintillation fluid (Ready-Solv ^{TM} EP, Beckman). Subsequently they are counted in the Beckman LS 9000 counter.

2.1.8. Determination of free and total cholesterol

The amount of free and total cholesterol present in the cells was determined according to the method of Gamble et al. (152). Cultured cells that had been incubated with either LDL or AcLDL were washed and harvested as in the ACAT assay (see Methods 2.1.7). The pellet was used for protein determination. Once this had been established, the supernatant was aliquoted into test-tubes. Standard solutions of 0.1 mg/ml cholesterol were distributed into test-tubes to yield standard curves (see Figure 2.1). The chloroform present in the various samples was evaporated under nitrogen, after which the samples were heated for a further 20 minutes at 105°C. To each test-tube, 200 µl of 95% ethanol was added, as well as 2 ml of premix 1 to the cholesteryl oleate standards and samples, and 2 ml of premix 2 to the cholesterol standards and samples. Premix 1 consists of 845 µl of 0.1 M potassium phosphate buffer, pH 7.4; 210 µl of 1 U/ml cholesterol oxidase; 10 µl 10 µl/ml peroxidase (horse radish); 0.5 U/ml cholesterol esterase; 105 µl of 0.5% Triton X-100; 20 mM sodium cholate; and 315 µl of 4 mg/ml ρ-hydroxyphenylacetic acid. Premix 2 was the same as premix 1, except that cholesterol esterase

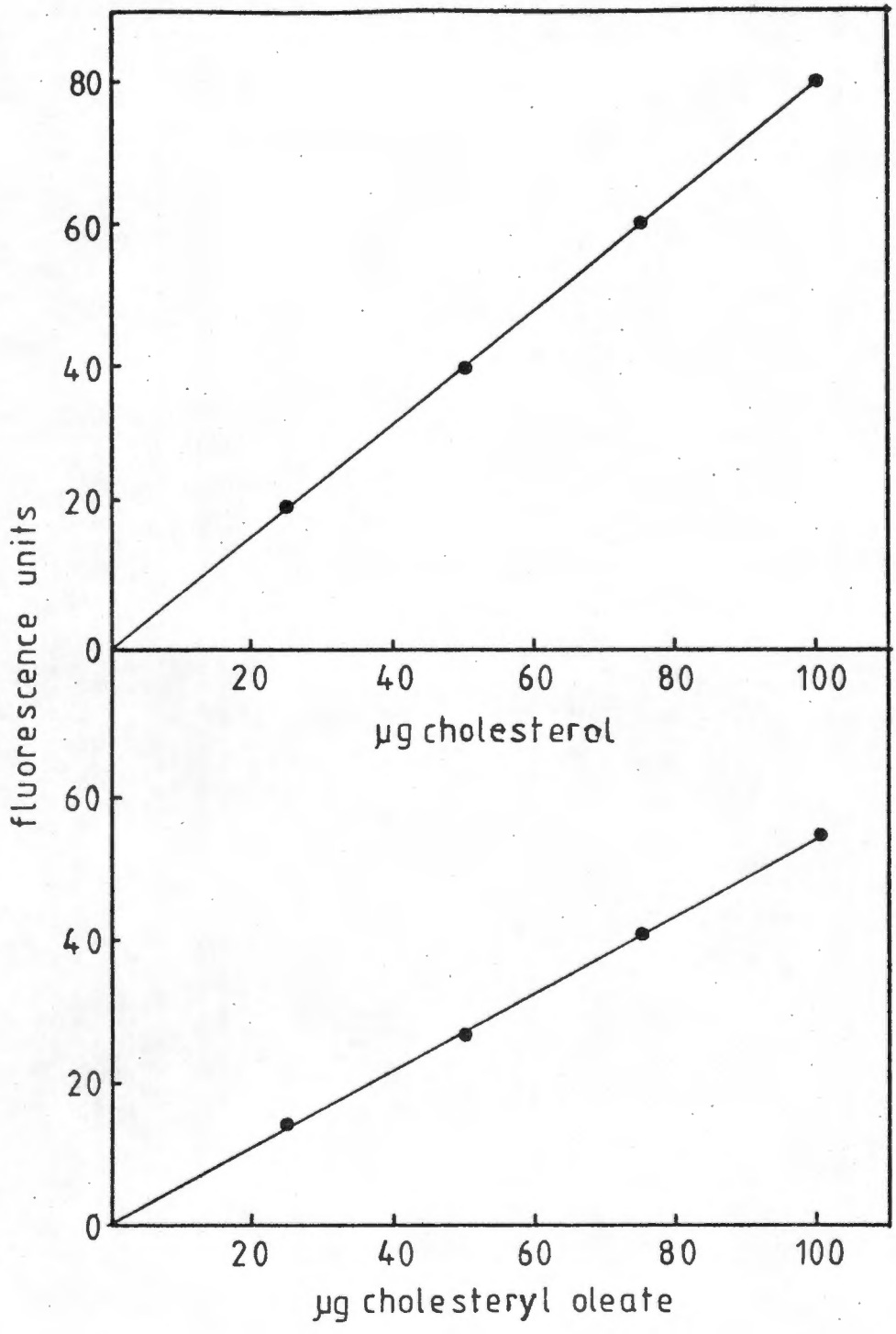


FIGURE 2.1: STANDARD CURVES OF FREE AND TOTAL CHOLESTEROL

was omitted and the equivalent amount of potassium phosphate buffer was added. All the enzymes were made up in buffer. Once the premixes were added to the correct samples, the test-tubes were placed in a waterbath at 37°C for an hour. The fluorescence was measured at an excitation wavelength of 324 nm and at an emitter wavelength of 415 nm on a Perkin Elmer 203 fluorescence spectrophotometer.

2.2. MATERIALS

Corning Ltd., Stone, England	: All culture plastic ware.
Gibco Laboratories, New York, USA	: Eagle's Minimum Essential medium buffered with Earle's salts.
Difco Laboratories, Detroit, USA	: Trypsin (1:250), Gelatin, Tryptose phosphate broth.
Hoechst Pharmaceuticals, Johannesburg, S.A.	: Penicillin G, Streptomycin sulphate.
State Vaccine Institute, Cape Town, S.A.	: Foetal calf serum, Rabbit serum.
Western Province Blood Transfusion Service, Cape Town, S.A.	: Normolipidaemic human blood.

Sigma Chemical Co., St. Louis, USA	: Chloroquine, EDTA, Sodium heparin, Hepes, Cholesterol, Cholesterol oxidase, Cholic acid, Cholesterol oleate.
Peptide Institute Inc., Osaka, Japan	: Leupeptin
BDH Chemicals, Poole, England	: Acrylamide, Bis-acrylamide (NN'-methylene-bisacrylamide), TEMED (NNN'H-tetramethylene-diamine).
Bio-rad Laboratories, Richmond, California	: Molecular weight markers (MW 200 000 to 14 400), Dextran sulphate.
Miles Laboratories, Goodwood, S.A.	: Agarose, Bovine serum albumin-fraction V, Cholesterol esterase, Peroxidase.
Behring Diagnostics, Marburg, W. Germany	: Rabbit anti-human factor VIII (AHG) associated protein, Goat anti-rabbit IgG conjugated to FITC.
Millipore Corp., Bedford, Massachusetts, USA	: Millipore filters, Whatman GFC filters.

New England Nuclear, Boston, Massachusetts, USA	: Thymidine |methyl-^{3}H|-
The Radiochemical Centre Amersham, England	: |^{125}|sodium iodide
E. Merck A.G. Darmstadt, Germany	: All other reagents (analytical grade).

3. RESULTS

3.1. ENDOTHELIAL CELL CULTURE

The cells chosen for this study were bovine aortic endothelial cells, which were isolated by mechanical scraping of the lumen of the aorta of a bovine foetus, and which were subsequently cloned (A_3Cl_2).

3.1.1. Morphology of cultured cells on different substrates

Endothelial cells *in vivo* are characterized by the formation of a monolayer of flat, polygonal cells which are contact-inhibited. Although it has been relatively easy to isolate and grow endothelial cells in culture, the maintenance of a contact-inhibited monolayer has proved to be more difficult. Hence, various substrates and agents have been employed to establish a cell monolayer resembling the *in vivo* morphology at post-confluence (see Introduction 1.2).

In this study, a comparison of cells grown on gelatin-coated Petri dishes to cells grown on uncoated plastic Petri dishes was made (Plate 3.1). The cells seeded at 10^5 cells/60 mm dish were sparsely distributed during the first 3 days. The plating efficiency was about 98%. These epithelioid cells with radiating protrusions were found in a number of foci on the dishes, which eventually converged to form a monolayer of polygonal cells. This sheet of cells, having a cobblestone appearance, had been formed by day 6. Thereafter, the cells

PLATE 3.1: CULTURED BOVINE AORTIC ENDOTHELIAL CELLS

Bovine aortic endothelial cells (A_3Cl_2) were seeded at 10^5 cells/60 mm Petri dish on day 0 in 4 ml of FCS-medium. The cells received fresh medium every 3-4 days.

A-D: Subconfluent layer of cells (day 1, 2, 3, 5 respectively) showing rapid proliferation of cells to confluence.

E: Confluent monolayer of cells (day 10) grown on gelatin-coated Petri-dishes.

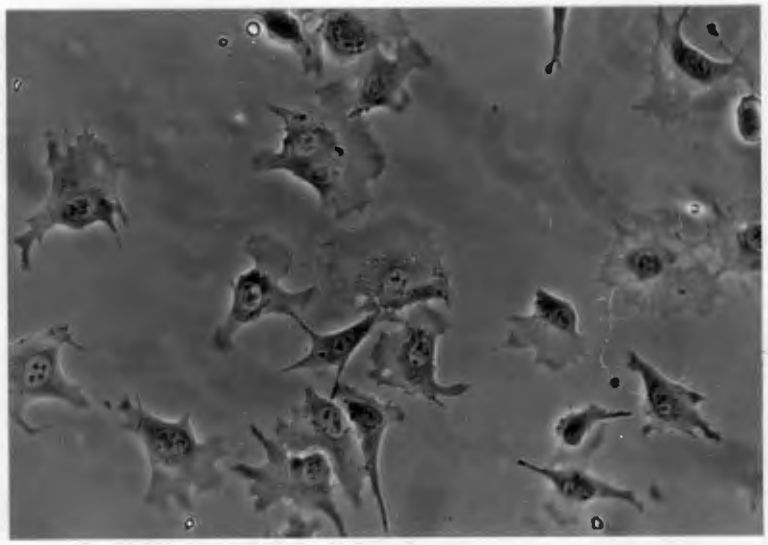
F: Multilayer of cells (day 10) grown on plastic Petri-dishes.

G: Post-confluent layer of cells (day 14) grown on gelatin-coated Petri-dishes showing a small percentage of "sprouters".

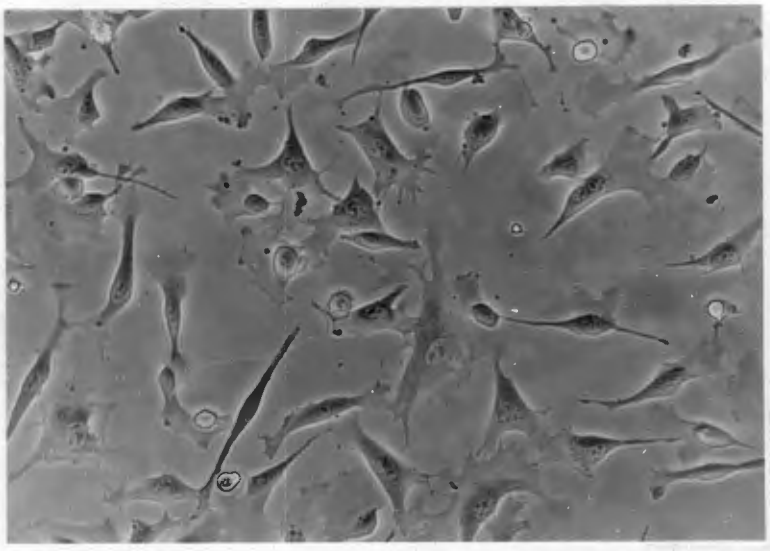
H: Confluent layer of cells treated with 70 µM chloroquine for 4 h. Note extensive vacuolation.

Magnification = x150.

A



B



C

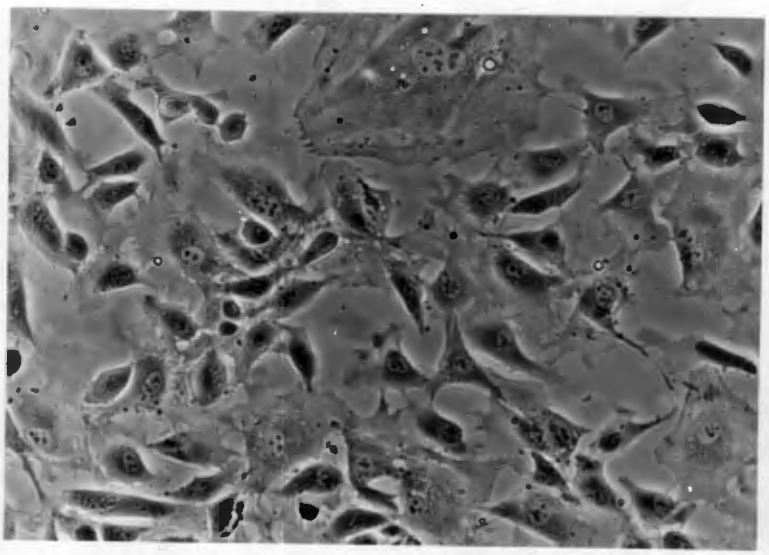
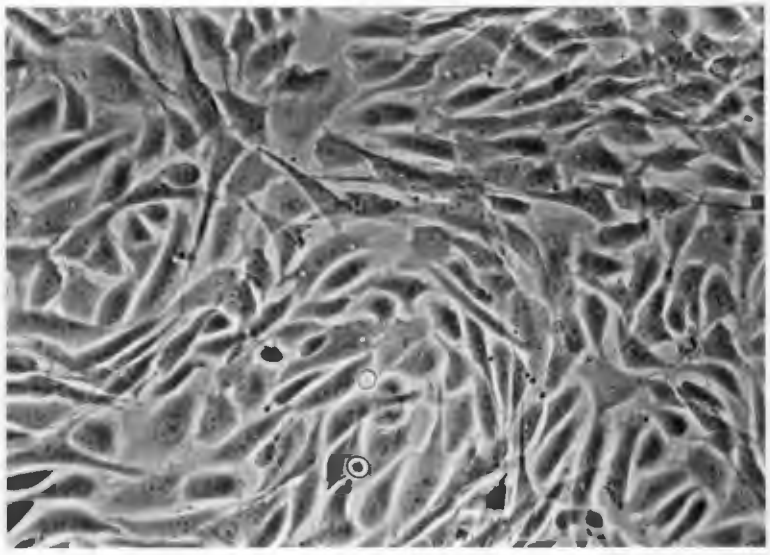
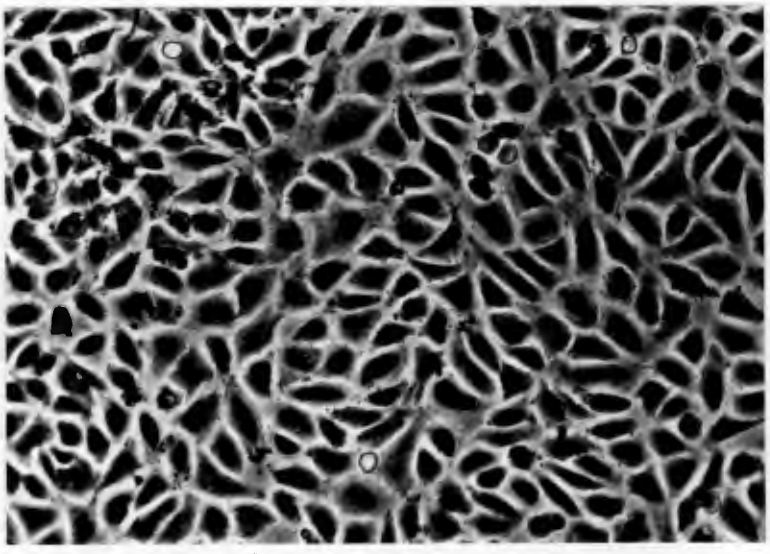


PLATE 3.1: CULTURED BOVINE AORTIC ENDOTHELIAL CELLS

D



E



F

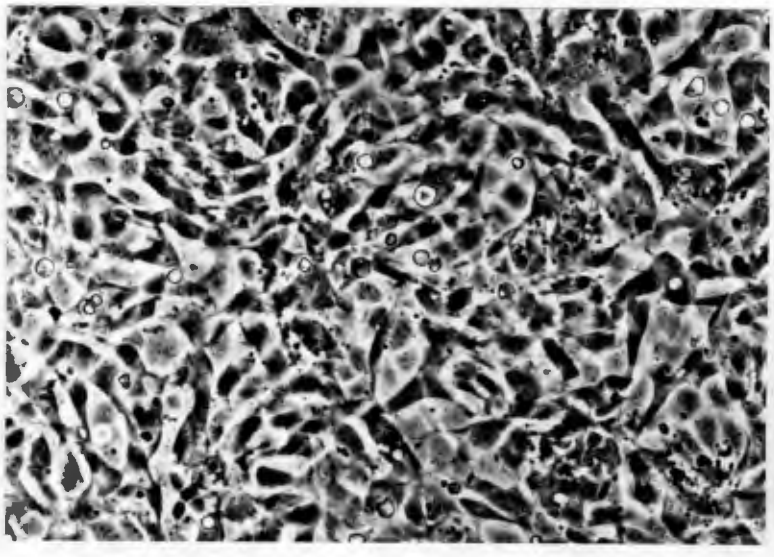
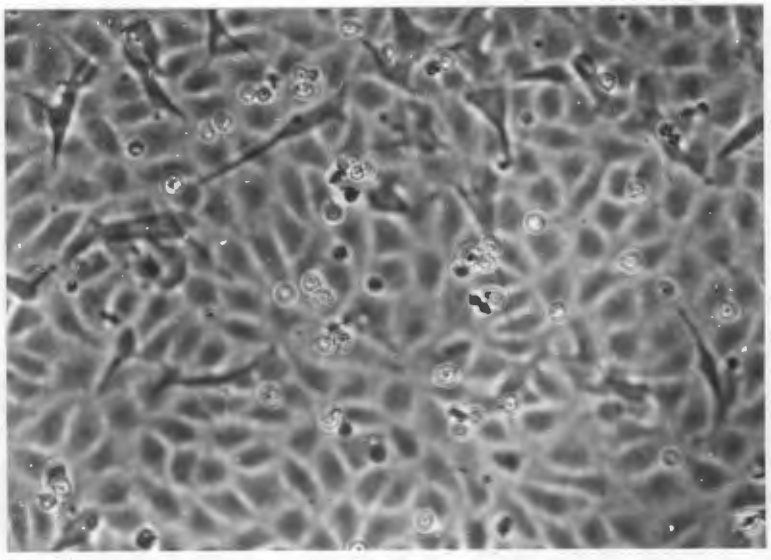


PLATE 3.1 (cont.)

G



H

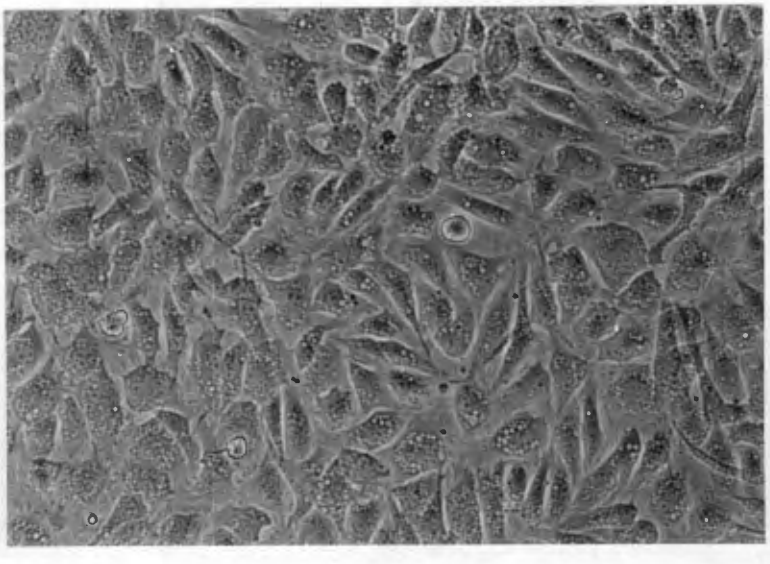


PLATE 3.1 (cont.)

grown on gelatin-coated dishes formed a tight monolayer of small round cells, whereas those grown on plastic dishes failed to maintain a contact-inhibited monolayer, and multi-layered in random directions. The monolayer on gelatin-coated dishes could be maintained for about 10-12 days, after which "sprouters" appeared. These "sprouters" are fusiform cells connected end-to-end, which have been shown to grow in focal areas as a single layer beneath the monolayer (19,43). The "sprouters" appear to proliferate to a limited extent, giving rise to a small number of cells (less than 1% of the total number of cells), even when cultures were maintained for 3 weeks.

In most subsequent experiments, cells were therefore cultured on gelatin-coated dishes because of their closer resemblance to the monolayer found *in vivo*. Sparse cultures were used in experiments before day 6 and confluent cultures were used between day 9 and day 12 to avoid the presence of sprouting cells.

3.1.2. Comparison of growth rate

Since the morphology of A_3Cl_2 differed, depending on the substrate to which they adhered, it was of interest to study the growth rate of the two cultures. This was done by investigating the protein content, number of cells and ^{3}H-thymidine incorporation with increasing age of culture.

The protein content per dish was measured at various time

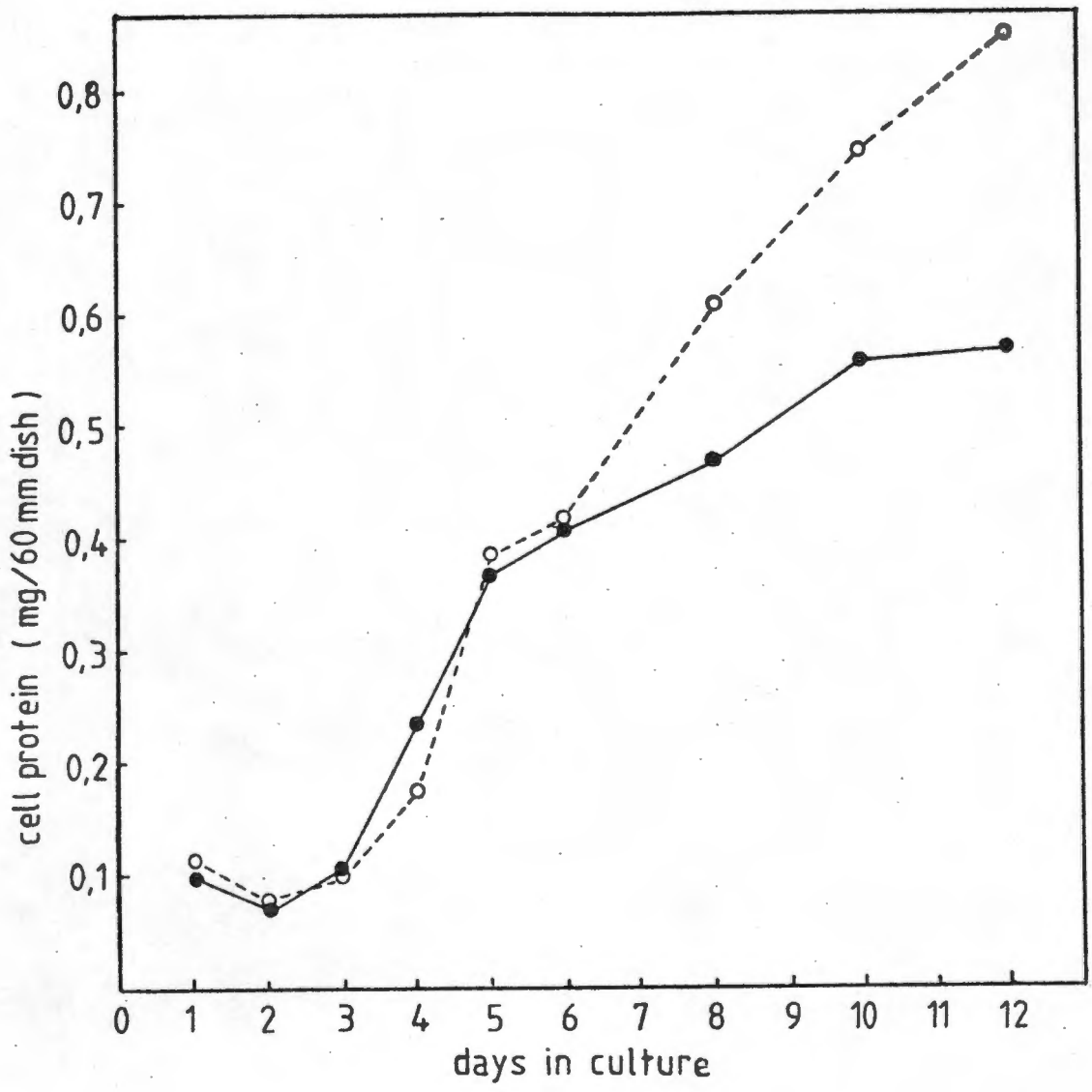


FIGURE 3.1: PROTEIN CONTENT IN ENDOTHELIAL CELLS WITH INCREASING AGE OF CULTURE.

Endothelial cells (A_3Cl_2) were plated at a seeding density of 10^5 cells/60 mm dish on day 0, and grown in FCS-medium on either gelatin-coated (●—●) or uncoated plastic (o--o) dishes. On each specified day, the protein content was determined. The medium was changed every 3 days. Each point is the mean value obtained from duplicate dishes.

intervals in cultures of increasing age (Figure 3.1). It was found that the cells grown on gelatin-coated dishes showed little protein content increase once a monolayer of cells had been formed. In contrast, cultures grown on plastic showed a continued steady increase in protein content, as would be expected with the formation of multilayers.

A similar pattern was observed when the number of cells per dish was determined in cultures of increasing age (Figure 3.2.A). The number of cells grown on gelatin-coated or plastic dishes did not differ substantially in sub-confluent cultures. However, once confluence had been attained, the number of cells grown on gelatin-coated dishes did not increase, reflecting a tight monolayer of cells. On the other hand, the number of cells grown on plastic continued increasing with days in culture, reflecting the multi-layering nature of A_3Cl_2 on plastic.

In the same experiment, in which the cell numbers per dish were determined, the incorporation of ^{3}H-thymidine was measured. This was used as an indication of DNA synthesis occurring in dividing cells. In contrast to the difference observed in the number of cells per dish in the two cultures, the incorporation of ^{3}H-thymidine did not differ. In both cases, the incorporation decreased by about 90% from day 1 to day 10 (Figure 3.2.B).

It may have been expected from the previous data that the

FIGURE 3.2: CELL NUMBER AND ^{3}H-THYMIDINE INCORPORATION IN ENDOTHELIAL CELLS WITH INCREASING AGE OF CULTURE

Endothelial cells (A_3Cl_2) were seeded at 10^5 cells/60 mm dish on day 0, and grown in FCS-medium on either gelatin-coated (●—●) or uncoated plastic (o---o) dishes. On the specified day, the cells were incubated with 2 µCi ^{3}H-thymidine in 2 ml of FCS-medium for 1 h. The medium was discarded and the remaining cells were trypsinized with 0.05% trypsin - 0.02% EDTA.

A: An aliquot of the cell suspension was used to determine the cell number by counting the cells in a Coulter counter.

B: The remaining cell suspension was precipitated with trichloroacetic acid (final concentration of 10%) and the radioactivity in the protein pellet was counted as a measure of ^{3}H-thymidine incorporation.

Each point represents the mean value obtained from duplicate dishes.

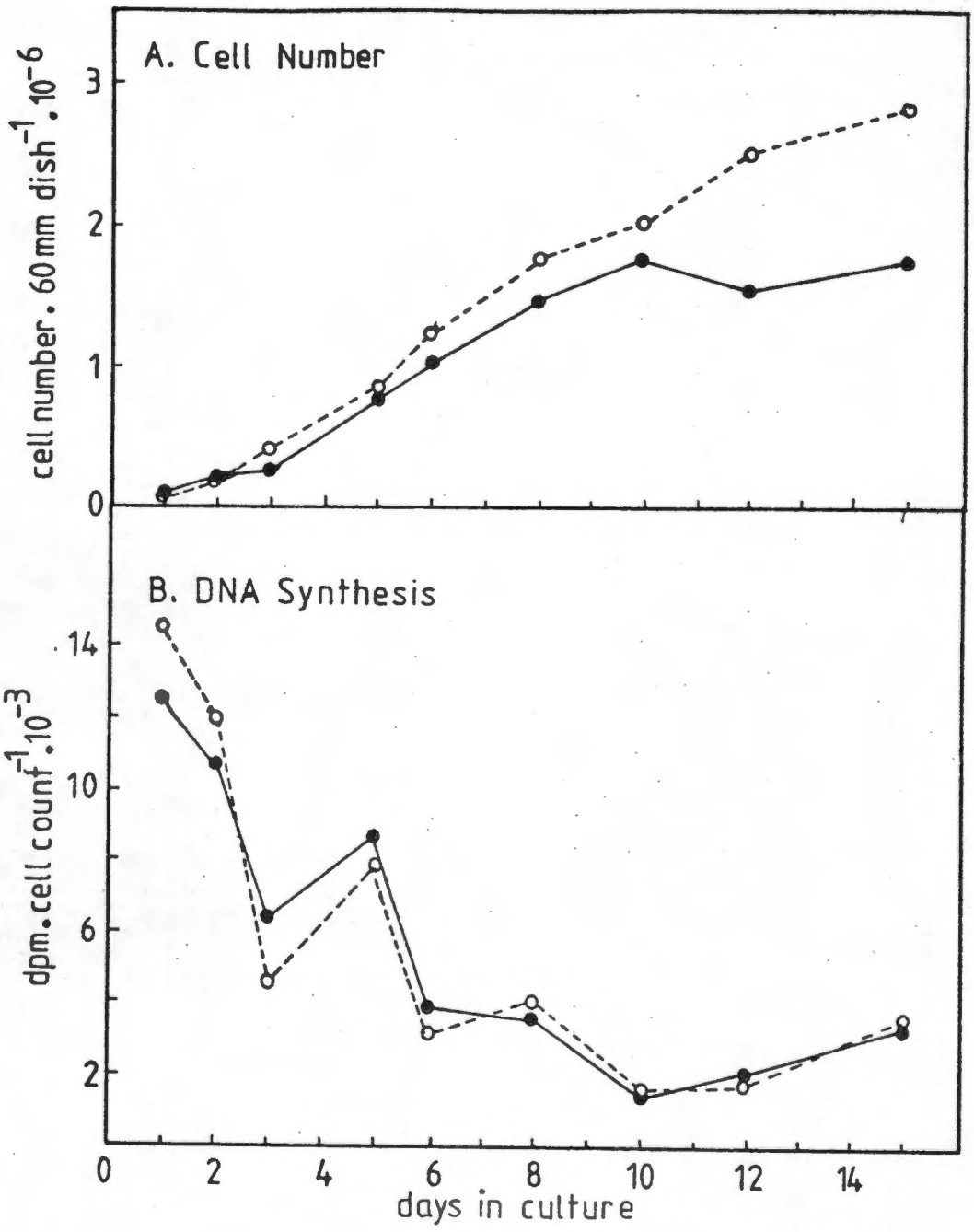

A. Cell Number
cell number . 60 mm dish^{-1} . 10^{-6}
0
1
2
3
B. DNA Synthesis
dpm . cell count^{-1} . 10^{-3}
2
6
10
14
0
2
4
6
8
10
12
14
days in culture

FIGURE 3.2

post-confluent multilayered culture (grown on uncoated plastic) would exhibit greater ^{3}H-thymidine incorporation than the post-confluent monolayer (grown on gelatin-coated dishes). However, ^{3}H-thymidine incorporation need not be an accurate reflection of DNA synthesis and thus replication of the cells. In addition, the method employed to measure ^{3}H-thymidine incorporation may not have been sensitive enough to distinguish between a 10% difference in proliferation rates of the cultures, once confluence had been attained. An alternative explanation could be that the monolayered culture was still dividing, with some cells being released into the medium rather than remaining attached to the substrate. For this reason, the medium of duplicate dishes in each culture was collected and counted on the specified days (Table 3.1). The number of cells released into the medium were expressed as a percentage of the total number of cells that remained attached in each dish. All the dishes received fresh medium every day. The number of cells found in the medium on the first day was 1.7% for cells grown on gelatin-coated dishes and 1.3% for those grown on plastic dishes. Thereafter, the number of cells released in each 24 h period was consistently less than 1% of those attached to the dish. As can be seen in Table 3.1, the number of cells released by cell cultures on gelatin-coated dishes are similar to the number released by the cell cultures on plastic dishes. It would therefore appear that the difference in the growth of the two cultures was merely not the result of a difference in the release of cells from the two substrates. This would imply

TABLE 3.1: CELLS RELEASED INTO THE MEDIUM

Endothelial cells were seeded at 10^5 cells/60 mm Petri dish in 4 ml of FCS medium. On the specified days, the medium was removed and the number of cells released into the medium counted in duplicate dishes of both cultures. The remaining cells in the dish were trypsinized and counted in the same way. FCS-medium was changed on all the dishes each day. G = gelatin-coated Petri dishes. P = plastic Petri dishes.

Culture age (days)	Number of attached cells per dish		Number of released cells per dish		% of released cells	
	G ($x10^6$)	P ($x10^6$)	G (10^4)	P (10^4)	G	P
1	0.179	0.177	0.302	0.226	1.7	1.3
2	0.316	0.314	0.220	0.272	0.7	0.9
3	0.712	0.607	0.175	0.419	0.3	0.7
4	0.934	1.175	0.410	0.605	0.4	0.5
5	1.058	1.350	0.585	0.622	0.5	0.5
6	1.815	2.100	0.847	0.725	0.5	0.3
9	2.040	2.885	1.790	0.875	0.9	0.3

that the cells grown on gelatin-coated dishes had a slower division rate than those grown on uncoated plastic dishes.

The observation that ^{3}H-thymidine incorporation did not decrease to zero in confluent cultures has also been observed by Gimbrone *et al* (153). These authors obtained a 90% decrease from a day 2-3 culture to a dense monolayer of human umbilical vein endothelial cells. The 10% ^{3}H-thymidine incorporation is most likely a reflection of residual radioactivity in the cells rather than an indication of replication. The increase observed after day 10 could possibly be due to the appearance of "sprouters" in the culture grown on gelatin-coated dishes. However, as mentioned earlier, "sprouters" were almost absent in confluent cultures up to day 10-12, and even thereafter comprised less than 1% of the total number of cells per dish. Another situation which could lead to an increased incorporation of ^{3}H-thymidine would be the peeling of the cell layer, with the subsequent proliferation of cells. This phenomenon, which has been found to occur *in vitro* after an injury of the endothelial layer, however, did not occur in this case.

3.2. VALIDATION OF EXPERIMENTAL PROCEDURES

3.2.1. ^{125}I-lipoprotein binding

Washing of cells: After incubating cells with ^{125}I-lipoproteins, it is important to remove the medium containing unbound radioactive material, to obtain a more accurate

value of bound ^{125}I-lipoproteins. This was done by sequential washing of the cells. Goldstein et al (114) investigated the number of washes required to remove unbound radioactive medium from fibroblasts and concluded that 8 washes were sufficient. The number of cell washes required could perhaps differ between different cell types and therefore this was investigated with endothelial cells (A_3Cl_2), using normal human skin fibroblasts (GM 0203) as a comparison. The cells were incubated with either ^{125}I-LDL or ^{125}I-AcLDL in LPDS-medium at 37°C for 4 h or in Hepes-medium at 4°C for 2 h. The two different temperatures were studied, since it was of interest to perform future experiments at both these temperatures.

The cells which had been incubated with the ^{125}I-lipoprotein at 4°C for 2 h received 12 sequential washes with phosphate buffered saline containing 0.2% bovine serum albumin (PBS-BSA) followed by 4 washes with phosphate buffered saline (PBS) (Figure 3.3). Each wash was collected and counted for radioactivity. When cells were incubated with ^{125} -lipoproteins at 37°C for 4 h, they were first cooled to 4°C, and then washed at this temperature. This temperature was employed to prevent movement of ^{125}I-lipoproteins across the cell membrane. A washing procedure of 8 sequential washes with PBS-BSA followed by 2 washes with PBS was used (Figure 3.4). At both temperatures recovery of unbound material appeared complete after about 6 to 8 washes. Since a long washing procedure could possibly result in dissociation of some bound material, it was decided to use a total of 8 washes (4 PBS-BSA;

FIGURE 3.3: CELL WASHES AFTER ^{125}I-LIPOPROTEIN BINDING AT 4^{o}C

On day 3, endothelial cells which had not been up-regulated, were incubated for 2 h at 4^{o}C with 2 ml of Hepes-medium containing either 10 µg protein/ml ^{125}I-LDL (A) or 10 µg protein/ml ^{125}I-AcLDL (B). Similarly, on day 6, human skin fibroblasts (GM0203) which had been up-regulated for 48 h with LPDS-medium, were exposed to 10 µg protein/ml ^{125}I-LDL (C). Subsequently, the medium was removed and the radioactivity counted. Each dish was then washed sequentially 12 times with 3 ml of phosphate buffered saline containing 0.2% bovine serum albumin (PBS-BSA) and 4 times with 3 ml of phosphate buffered saline (PBS). Each wash was collected and the radioactivity was counted. The dotted line represents the background count of the counter.

Each point represents the mean value obtained from duplicate dishes.

FIGURE 3.3

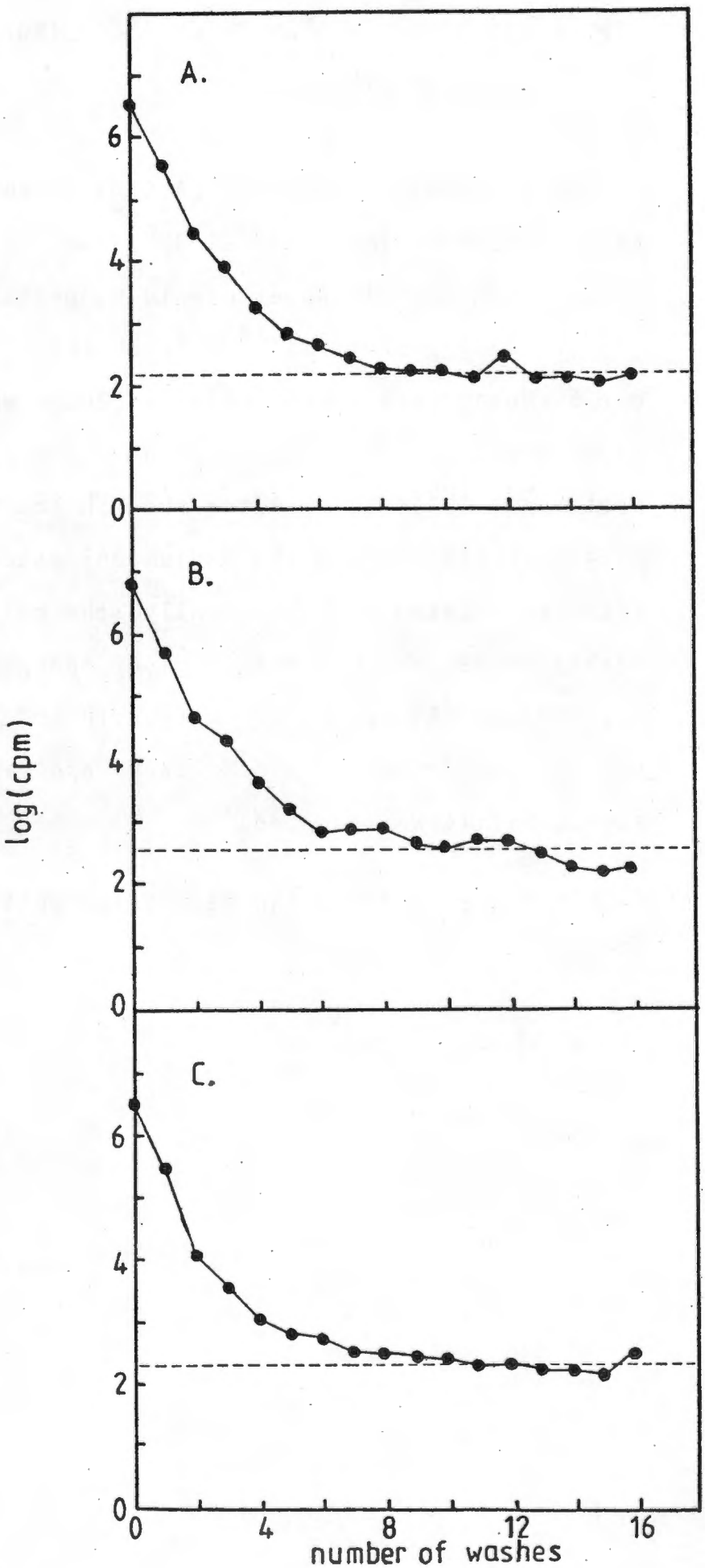
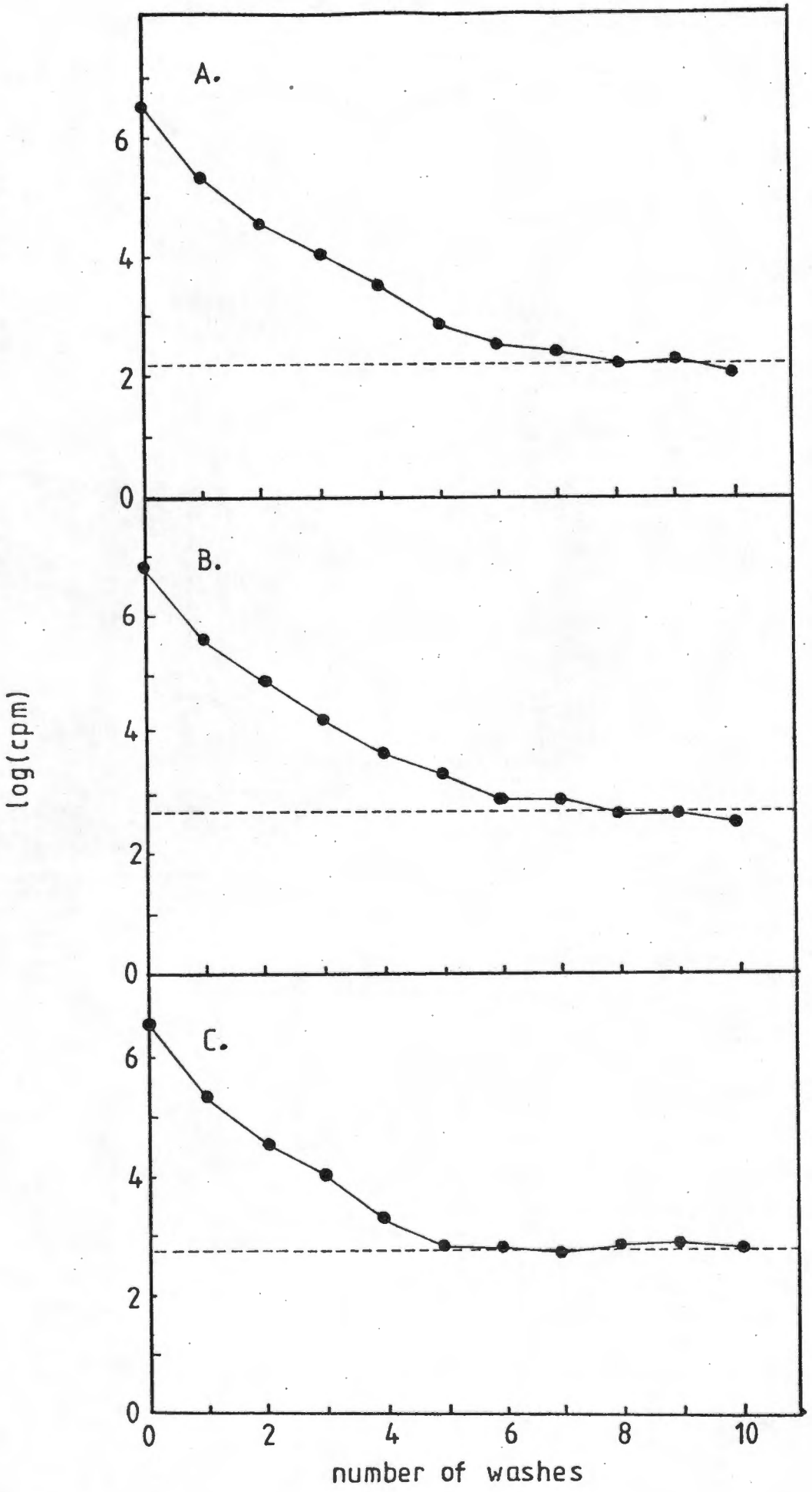


FIGURE 3.4: CELL WASHES AFTER ^{125}I-LIPOPROTEIN BINDING AT 37°C

On day 3, endothelial cells (A_3Cl_2) which had not been up-regulated, were incubated at 37°C for 4 h with 2 ml of LPDS-medium containing either 10 µg protein/ml of ^{125}I-LDL (A) or 10 µg protein/ml ^{125}I-AcLDL (B). Similarly, on day 6, human skin fibroblasts (GM0203), which had been up-regulated for 48 h in LPDS-medium, were exposed to 10 µg protein/ml ^{125}I-LDL. After the 4 h incubation, the cells were cooled to 4°C and the medium collected and the radioactivity counted. Subsequently, the cells were sequentially washed 8 times with 3 ml of phosphate buffered saline containing 0.2% bovine serum albumin and twice with 3 ml of phosphate buffered saline. Each wash was collected and the radioactivity was counted.

Each point represents the mean value obtained from duplicate dishes.

FIGURE 3.4



4 PBS) in future experiments.

Release of bound material: The release of bound ^{125}I-lipoproteins was studied using 3 different agents, namely, heparin, dextran sulphate and trypsin, to dissociate ligand from cells. Binding at 4^{o}C was investigated because it has been shown that lipoproteins at this temperature are bound to cells, but not internalized (114).

Initially a comparison of release of bound ^{125}I-lipoproteins with time was studied using dextran sulphate and trypsin (Table 3.2). The standard procedure involved the incubation of endothelial cells with ^{125}I-lipoproteins for 2 h at 4^{o}C, followed by 8 washes with PBS-BSA and PBS. Thereafter, the cells were incubated for different time intervals with either trypsin or dextran sulphate. This solution was then collected, and the radioactivity counted and compared to the radioactivity remaining associated with the cells. From the data obtained, it could be concluded that a 20 min incubation with trypsin was sufficient to maximally release bound ^{125}I-lipoproteins. A 20 min incubation with trypsin released 81% of ^{125}I-LDL and 68% of ^{125}I-AcLDL initially bound to the cells. At this stage all the cells had detached from the dish. The generally used incubation time period of cells with dextran sulphate resulted in a release of 60% of the total amount of bound ^{125}I-AcLDL after 60 mins, which did not increase substantially with a longer incubation period. However, the release of the total amount of bound ^{125}I-LDL after 60 min was 60% and increased to 71% after 120 min. Even

TABLE 3.2: RELEASE OF BOUND ^{125}I-LIPOPROTEINS BY TRYPSIN AND DEXTRAN SULPHATE AT 4°C

Endothelial cells were treated for 48 h in LPDS-medium. On day 6, the cells were incubated at 4°C for 2 h with 2 ml of Hepes-medium containing 10 µg protein/ml of ^{125}I-lipoproteins. After washing the cells, they were incubated with 0.05% trypsin - 0.02% EDTA or 10 mg/ml dextran sulphate. The amounts of released and cell-associated ^{125}I-lipoproteins were determined and from these values the fraction of ^{125}I-lipoprotein released was calculated as a percentage of the total ^{125}I-lipoprotein initially bound to the cells. Data are the mean of duplicate dishes used in the same experiment.

Treatment	Time (min)	^{125}I-lipoprotein released (ng protein/mg cell protein)		^{125}I-lipoprotein cell-associated (ng protein/mg cell protein)		% of ^{125}I-lipoproteins released	
		AcLDL	LDL	AcLDL	LDL	AcLDL	LDL
Trypsin	5	16	31	10	12	62	72
	10	16	28	10	8	62	78
	20	17	35	8	8	68	81
	30	18	30	10	8	64	79
	60	18	29	7	8	69	78
Dextran sulphate	30	11	19	11	16	50	54
	60	14	18	10	16	58	53
	120	14	25	8	10	64	71

with this latter time period, the release of bound ^{125}I-LDL was less than that released by trypsin.

In order to further investigate the release by trypsin, it was of interest to see whether cells incubated at 37°C with trypsin would release more bound ^{125}I-lipoprotein than a 4°C incubation with trypsin (Table 3.3). The possibility of internalization of ^{125}I-lipoproteins at 37°C had to be considered, and thus a short incubation period would be preferable. After 5 min at 37°C with trypsin, 51% of ^{125}I-AcLDL and 80% of ^{125}I-LDL of the total amount bound lipoprotein had been released. The amount released did not change substantially in the next 30 min. Since the release of bound ^{125}I-lipoproteins was not improved in comparison to the release at 4°C, this method was not used in future experiments.

A comparison of the release of the total amount of bound ^{125}I-lipoproteins by the three agents, trypsin, heparin and dextran sulphate, is shown in Table 3.4. Similar results to those shown in Table 3.2 were obtained in the case of trypsin and dextran sulphate. The release of bound material by heparin was similar to that of dextran sulphate, but less than that of trypsin. This was more markedly observed with the release of 10 µg/ml of ^{125}I-lipoproteins bound than 20 µg/ml of ^{125}I-lipoproteins bound. For example, at 10 µg/ml dextran sulphate and heparin released about 30% of ^{125}I-AcLDL bound and trypsin released about 70%. The situation with ^{125}I-LDL bound was similar. However, under all the

TABLE 3.3: RELEASE OF BOUND ^{125}I-LIPOPROTEIN USING TRYPSIN AT 37°C

Endothelial cells (A_3Cl_2) were incubated with 2 ml of Hepes-medium containing 10 µg protein/ml of ^{125}I-lipoproteins at 4°C for 2 h. After the cells had been washed, 0.05% trypsin - 0.02% EDTA, which had been warmed to 37°C, was added to the cells and incubated for increasing periods at 37°C. The amounts of released and cell-associated ^{125}I-lipoproteins were determined, and the percentage of the total amount of bound ^{125}I-lipoproteins which were released was calculated. Data are the mean of duplicate dishes.

Time (min)	^{125}I-lipoprotein released (ng protein/mg cell protein)		^{125}I-lipoprotein cell-associated (ng protein/mg cell protein)		% of ^{125}I-lipoproteins released	
	AcLDL	LDL	AcLDL	LDL	AcLDL	LDL
5	20	51	19	13	51	80
20	23	53	23	15	50	78
30	22	52	21	13	51	80

TABLE 3.4: COMPARISON OF RELEASE OF BOUND ^{125}I-LIPOPROTEINS BY HEPARIN, DEXTRAN SULPHATE AND TRYPSIN

Endothelial cells were pre-incubated for 48 h in LPDS-medium. Subsequently the cells were incubated at 4°C for 2 h in Hepes medium containing either 10 or 20 µg ^{125}I-LDL protein/ml or 10 or 20 µg ^{125}I-AcLDL protein/ml in the presence or absence of 200 µg protein/ml of unlabelled LDL or AcLDL, respectively. Bound ^{125}I-lipoproteins were released by 0.05% trypsin-0.02% EDTA (20 min), or 10 mg/ml heparin (60 min) or 10 mg/ml dextran sulphate (60 min).
T = total ^{125}I-LDL or ^{125}I-AcLDL released.
S = amount of ^{125}I-LDL or ^{125}I-AcLDL released from high affinity sites.
Each experiment was performed using duplicate dishes. Data are either the mean ± SEM of more than one experiment or the mean of 2 experiments.

Treatment	^{125}I-lipoproteins bound (µg protein/ml)	Number of experiments				% Release of bound ^{125}I-lipoproteins			
		AcLDL		LDL		AcLDL		LDL	
		T	S	T	S	T	S	T	S
Trypsin	10	9	7	9	8	58±3.0	36±6.7	64±3.0	62±4.4
	20	6	3	5	3	72±2.4	54±13.4	79±1.8	82±3.7
Heparin	10	3	2	3	2	27±1.7	14	34±1.7	28
	20	2	2	2	2	54	57	67	66
Dextran sulphate	10	4	2	4	2	31±4.1	26	33±4.6	36
	20	2	2	2	2	57	62	75	86

same various conditions, the percentage release of bound ^{125}I-LDL was larger than that of bound ^{125}I-AcLDL.

At this stage it was decided to determine how much of the total amount of released ^{125}I-lipoproteins was actually bound by a saturable, high-affinity process, i.e. material bound to high-affinity receptor sites which recognize and bind the protein moiety of the lipoproteins. Saturable, high-affinity binding was calculated from the difference between the values obtained in the presence or absence of unlabelled lipoproteins. In Table 3.4, a comparison has been made of the percentage of total and receptor-bound ^{125}I-lipoproteins released by either trypsin, heparin or dextran sulphate. In all cases, the percentage release of receptor-bound (high-affinity) ^{125}I-LDL was larger than that of ^{125}I-AcLDL (the absolute amounts of labelled lipoproteins released from high-affinity sites are shown in Table 3.7). This could possibly be a reflection from the observation that the percentage release of receptor-bound LDL to non-receptor bound LDL was between 40-60%, while the percentage release of receptor-bound AcLDL to non-receptor-bound AcLDL was only 30-40%. Furthermore, it was observed that when 20 µg protein/ml ^{125}I-lipoproteins were bound to the cells, the percentage release from high-affinity sites was more than when 10 µg protein/ml ^{125}I-lipoproteins were bound to the cells. For example, in the case of trypsin, upon incubation of the cells with 10 µg protein/ml of ^{125}I-AcLDL, the percentage release from high-affinity sites was about 35% and with 20 µg protein/

ml of ^{125}I-AcLDL the percentage release was about 55%. Similarly for LDL, upon incubation of the cells with 10 µg protein/ml of ^{125}I-LDL, the percentage release was about 60%, while at 20 µg protein/ml of ^{125}I-LDL, the percentage release was about 80%. The difference between the percentage release of 10 µg protein/ml and 20 µg protein/ml of bound ^{125}I-lipoproteins is consistent with a greater release of material bound to sites other than high-affinity receptors.

In view of the above findings, most of the subsequent experiments made use of cells incubated with 20 µg protein/ml ^{125}I-lipoproteins and trypsin was used to release bound material.

3.2.2. ^{125}I-lipoprotein degradation

Deiodination: Since the measure of degradation is dependent on the determination of ^{125}I-apolipoprotein B degradation products, which are mostly in the form of monoiodotyrosine (154), the amount of deiodination which might be occurring was studied, as any free-iodide is not taken for measurement (free-iodide present in the process of labelling the lipoproteins would result in a very large background value). Deiodination of tyrosine residues could possibly occur before or after degradation of the ^{125}I-lipoproteins has taken place in the lysosomes. Chloroquine, which prevents lipoprotein degradation (see Results 3.3.5), was used as a means of determining at what stage deiodination was occurring.

After cells were incubated with ^{125}I-lipoproteins at 37°C for 4 h, the protein in the medium was precipitated with trichloroacetic acid (TCA). The TCA-soluble fraction represented both the free-iodide and the non-iodide material (Table 3.5). The non-iodide material was determined by converting the free-iodide to I_2 by the addition of hydrogen peroxide via the standard method (see Methods 2.1.4). When the cells had been incubated for 4 h with ^{125}I-LDL, the amount of free-iodide obtained in the TCA-soluble fraction was 26% of the total amount of radioactivity determined in this fraction. Similarly, when cells had been incubated with ^{125}I-AcLDL, the amount of free-iodide was 32% of the total amount of radioactivity in the TCA-soluble fraction.

Under the same experimental conditions, but in the presence of chloroquine, no TCA-soluble counts were obtained. This could possibly be explained by two phenomena: either that chloroquine inhibits deiodinase activity, or else deiodination occurs only once degradation products have been formed. If the latter were the case, then the amount of degradation measured would be an underestimation of the true values.

3.2.3. Incubation time courses

To learn more about the metabolism of lipoproteins in endothelial cells, the kinetics of cell uptake and degradation of lipoproteins was determined at either 37°C or 4°C. Hence, the cells were incubated with lipoproteins for increasing time intervals until a steady state was reached. This steady

TABLE 3.5: MEASUREMENT OF DEIODINATION

Endothelial cells (A_3Cl_2) were treated with LPDS-medium for 48 h. On day 6, cells were incubated with 2 ml of LPDS-medium containing 10 µg protein/ml of ^{125}I-lipoprotein in the presence or absence of 70 µM chloroquine at 37°C for 4 h. Thereafter, the medium was collected and the protein was precipitated with 50% trichloroacetic acid (final concentration of 12.5% TCA). The amount of total iodide and non-iodide material found in the TCA-soluble fraction was determined. Data are the mean of duplicate dishes.

TCA-soluble fraction	Treatment	^{125}I-LDL		^{125}I-AcLDL	
		cpm/dish	% of total	cpm/dish	% of total
Total	- chloroquine	60 982	100	57 352	100
	+ chloroquine	-	-	-	-
Non-iodide	- chloroquine	45 136	74	38 832	68
	+ chloroquine	-	-	-	-
Free-iodide	- chloroquine	15 846	26	18 520	32
	+ chloroquine	-	-	-	-

state was reflected by the levelling off of bound or cell-associated material.

At 37°C: Endothelial cells were incubated at 37°C for varying lengths of time with 10 µg protein/ml ^{125}I-lipoprotein in LPDS-medium (Figure 3.5A). The amount of cell-associated ^{125}I-LDL reached a plateau by about 2 h, and proceeded to decrease rather rapidly thereafter, exhibiting a 76% drop from 4 h to 24 h. In contrast, the amount of cell-associated ^{125}I-AcLDL reached and maintained a plateau between 20 min and 2 h, and dropped by only 19% from 4 h to 24 h. The decline in ^{125}I-lipoprotein associated material could be due to the down-regulation of the receptors. The greater drop in ^{125}I-LDL compared to ^{125}I-AcLDL cell-associated material was consistent with results obtained regarding receptor regulation, which showed that the LDL receptor was far more susceptible to regulation than the receptor for AcLDL (see Results 3.3.3).

There was an initial lag of approximately 30 min before the amounts of ^{125}I-LDL and ^{125}I-AcLDL degraded, increased at a linear rate (Figure 3.5B). The rate of degradation (given in all following experiments as ng lipoprotein/mg cell protein/4 h) therefore tends to underestimate the actual degradation rate. However, the degree of underestimation is small. For example, the rates calculated by measuring the total amount of degradation over the first 4 h period were 117 ng ^{125}I-AcLDL/mg cell protein/h and 243 ng ^{125}I-LDL/mg cell protein/h, whereas the rates calculated from the degradation occurring during the last 3.5 h period of the 4 h incubation were

FIGURE 3.5: INCUBATION OF ENDOTHELIAL CELLS WITH ^{125}I-LIPOPROTEINS AS A FUNCTION OF TIME AT 37°C

Endothelial cells were pre-treated with LPDS-medium for 48 h. On day 5, they were incubated at 37°C with LPDS-medium containing 10 µg protein/ml of ^{125}I-AcLDL (open markings) and ^{125}I-LDL (closed markings), for the specified times.

A. After removal of the medium, the cells were washed and then dissolved in 1 ml of 1 M NaOH overnight at 37°C (see Methods 2.1.4). The radioactivity obtained in this solution was taken to represent cell-associated ^{125}I-lipoproteins.

B. After the various times of incubation, the medium on the cells was collected and precipitated with trichloroacetic acid (TCA). The TCA-soluble material was treated with potassium iodide and hydrogen peroxide, followed by a chloroform extraction. The radioactivity in the aqueous layer was counted as a measure of degraded ^{125}I-lipoproteins.

In each case, an expanded scale from 0 - 4 h has been shown. Each point represents the mean value obtained from duplicate dishes.

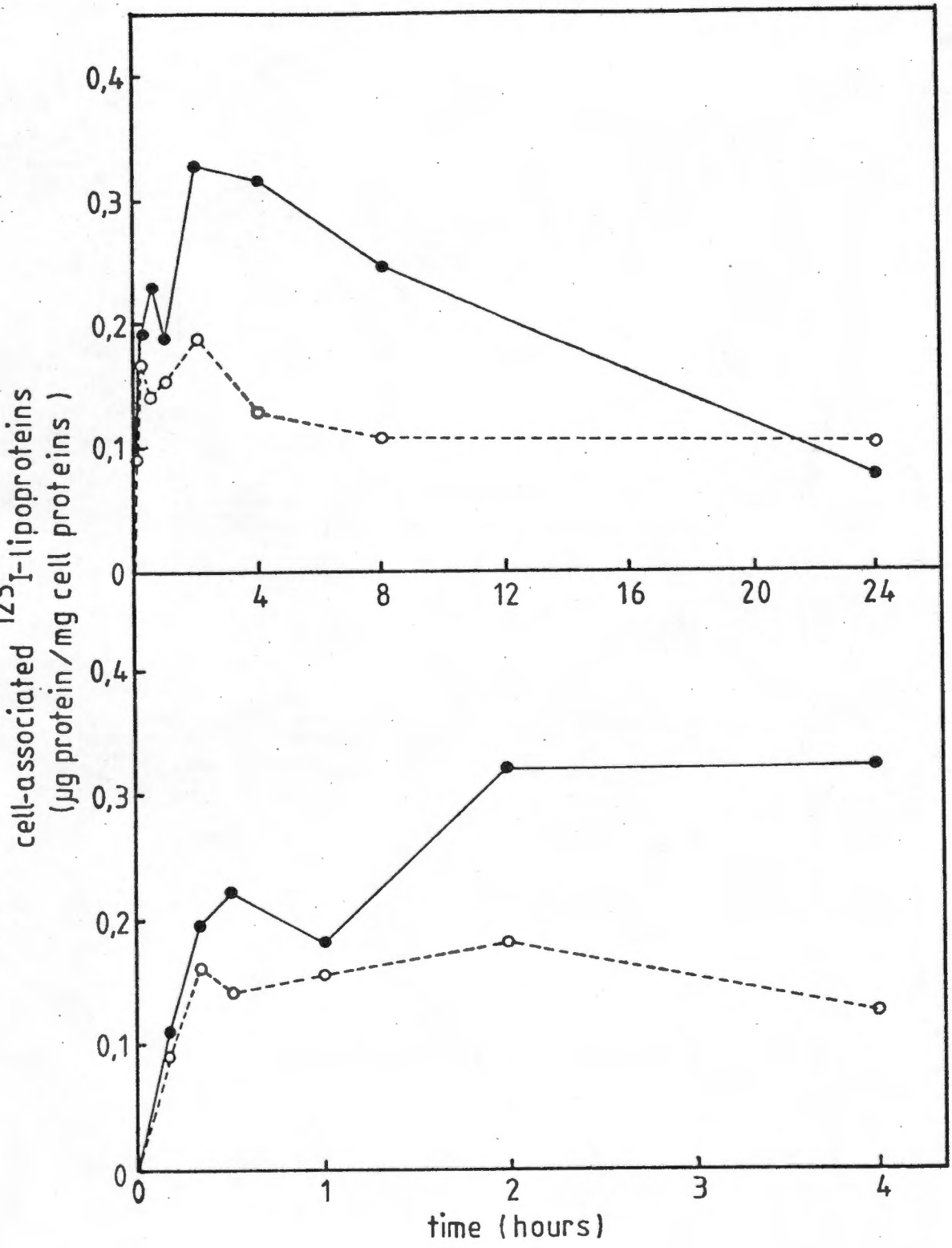
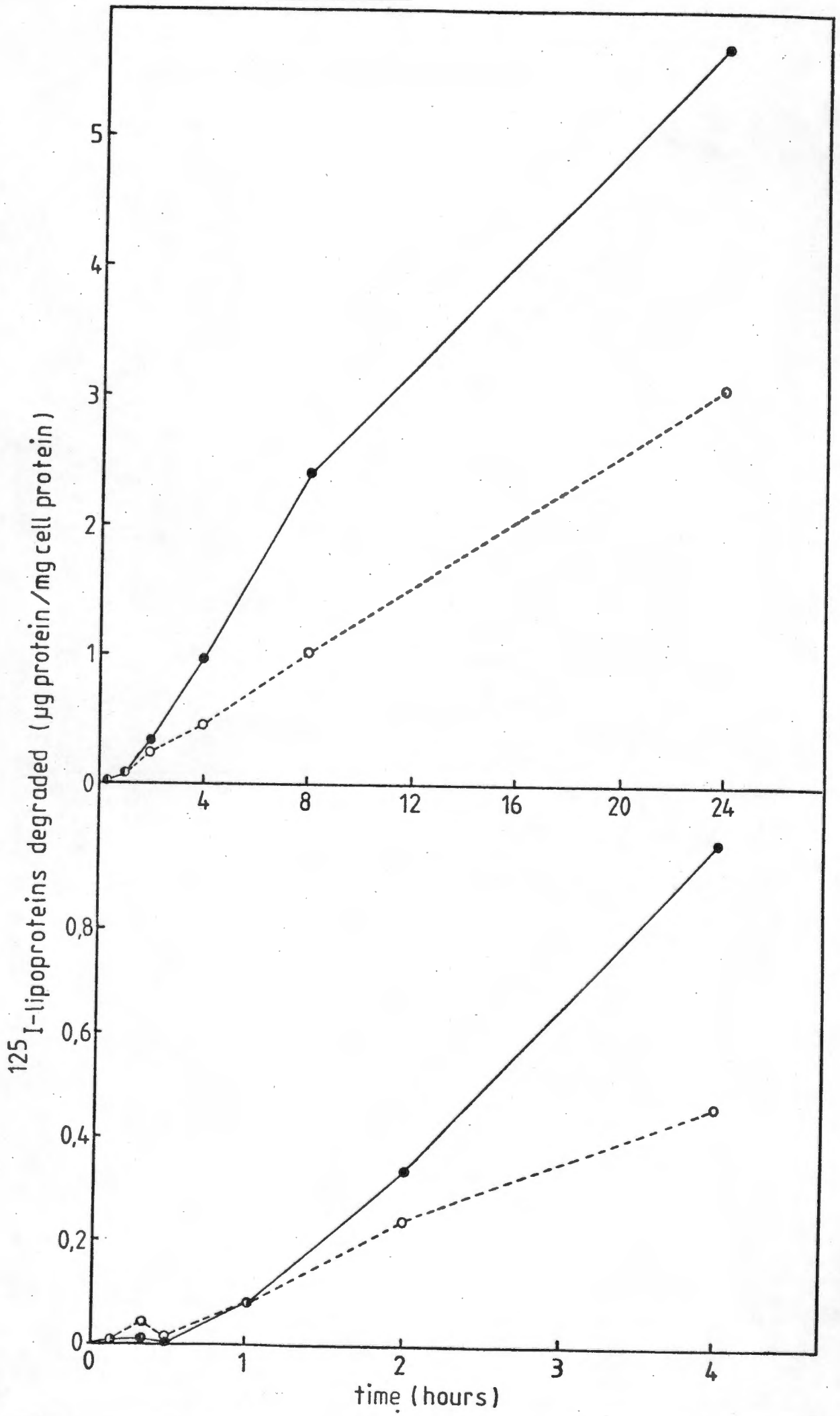

cell-associated ^{125}I-lipoproteins
(µg protein/mg cell proteins)
0,4
0,3
0,2
0,1
0
4
8
12
16
20
24
0,4
0,3
0,2
0,1
0
0
1
2
3
4
time (hours)

FIGURE 3.5 A

TABLE 3.5 B


^{125}I-lipoproteins degraded (µg protein/mg cell protein)
0
1
2
3
4
5
4
8
12
16
20
24
0
0,2
0,4
0,6
0,8
1
2
3
4
time (hours)

113 ng ^{125}I-AcLDL/mg cell protein/h and 240 ng ^{125}I-LDL/mg cell protein/h. Degradation rates were thus underestimated by 3.4% and 4.2% in the case of AcLDL and LDL, respectively.

In all future experiments, cells were incubated at 37°C with ^{125}I-lipoproteins for 4 h. This time period was chosen as it allowed for a relatively substantial amount of degradation products to form, which thus allowed for a sensitive and accurate assay. Although a longer time period resulted in increasing amounts of degradation products, it could be seen that longer periods resulted in a significant down-regulation of receptors (Fig. 3.5A).

At 4°C: Endothelial cells (A_3Cl_2) were incubated at 4°C for varying time intervals in Hepes-medium containing 10 µg protein/ml of ^{125}I-lipoproteins in the presence or absence of 200 µg protein/ml of unlabelled lipoprotein. The amounts of receptor-bound lipoproteins were calculated as previously described (see Results 3.2.1). It was found that a plateau was obtained after about 1 h for both ^{125}I-LDL and ^{125}I-AcLDL (Figure 3.6). Only a slight increase (10-20%) occurred with a further incubation to 2 h. To ensure that binding of lipoproteins at 4°C had reached saturation, all future experiments were performed using a 2 h incubation period. Longer incubation periods, as studied at 37°C, were not investigated as the cells are not favourably disposed to Hepes-medium for very long periods of time.

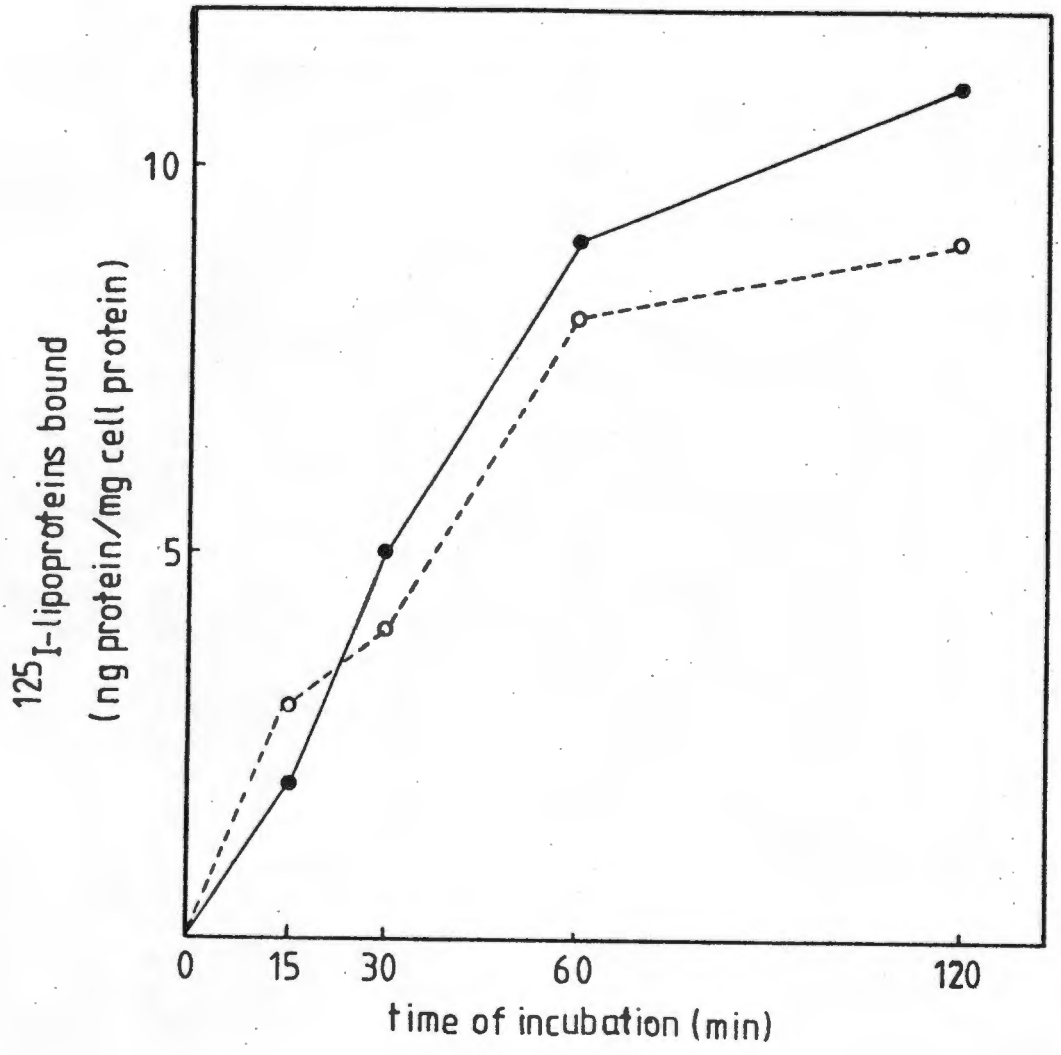


FIGURE 3.6: HIGH AFFINITY BINDING OF ^{125}I-LIPOPROTEINS AS A FUNCTION OF INCUBATION TIME AT 4°C

On day 2, endothelial cells (A_3Cl_2) were incubated at 4°C for increasing time periods with 2 ml of Hepes-medium containing either 10 µg protein/ml of ^{125}I-LDL (●—●) or ^{125}I-AcLDL (o---o) in the absence or presence of 200 µg protein/ml of unlabelled LDL or AcLDL respectively. After the specified times, the medium was discarded and the cells were washed with PBS-BSA and PBS (see Methods 2.1.4). The amounts of bound ^{125}I-lipoproteins were released by incubating the cells for 20 min with 0.05% trypsin - 0.02% EDTA. High affinity binding was taken to be the difference between the values obtained in the absence and presence of unlabelled lipoproteins. Each point represents the mean value obtained from duplicate dishes.

3.3. CHARACTERISTICS OF ENDOTHELIAL CELL LIPOPROTEIN RECEPTORS

3.3.1. Receptor affinity and capacity for lipoproteins

The affinity and capacity of receptors for LDL and AcLDL in endothelial cells was investigated at both 37°C and 4°C.

At 37°C: The receptor affinity of the cells (A_3Cl_2) for LDL and AcLDL was determined by incubating the cells with increasing amounts of ^{125}I-lipoproteins in LPDS-medium at 37°C. After a 4 h incubation, the medium was removed for determination of non-iodide TCA-soluble ^{125}I-degraded products (see Methods 2.1.4). After washing the cells, the amount of ^{125}I-lipoproteins bound to the cells was released by a 20 min incubation at 4°C with 0.05% trypsin - 0.02% EDTA. The radioactivity in the trypsin supernatant was determined as a measure of bound ^{125}I-lipoprotein, and the radioactivity remaining associated with the cells was taken as the intracellular ^{125}I-lipoproteins.

The resulting quantities of bound, internalized and degraded ^{125}I-lipoproteins were represented by two-component curves (Figure 3.7). This indicates the involvement of both a receptor-mediated (saturable) and non-receptor-mediated (non-saturable) processing of ^{125}I-LDL and ^{125}I-AcLDL by endothelial cells. The saturable, high-affinity component of the curve was obtained by subtracting from each lipoprotein concentration on the experimental curve, the corresponding non-saturable, low-affinity component value. The non-

FIGURE 3.7: BINDING, INTERNALIZATION AND DEGRADATION OF ^{125}I-LIPOPROTEINS AT 37°C

Endothelial cells (A_3Cl_2) were pre-treated with LPDS-medium for 48 h. On day 9, the cells were incubated with 2 ml of LPDS-medium containing increasing concentrations of ^{125}I-LDL (●—●) and ^{125}I-AcLDL (o--o) at 37°C. After 4 h, the cells were cooled to 4°C and the medium removed and analysed for degradation products. The cells were extensively washed and incubated for 20 min at 4°C with 0.05% trypsin - 0.02% EDTA to remove bound ^{125}I-lipoproteins. The remaining cell pellet was analysed for internalized ^{125}I-lipoproteins, and protein content.

Each point is the mean value obtained from duplicate dishes.

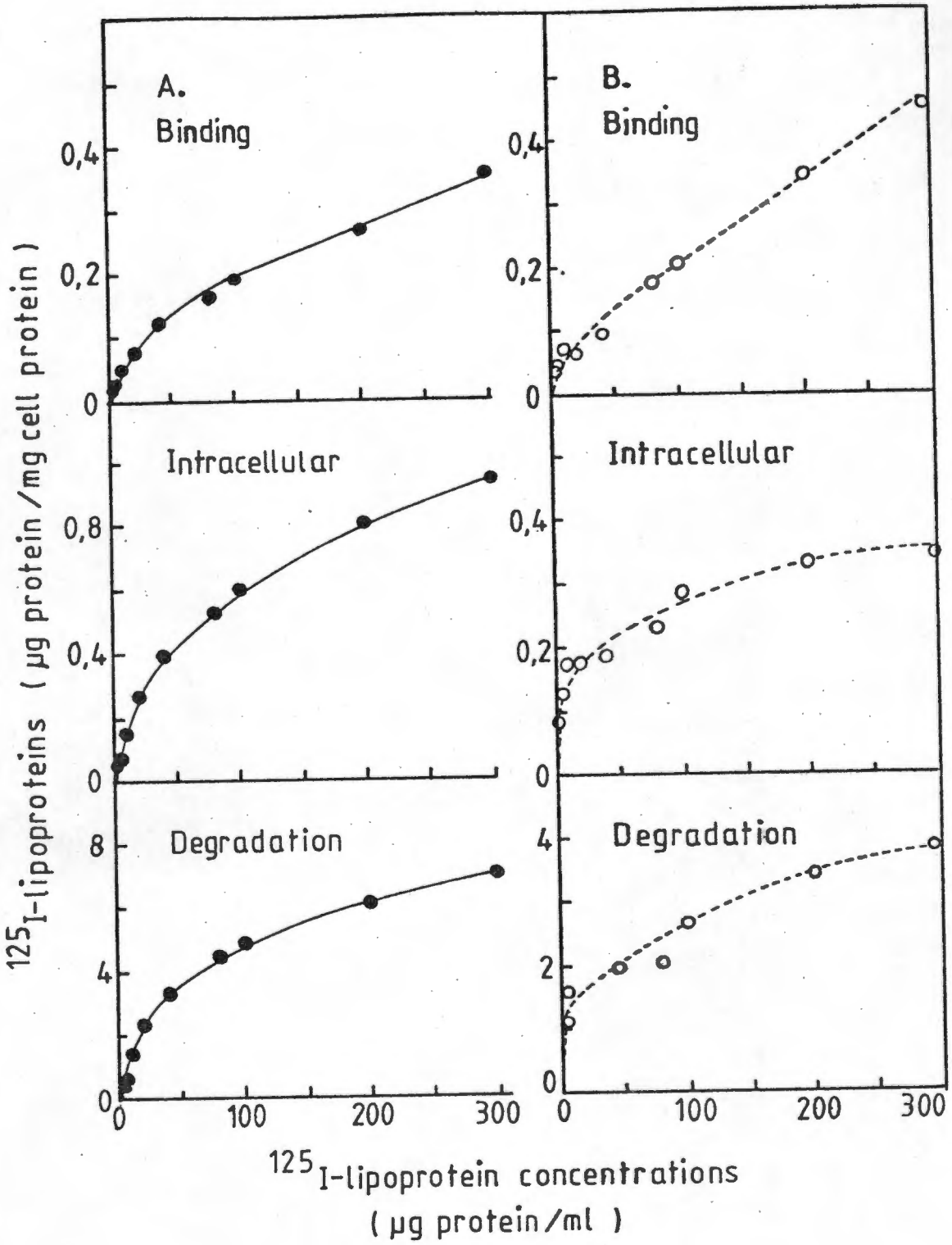

A.
Binding
B.
Binding
Intracellular
Intracellular
Degradation
Degradation
0,4
0,2
0
0,8
0,4
0
8
4
0
0,4
0,2
0
4
2
0
0
100
200
300
125I-lipoproteins (µg protein /mg cell protein)
125I-lipoprotein concentrations
(µg protein/ml)

FIGURE 3.7

saturable component was derived from the linear portion of the curve at the higher lipoprotein concentrations. The saturation curve was used for Scatchard (136) and Riggs (151) analyses of the equilibrium dissociation constants (k_δ) and maximal capacity values (V_{max}) respectively (see Methods 2.1.5).

In Figure 3.7B, it was observed that the binding curve for AcLDL had a relatively large non-saturable, high-affinity component. Considering the values found for intracellular amounts and degradation rates, it is evident that AcLDL was taken up and catabolized largely by a high-affinity process. Thus, an experiment in which lower concentrations of ^{125}I-AcLDL were added to the medium was performed to obtain a more accurate equilibrium dissociation constant (Figure 3.8). Generally, a k_δ value is obtained from the affinity of the lipoprotein for its receptor (i.e. binding data). However, since the intracellular content and degradation rates depend on and reflect the lipoprotein binding as a result of the saturable process, the k_δ values were alternatively calculated from the saturation curves obtained for intracellular levels and degradation of the lipoproteins. As can be seen in Figure 3.8A, the binding curve again showed a very large non-saturable component which tended to mask the saturable high-affinity component. However, the intracellular and degradation curves provided no such problem, and were in agreement with Figure 3.7B, in that the receptor affinity for AcLDL was very high.

FIGURE 3.8: BINDING, INTERNALIZATION AND DEGRADATION OF ^{125}I-AcLDL AT 37°C

Endothelial cells were pre-treated with LPDS-medium for 48 h. On day 8, they were incubated at 37°C with 2 ml of LPDS-medium containing increasing concentrations of ^{125}I-AcLDL. The amount of bound, internalized and degraded ^{125}I-AcLDL was determined as described in Methods 2.1.4.

Each point represents the mean value obtained from duplicate dishes.

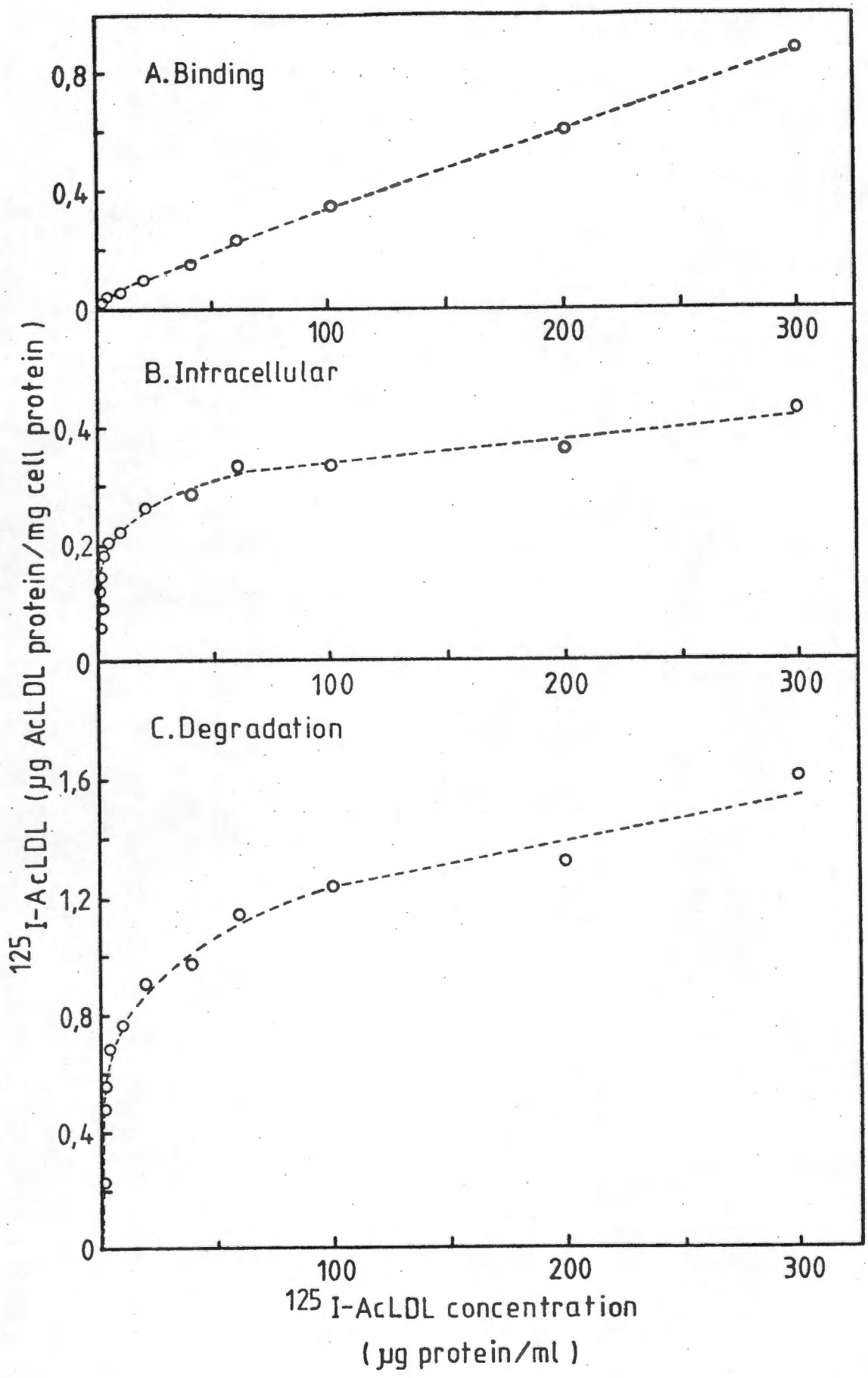

A. Binding
B. Intracellular
C. Degradation
0,8
0,4
0
100
200
300
0,2
1,6
1,2
0,8
0,4
125 I-AcLDL (µg AcLDL protein/mg cell protein)
125 I-AcLDL concentration
(µg protein/ml)

FIGURE 3.8

A number of experiments were performed in order to obtain k_δ values for the affinity at 37°C of receptors on endothelial cells for LDL and AcLDL (Table 3.6). The half-maximal values obtained for LDL binding, intracellular content and degradation rates were all in the range of 22-32 µg LDL protein/ml. In contrast, the processing of AcLDL showed considerable variability. The half-maximal values ranged from 1.77 to 5.58 µg AcLDL protein/ml, using degradation and binding data respectively. This difference in half-maximal values could possibly be due to the large non-saturable binding component which prevents an accurate estimation of the saturable high-affinity component. For example, the three experiments (Expts. 5, 6b and 7) in which k_δ values were determined using the binding data, the degree of accuracy is questionable, as a slight variation in the slope of the non-saturable component would result in a large difference in k_δ values. In contrast, the range of k_δ values obtained from intracellular content curves of AcLDL, the values were rather similar with an average k_δ value of 2.99 $\pm$ 0.44 µg protein/ml. However, in obtaining degradation saturation curves for AcLDL, a problem arose in two experiments (Expts 5 and 6a). As described in Methods 2.1.4, to obtain degradation values, controls of empty dishes with the labelled lipoprotein were used to control for cell independent breakdown products present in the ^{125}I-lipoprotein before use. In these two cases, the control values were somewhat higher than usual, so that at higher concentrations of ^{125}I-AcLDL a relatively small error in the control value resulted in relatively large errors in degradation values.

TABLE 3.6: AFFINITY CONSTANTS OF RECEPTORS AND BINDING CAPACITY OF ENDOTHELIAL CELLS AT 37°C

Equilibrium dissociation constants (k_δ) and maximum binding capacity (V_{max}) were calculated from linearization plots of saturable ^{125}I-lipoprotein data at 37°C. K_δ values were obtained using the Scatchard analysis method, and V_{max} values were obtained from the Riggs analysis method (see Methods 2.1.5).

Data in parentheses are the means ± S.E.M. of k_δ values obtained in the various experiments performed.

* Cells had been up-regulated for 48 h in LPDS-medium.

\+ Cells had not been up-regulated.

	Binding		Intracellular		Degradation	
	k_δ (μg/ml)	V_{max} (ng/mg)	k_δ (μg/ml)	V_{max} (ng/mg)	k_δ (μg/ml)	V_{max} (ng/mg)
LDL						
Expt. 1: sparse+	41	79	19	150	25	924
Expt. 2a: sparse*	27	193	28	582	17	4325
b: confluent*	28	125	36	490	25	4227
	(32±4.51)		(28±4.91)		(22±2.67)	
AcLDL						
Expt. 3: sparse+	-	-	3.86	191	1.13	379
Expt. 4: sparse*	-	-	-	-	0.66	336
Expt. 5: sparse*	8.35	32	2.96	47	-	-
Expt. 6a: sparse*	-	-	2.26	48	-	-
b: confluent*	4.64	58	4.06	166	2.76	2152
Expt. 7: confluent*	3.76	19	1.82	278	2.53	1007
	(5.58±1.41)		(2.99±0.44)		(1.77±0.52)	

Reliable k_δ determinations were not possible from these values and are therefore not shown. In the degradation curves used to obtain k_δ values, the duplicates did not vary by more than 10%. The half-maximal values obtained were between 0.66 and 2.76 µg protein/ml. Expts. 6b and 7 were the only experiments in which a complete set of half-maximal values (i.e. binding, intracellular and degradation) for AcLDL was obtained. It can be seen that in these cases, the half-maximal values are similar for the three different parameters. However, because of the possible inaccuracies in obtaining half-maximal values from the binding saturable curves, degradation data are more likely to be a true reflection of the k_δ value.

LDL, on the other hand, had similar half-maximal values, obtained from either binding, intracellular or degradation data. The average k_δ value obtained from binding data was about 32 µg protein/ml, while the average half-maximal values obtained from intracellular and degradation data were 28 and 22 µg protein/ml, respectively. Regardless of the variability in the half-maximal values obtained for AcLDL, it can be concluded that endothelial cells have a higher affinity at 37°C for AcLDL ($k_\delta \sim$ 2-5 µg protein/ml) than for LDL ($k_\delta \sim$ 20-30 µg protein/ml).

The maximum number of receptors (V_{max}) for LDL as determined by all three parameters in sparse cultures, was found to be lower if the cells had not been up-regulated (up-regulation

has been further examined in Results 3.3.3). In comparing the V_{max} for LDL in sparse and confluent up-regulated cultures, it was found in this one experiment (Expt. 2a and 2b) that the values for each parameter were very similar, being only slightly less in confluent cultures. However, in subsequent experiments (see Table 3.7), it has been consistently found that sparse cultures catabolize more LDL than confluent cultures. The similarity in this experiment of the V_{max} for LDL in sparse and confluent cultures cannot be explained. In contrast to LDL, the V_{max} for AcLDL was not affected by up-regulation. However, the V_{max} for AcLDL in up-regulated cultures was found to be lower in sparse cultures than in confluent cultures. These results are consistent with the findings presented in Results 3.3.2, which showed that more AcLDL was catabolized in confluent cultures than in sparse cultures.

A different approach was employed to determine high-affinity binding, intracellular content and degradation rates. These data were obtained by competition experiments in which unlabelled lipoproteins competed with labelled lipoproteins for interaction with the cell surface. In Table 3.7, a comparison was drawn between the uptake and degradation of LDL and AcLDL by high-affinity receptors. Up-regulated sparse cultures metabolize more LDL than do up-regulated confluent cultures. In contrast, up-regulated sparse cultures metabolize less AcLDL than do confluent cultures. Once again, up-regulation of the LDL receptor results in an

TABLE 3.7: HIGH AFFINITY BINDING, INTRACELLULAR CONTENT AND RATES OF DEGRADATION OF ^{125}I-LIPOPROTEINS IN CULTURED ENDOTHELIAL CELLS.

Endothelial cells were either up-regulated in LPDS-medium for 48 h or maintained in FCS-medium, i.e. the cells were not up-regulated. Thereafter, the cells were incubated with either 10 or 20 µg ^{125}I-lipoprotein/ml in the presence or absence of 200 µg/ml of unlabelled lipoprotein, for 4 h at 37°C. The medium and cells were subsequently analysed for binding, internalization and degradation data. Each parameter was corrected for non-saturable material. Each experiment was performed using duplicate dishes. Data are the mean ± S.E.M. of the various experiments executed, or the mean of two experiments.

Treatment	^{125}I-lipoprotein (µg/ml)	Number of Experiments		Receptor bound (ng/mg)		Intracellular (ng/mg)		Degradation (ng/mg)	
		AcLDL	LDL	AcLDL	LDL	AcLDL	LDL	AcLDL	LDL
Sparse cultures:									
Up-regulated	10	3	4	16±2	19±4	72±9	116±25	289±15	377±63
	20	4	4	20±1	64±8	60±9	202±44	474±127	1381±205
not up-regulated	10	2	5	10	5±1	52	27±3	284	86±26
Confluent cultures:									
Up-regulated	10	2	2	28	9	158	66	563	184
	20	6	4	20±6	29±6	119±17	68±6	1055±70	663±57
not up-regulated	10	3	3	12±1	5±1	134±28	26±4	474±103	36±12

increased metabolism of LDL, whereas the receptor-mediated uptake and degradation of AcLDL was not affected by up-regulation. The maximum number of LDL receptors per cell was estimated to be about 98 000, while the maximum number of AcLDL receptors per cell was about 31 000 (see Methods 2.1.5). In comparison, human fibroblasts produce a maximum of 20 000 - 50 000 LDL receptors per cell (113) and macrophages have about 20 000 - 40 000 receptors per cell for AcLDL (78).

4°C: An alternative approach to measure the k_δ values of LDL and AcLDL was to incubate cells at 4°C with Hepes-medium containing increasing amounts of ^{125}I-lipoproteins. After 2 h, the cells were washed, dissolved in 1 M NaOH and counted. This solution was assumed to represent the amount of surface bound ^{125}I-lipoproteins, since at 4°C no internalization of the lipoproteins occurs (114) (Figure 3.9). Two-component curves were obtained for both ^{125}I-LDL and ^{125}I-AcLDL at different cell densities and states of receptor regulation. The half-maximal values, determined using the Scatchard method (see Methods 2.1.5) were about 4-6 µg ^{125}I-AcLDL protein/ml in cells which had not been pre-treated with LPDS-medium for 48 h, regardless of cell density (Table 3.8). In an experiment in which confluent cells had been up-regulated, the half-maximal value was 13 µg ^{125}I-AcLDL protein/ml. Therefore, these results obtained at 4°C confirm the high-affinity of endothelial cell receptors for AcLDL, and are consistent in that this affinity was not affected by regulation or with

FIGURE 3.9: ENDOTHELIAL CELL BINDING OF ^{125}I-LIPOPROTEINS AT 4°C

Endothelial cells (A_3Cl_2) were incubated with increasing amounts of ^{125}I-LDL (●——●) and ^{125}I-AcLDL (●---●) in 2 ml of Hepes-medium at 4°C. After 2 h, the medium was discarded and the cells were extensively washed. Subsequently, the cells were solubilized in 1 M NaOH overnight at 37°C. The radioactivity (binding) and the protein contents were then determined.

A. The cells were not up-regulated and the experiment was performed on day 2.

B. The cells were not up-regulated and were used on day 9.

C. Cells had been up-regulated for 24 h in 2 ml of LPDS-medium. On day 12, the cells were used for experimentation.

Each point is the mean value obtained from duplicate dishes.

FIGURE 3.9

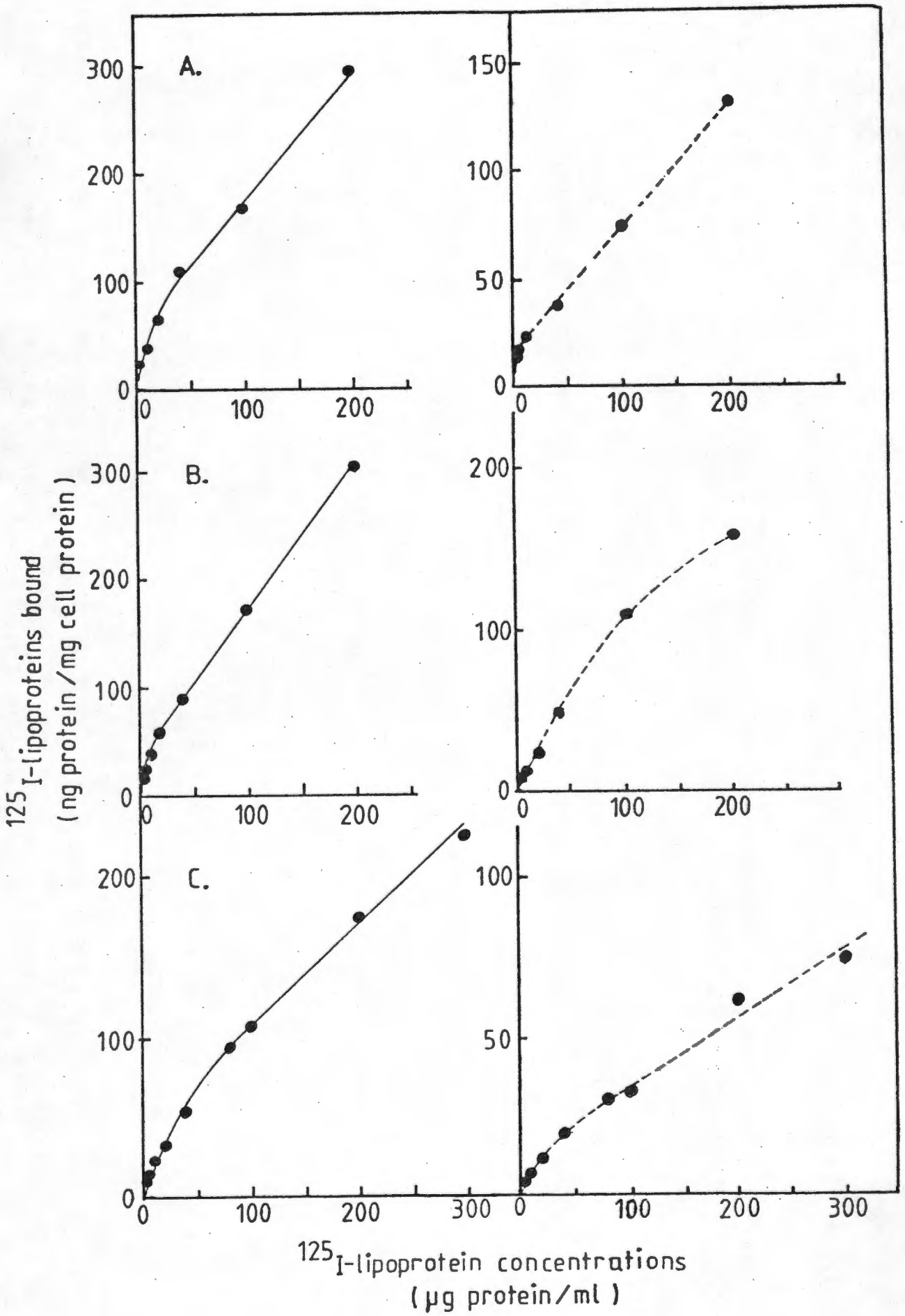

A.
B.
C.
300
200
100
0
150
50
100
200
^{125}I-lipoproteins bound
(ng protein/mg cell protein)
^{125}I-lipoprotein concentrations
(µg protein/ml)

TABLE 3.8: AFFINITY CONSTANTS, BINDING CAPACITY AND RECEPTOR NUMBERS PER CELL.

Equilibrium dissociation constants (k_δ), maximum binding capacity (V_{max}) and receptor numbers per cell were determined from the linearization plots of saturable ^{125}I-lipoprotein binding data obtained at 4°C (Figure 3.9). k_δ values were calculated using the Scatchard analysis method (see Methods 2.1.5).

Experiments	Age of culture (days)	k_δ (µg protein/ml)	
		LDL	AcLDL
1+	2	3.1	5.6
2+	9	20.4	4.1
3*	12	18.5	13.0

+ Cells had not been up-regulated.

* Cells had been up-regulated for 24 h in LPDS-medium.

age. For LDL, the half-maximal binding values at 4°C were about 20 µg ^{125}I-LDL protein/ml in two experiments which are consistent with the results obtained at 37°C. However, in another experiment the half-maximal binding value was 3.1 µg protein/ml. This result could arise from the relatively high non-saturable component which would tend to mask the saturable component and give rise to inaccuracies in obtaining half-maximal values.

In view of the studies on receptor affinity at 37°C and 4°C, it can be concluded that temperature has no effect on the binding of lipoproteins to the receptors. The k_δ values obtained for receptor affinity of AcLDL at 37°C (2-5 µg protein/ml) and 4°C (4-6 µg protein/ml) were in a similar range. The same similarity of k_δ values for LDL receptor affinity at 4°C and 37°C were observed, falling in the range of about 20-30 µg protein/ml. In addition to the receptor affinity being temperature independent, it was shown that the age of the culture does not affect the receptor affinity for either LDL or AcLDL.

3.3.2. Receptor activity with culture age

Considering the importance of the role of endothelial repair after damage to the intact monolayer (see Introduction 1.4), it was of relevance to study the receptor activities of these cells for LDL and AcLDL in cultures of increasing age.

The cells were pre-incubated for 48 h in LPDS-medium,

followed by a 4 h incubation at 37°C in LPDS-medium containing ^{125}I-LDL or ^{125}I-AcLDL in the presence or absence of unlabelled LDL or AcLDL, respectively. The degradation rates, dependent on high-affinity processes of both ^{125}I-LDL and ^{125}I-AcLDL, were measured on the specified days. This parameter was corrected for low-affinity non-saturable degradation by subtracting the values obtained in the presence of unlabelled lipoprotein from those obtained in the absence of unlabelled lipoproteins. Degradation rates have been used to illustrate the results obtained. Since the amount of lipoprotein bound is the rate limiting step in the degradation pathway, the rate of degradation is a reflection of the number of lipoprotein receptors. Because of the sensitivity and reproducibility of the assay employed to measure the degradation of lipoproteins (degradation values of duplicate dishes were always within 10% of each other), this parameter was often used in preference to the binding data to investigate receptor activity. This approach avoids the problems encountered in obtaining binding data, where the large amounts of non-saturable lipoproteins bound tended to mask high-affinity bound material (see Results 3.3.1). Since it has been found by Goldstein et al (155) that LDL binds in a saturable high-affinity manner to glass beads, there is a possibility that perhaps the lipoproteins bind to the plastic dishes in a similar manner. In addition, the degradation assay avoids manipulation and possible loss of cells. The degradation rates are also obtained independently of the problems involved in obtaining quantitative releases of bound lipoproteins to the cells.

The pattern that emerged for the degradation rates of ^{125}I-AcLDL with increasing days in culture was quite different to that obtained for ^{125}I-LDL (Figure 3.10). Although comparatively little ^{125}I-AcLDL was degraded in sparse cultures, the degradation rate increased steadily with increasing age of culture. In contrast, the largest amount of ^{125}I-LDL degraded was obtained in sparse cultures, and this amount decreased with increasing culture age. This phenomenon was consistently found in two other experiments, with the amount of ^{125}I-AcLDL degraded from day 4 to day 10, increasing by 344 $\pm$ 84% and that of ^{125}I-LDL, during the same period, decreasing by 46 $\pm$ 7% (Table 3.9). At low concentrations (10 - 20 μg protein/ml) of the ^{125}I-lipoproteins, sparse cultures degrade more ^{125}I-LDL than ^{125}I-AcLDL. In contrast, confluent cultures degrade more ^{125}I-AcLDL than ^{125}I-LDL. However, Figure 3.10 does not represent maximum levels of degradation since the cells were not exposed to saturating concentrations of the lipoproteins.

The internalization index (obtained by the addition of degradation and intracellular values divided by the binding values) provides a measure of the efficiency or rate of internalization of bound lipoproteins (156). In sparse and confluent up-regulated cell cultures, the internalization index for LDL and AcLDL was similar (Table 3.10), being about 26. In addition, the internalization index remains the same for both LDL and AcLDL with increasing culture age. There is no indication of a decrease in the internalization index with increasing confluence of the cell culture. These

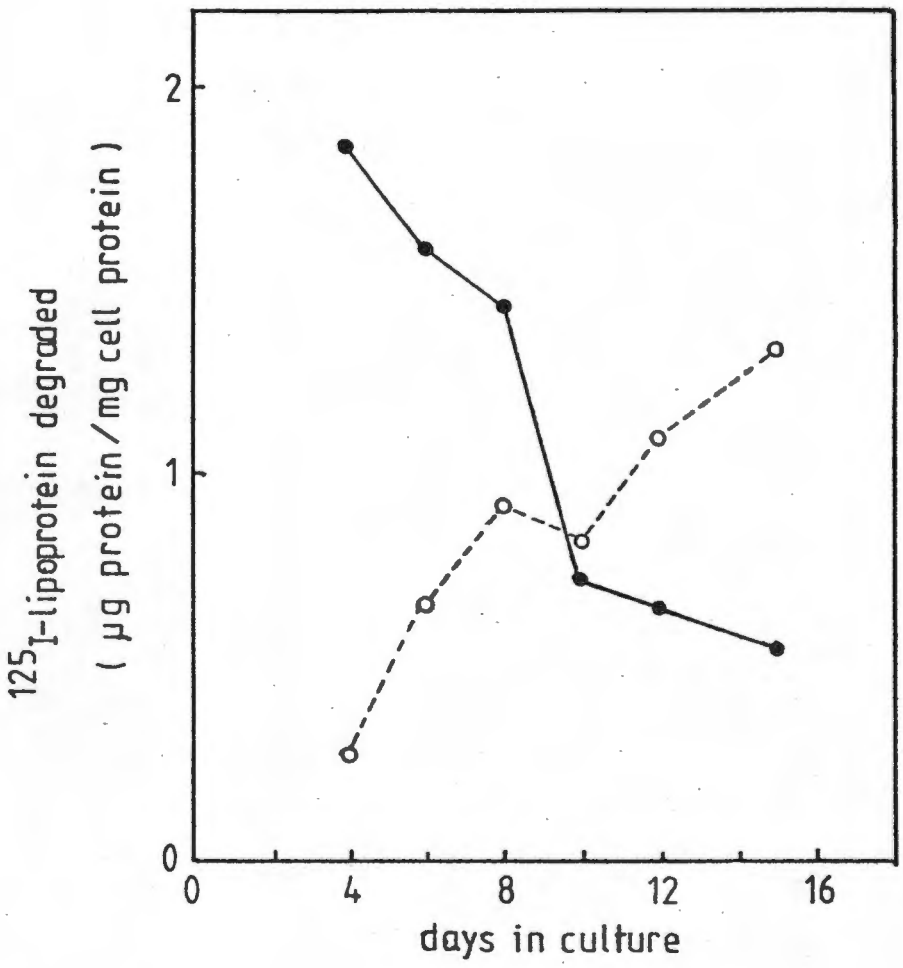


FIGURE 3.10: HIGH AFFINITY DEGRADATION OF ^{125}I-LIPOPROTEINS BY ENDOTHELIAL CELLS AS A FUNCTION OF CULTURE AGE

Endothelial cells (A_3Cl_2) were up-regulated with 2 ml of LPDS-medium for 48 h, prior to incubation with 20 µg protein/ml of ^{125}I-LDL (●——●) or ^{125}I-AcLDL (o---o) in the presence or absence of 200 µg protein/ml of unlabelled lipoproteins at 37°C. After 4 h, the medium was removed and precipitated with trichloroacetic acid (TCA). The TCA-soluble material was analysed for ^{125}I-lipoprotein degradation products. High affinity degradation was determined by the difference between the values obtained in the absence and presence of unlabelled lipoproteins.

Each point is the mean value obtained from duplicate dishes.

TABLE 3.9: EFFECT OF CULTURE AGE ON ^{125}I-LIPOPROTEIN DEGRADATION

Endothelial cells (A_3Cl_2) were up-regulated in LPDS-medium for 48 h. On day 4 and day 10, the cells were incubated with 10 µg protein/ml (+) or 20 µg protein/ml (*) of ^{125}I-lipoproteins in the presence or absence of 200 µg protein/ml of unlabelled lipoproteins, for 4 h at 37°C. The ^{125}I-lipoprotein degradation rates were corrected for non-saturable material. The differences in the degradation rates of ^{125}I-LDL and ^{125}I-AcLDL from a day 4 to a day 10 culture are expressed as a percentage change. Data are the mean of duplicate dishes for each experiment.

Experiments	High-affinity degradation of ^{125}I-lipoproteins (ng protein/mg cell protein)				% Change of high-affinity degradation of ^{125}I-lipoproteins	
	AcLDL		LDL		AcLDL	LDL
	Day 4	Day 10	Day 4	Day 10	increase	decrease
1+	227	1154	1386	816	508	59
2+	280	826	1861	733	295	39
3*	273	625	554	222	229	40
					(344 ± 84)	(46 ± 7)

TABLE 3.10: INTERNALIZATION INDEX

Endothelial cells were either up-regulated for 48 h or maintained in FCS-medium (i.e. no up-regulation). Subsequently, cells were incubated for 4 h at 37°C with 10 µg protein/ml of ^{125}I-lipoprotein in the presence or absence of 200 µg protein/ml of unlabelled lipoprotein. The medium and cells were analysed for the amounts of bound (B), internalized (I) and degraded (D) material. Each parameter was corrected for the low-affinity component, and high-affinity values (shown in Table 3.7) were used to determine the internalization index.

Treatment	Internalization index $(\frac{D+I}{B})$			
	Sparse cultures		Confluent cultures	
	AcLDL	LDL	AcLDL	LDL
up-regulation	23	26	26	28
no up-regulation	34	23	51	12

results indicate that endothelial cells at confluence do internalize and degrade LDL bound to the surface. This is in contradiction to Fielding et al (163) and Vlodavsky et al (66), but is in accord with the findings of Coetzee et al (67) (see also Discussion).

3.3.3. Receptor regulation

Early work had shown that the receptors for LDL on various cell types could be up-regulated (i.e. an increased number of surface receptors due to cellular receptor synthesis following removal of cholesterol delivery to the cells) and down-regulated (i.e. a decreased number of surface receptors due to inhibition of receptor synthesis upon delivery of excess cholesterol to the cells). Incubation of the cells with LPDS-medium is a well-established means of up-regulating LDL receptors on cells (145,146). In contrast, the addition of medium containing cholesterol derivatives which freely traverse the cell membrane, such as 25-hydroxycholesterol, have been found to down-regulate the LDL receptors of cells (170).

Up-regulation with LPDS-medium time course: Endothelial cells (A_3Cl_2) were up-regulated by the addition of 2 ml of LPDS-medium for varying incubation times at 37°C. Thereafter, the cells were incubated with ^{125}I-lipoproteins for 4 h and subsequently the amounts bound and internalized and the rates of degradation were determined (Figure 3.11). In the case of ^{125}I-LDL, both the amount of cell-associated

FIGURE 3.11: UP-REGULATION TIME COURSE OF ENDOTHELIAL CELL RECEPTORS.

Endothelial cells were up-regulated in 2 ml of LPDS-medium for the specified time periods. The medium was changed every 24 h. Subsequently on day 6, cells were incubated with 10 µg protein/ml of either ^{125}I-LDL (●——●) or ^{125}I-AcLDL (o---o) at 37°C. After 4 h, the cells were cooled to 4°C, and the medium was collected for determination of ^{125}I-lipoprotein degraded products (see Methods 2.1.4). The cells were extensively washed and then analysed for cell-associated ^{125}I-lipoproteins and protein content. Each point represents the mean value obtained from duplicate dishes.

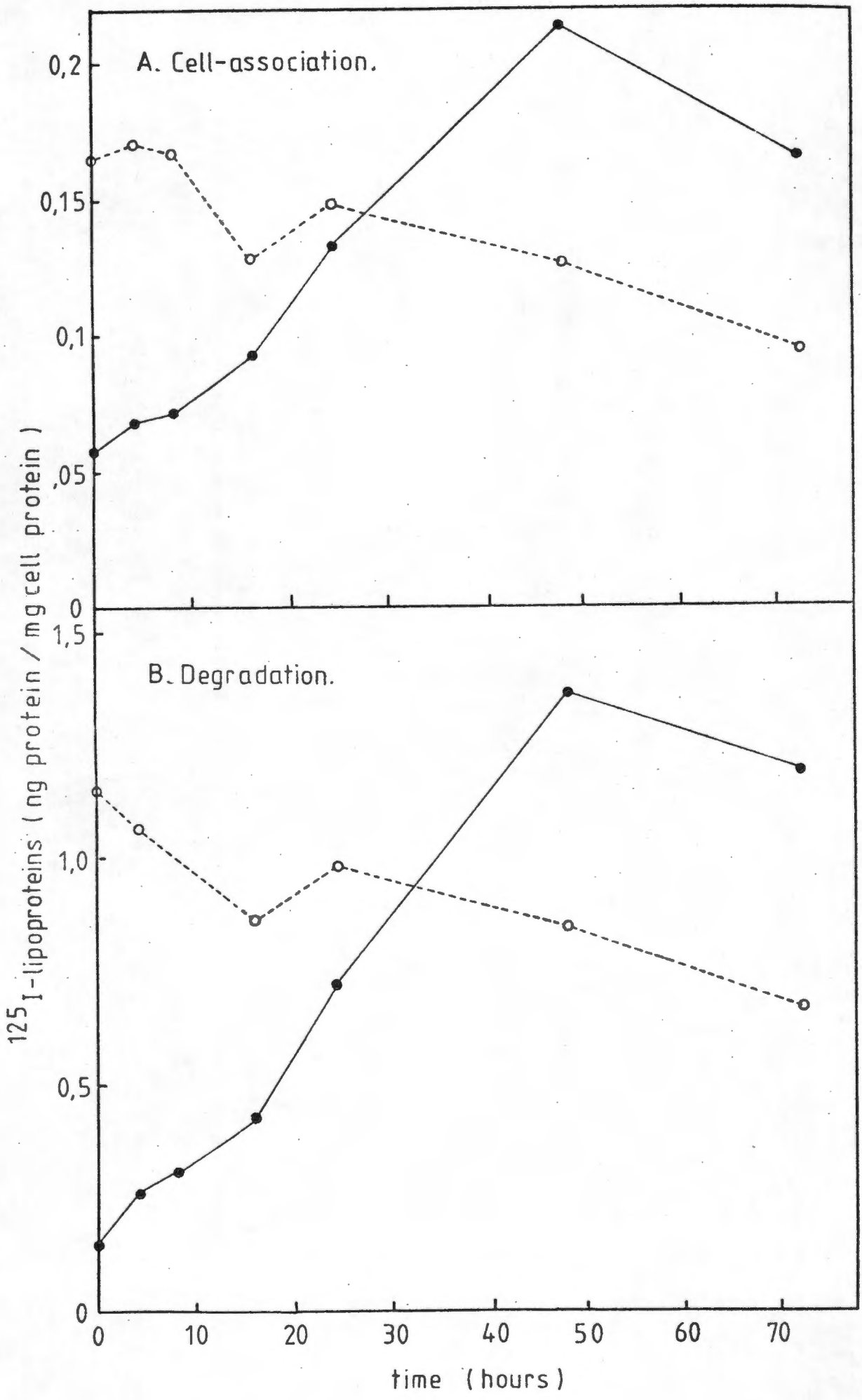

A. Cell-association.
B. Degradation.
0,2
0,15
0,1
,05
0
1,5
1,0
0,5
0
0
10
20
30
40
50
60
70
time (hours)
125I-lipoproteins (ng protein / mg cell protein)

FIGURE 3.11

and the rate of degradation reached a peak after a 48 h incubation with LPDS-medium. The increase in degradation rate, from cells which had not been incubated with LPDS-medium to cells which had had a 48 h incubation with LPDS-medium, was 9-fold. Upon longer incubation of the cells (for 72 h) with LPDS-medium a decrease in receptor activity resulted. The reason for this is not clearly understood. A possible explanation relates to the long exposure of the cells to a depleted growth medium (LPDS-medium) and a possible decreased ability of the cells to maintain normal functions under these conditions.

In contrast, an up-regulation of the AcLDL receptor was not evident. Cells which had been incubated with LPDS-medium were compared to those which had not received LPDS-medium prior to incubation with ^{125}I-AcLDL. It was found that regardless of the time of incubation with LPDS-medium (up to 22 h), the amounts of cell-associated and degraded material remained relatively unchanged. In a number of similar experiments (Table 3.11) no significant up-regulation was observed as a result of a 48 h exposure to LPDS-medium.

Down-regulation with 25-hydroxycholesterol time course:
Endothelial cells were incubated with LPDS-medium containing 25-hydroxycholesterol for increasing periods of incubation. The decrease in the receptors was determined by subsequently incubating the cells with ^{125}I-lipoproteins and determining the rates of degradation. As seen in Table 3.12, a decrease

TABLE 3.11: A 48 h UP-REGULATION OF THE LIPOPROTEIN RECEPTORS

Endothelial cells were either up-regulated for 48 h in LPDS-medium or maintained for 48 h in FCS-medium. The medium was changed after 24 h. The cells which had not received LPDS-medium were washed once with PBS, before addition of 10 µg protein/ml of ^{125}I-lipoprotein in LPDS-medium for 4 h at 37°C. Subsequently the amounts of cell-associated and degraded products of ^{125}I-lipoproteins were determined (see Methods 2.1.4). The ratios of values obtained for either cell-associated or degraded ^{125}I-lipoproteins with cells which had been up-regulated to those which had not been up-regulated, were determined. Data in parentheses are the means ± S.E.M. of the ratios obtained in the different experiments.

Experiments	Age of culture (days)	Ratio of lipoprotein up-regulated ($\frac{48}{0}$ h)			
		Cell-association		Degradation	
		AcLDL	LDL	AcLDL	LDL
1	5	0.72	1.90	0.56	2.19
2	10	0.99	1.49	0.93	3.43
3	5	1.17	3.56	1.02	5.74
4	5	-	2.00	-	3.58
5	5	1.14	1.98	1.16	3.87
6	8	1.09	-	0.98	-
		(1.02±0.08)	(2.19±0.36)	(0.93±0.10)	(3.76±0.57)

TABLE 3.12: DOWN-REGULATION TIME COURSE OF THE LIPOPROTEIN RECEPTORS

Endothelial cells (A_3Cl_2) were incubated for the specified times with 20 µg/ml of 25-hydroxycholesterol in 2 ml of LPDS-medium. The remaining cells were maintained in FCS-medium. On day 9, the cells were incubated with 10 µg protein/ml of ^{125}I-lipoproteins at 37°C for 4 h. Degradation values were obtained as described in Methods 2.1.4. Data are the mean of duplicate values.

Time (h)	Degradation of ^{125}I-lipoproteins (ng protein/mg cell protein)		% Down-regulation	
	AcLDL	LDL	AcLDL	LDL
0	1143	49	-	-
12	1213	3	-	94
24	916	4	20	92
48	650	3	43	94

in receptors of both LDL and AcLDL occurs. However, the down-regulation of the LDL receptor was far more rapid. Within the first 12 h, the LDL receptor was down-regulated by more than 90%, while the regulation of the AcLDL receptor was not affected during this short exposure to 25-hydroxycholesterol. Even after 24 h, the receptor for AcLDL was down-regulated by only 20%. Although in this experiment the cells looked viable even after a 48 h exposure to 25-hydroxycholesterol, on another occasion cell death had occurred shortly after 24 h. Thus, in subsequent experiments, cells were incubated for only 24 h with 25-hydroxycholesterol. Similar findings on down-regulation were found in a number of different experiments (Table 3.13). The receptor activity for LDL was down-regulated by about 96% as a result of a 24 h exposure to 25-hydroxycholesterol, while that for AcLDL was down-regulated by about 30% during the same period. Whether 25-hydroxycholesterol is causing a down-regulation of receptors or whether its toxic effect is somehow interfering with the uptake and degradation of ^{125}I-lipoproteins, was a matter to consider. A more reliable method to down-regulate the receptors was by incubating the cells with LPDS-medium containing either LDL or AcLDL (see Table 3.14).

Initially the up- and down-regulation of the receptor activities for LDL and AcLDL were determined using the total degradation rates. Since the degradation of receptor-mediated lipoproteins consistently made up 85-98% of the total degradation rates, these two values were similar. In

TABLE 3.13: A 24 h DOWN-REGULATION OF LIPOPROTEIN RECEPTORS

Endothelial cells were incubated either with 2 ml of FCS-medium for 24 h or LPDS-medium contai ing 20 µg/ml 25-hydroxycholesterol. Subsequently the cells were incubated with 10 µg protein/ml of ^{125}I-lipoproteins in 2 ml of LPDS-medium at 37°C. After 4 h, the medium was removed and analysed for degraded ^{125}I-lipoprotein material as described in Methods 2.1.4. Each experiment was performed using duplicate dishes.

Experiments	Age of culture (days)	^{125}I-lipoprotein degradation (ng protein/mg cell protein)				% of Down-regulation	
		AcLDL		LDL			
		Down-regulation time		Down-regulation time			
		0 h	24 h	0 h	24 h	AcLDL	LDL
1	5	304	201	476	4	33.9	99.2
2	4	844	621	1460	57	26.4	96.1
3	5	-	-	161	16	-	90.1
4	5	1366	1060	425	15	22.4	96.5
5	5	1134	825	333	9	27.2	97.3
6	10	759	499	288	17	34.3	94.1
7	9	1115	733	910	25	34.3	97.3
						(29.8±2.1)	(95.8±1.1)

other experiments (see Table 3.14), high-affinity degradation rates were determined leading to the same conclusions regarding the regulation of the two receptors.

Effect of culture age on up- and down-regulation: It has so far been established that the LDL receptor was susceptible to up- and down-regulation (Figure 3.11 and Table 3.12). However, although the AcLDL receptor was not susceptible to up-regulation by LPDS-medium (Figure 3.11), about a 30% down-regulation of the AcLDL receptor occurred upon expsure to 25-hydroxycholesterol (Table 3.13). Furthermore, it has been established that culture age (Table 3.14) has no effect on the regulation of receptors, except perhaps in the case of AcLDL regulation of the LDL receptor.

When the down-regulated state of the LDL receptor (obtained by incubation of the cells with LPDS-medium + LDL) was compared to the up-regulated state (the cells were exposed to LPDS-medium only), there was an 82% increase in receptor activity in sparse cultures. Under the same conditions, using confluent cultures, the increase in receptor activity was 80%. Thus there appears to be no culture age effect on the regulation of the LDL receptor if the down-regulated state of receptors is obtained by exposing the cells to LPDS-medium containing LDL.

A new finding was obtained in that the LDL receptor could be regulated by exposing the cells to AcLDL. In this case

TABLE 3.14: EFFECT OF CULTURE AGE ON UP- AND DOWN-REGULATION OF BOTH SPARSE AND CONFLUENT CULTURES

Endothelial cells, of both sparse and confluent cultures were treated for 48 h with 2 ml of either FCS-medium, LPDS-medium, LPDS-medium containing either 100 µg protein/ml of LDL or AcLDL or for 24 h with LPDS-medium containing 20 µg/ml of 25-hydroxycholesterol (25-OH-C). The various incubation media were changed every 24 h. The cells were then washed twice with phosphate buffered saline. Subsequently they were incubated with 20 µg protein/ml of ^{125}I-lipoproteins in the presence or absence of 200 µg protein/ml of unlabelled lipoproteins. The amount of ^{125}I-lipoprotein degraded was determined and corrected for non-saturable material. These two experiments were carried out independently, using different cell cultures. Data are the mean of duplicate dishes.

Cell cultures	Treatment	High-affinity degradation of ^{125}I-lipoproteins (ng protein/mg cell protein)	
		LDL	AcLDL
Sparse (day 4)	LPDS-medium	1061	711
	LPDS-medium + LDL	194	1031
	LPDS-medium + AcLDL	477	604
	LPDS-medium + 25-OH-C	57	621
	FCS-medium	309	751
Confluent (day 9)	LPDS-medium	643	1029
	LPDS-medium + LDL	131	1184
	LPDS-medium + AcLDL	79	927
	LPDS-medium + 25-OH-C	8	688
	FCS-medium	85	1029

a difference in the extent of receptor regulation between sparse and confluent cultures was observed. When the LDL receptor was down-regulated by exposure to LPDS-medium containing AcLDL, an increase in receptor activity of 55% occurred in sparse cultures when the cells were incubated with LPDS-medium only. However, under the same conditions, using confluent cultures, the receptor activity increased by 88%. This increase in LDL receptor activity with culture age could possibly be related to the increased catabolism of AcLDL by confluent cells (see Results 3.3.2).

In the case of the AcLDL receptor, the increase in receptor activity between cells incubated with LPDS-medium containing AcLDL to those incubated with LPDS-medium only, was 15% for sparse cultures. Confluent cultures under the same conditions exhibited a 10% increase in receptor activity. In contrast to the ability of the LDL receptor being regulated by AcLDL, the AcLDL receptor was not regulated by LDL under similar circumstances, in either sparse or confluent cultures.

Using cells incubated with LPDS-medium only as a measure of maximal LDL receptor activity, the down-regulation of the LDL receptor by 25-hydroxycholesterol was greater than by LPDS-medium containing LDL (i.e. 95% in sparse cultures and 99% in confluent cultures). By comparison, the down-regulation of the AcLDL receptor by 25-hydroxycholesterol was 13% in sparse cultures and 33% in confluent cultures.

This regulation of the AcLDL receptor is thus relatively small when compared to the regulation of the LDL receptor.

3.3.4. Receptor competition studies

From the data obtained concerning receptor regulation (see Results 3.3.3) and receptor activity with culture age (see Results 3.3.2), it was suggested that endothelial cells had two distinct receptors for LDL and AcLDL. This was established by incubating the cells with ^{125}I-AcLDL in the presence of increasing amounts of either unlabelled AcLDL or unlabelled LDL. Alternatively, cells were incubated with ^{125}I-LDL in the presence of increasing amounts of either unlabelled LDL or unlabelled AcLDL. The results of the competition experiments shown in Figure 3.12 indicate that whereas unlabelled AcLDL competed effectively with ^{125}I-AcLDL, unlabelled LDL did not compete, even after an addition of 300 µg protein/ml. Conversely, unlabelled AcLDL did not compete with ^{125}I-LDL, but unlabelled LDL did. In comparing Figure 3.12A to Figure 3.12B, it can be seen that the affinity of endothelial receptors for AcLDL was greater than the affinity of receptors for LDL. Addition of 50 µg protein/ml of unlabelled AcLDL resulted in a 73% competition with ^{125}I-AcLDL, while 50 µg protein/ml of unlabelled LDL resulted in only a 35% competition with ^{125}I-LDL. These results are consistent with those found in Results 3.3.1, where the half-maximal values for AcLDL were lower than those for LDL in experiments performed at 37°C.

FIGURE 3.12: COMPETITION STUDIES OF LIPOPROTEIN RECEPTORS IN ENDOTHELIAL CELLS

Endothelial cells were up-regulated for 24 h with 2 ml of LPDS-medium.

A. On day 5, cells were incubated with 2 ml of LPDS-medium containing 5 µg protein/ml of ^{125}I-LDL in the presence of the increasing specified amounts of unlabelled LDL (●——●) or unlabelled AcLDL (o---o).

B. On day 5, cells were incubated with 2 ml of LPDS-medium containing 5 µg protein/ml of ^{125}I-AcLDL in the presence of the increasing specified amounts of unlabelled AcLDL (o---o) or unlabelled LDL (●——●).

The amounts of ^{125}I-lipoproteins were measured as a function of increasing concentrations of unlabelled lipoproteins.

Each point represents the mean value obtained from duplicate dishes.

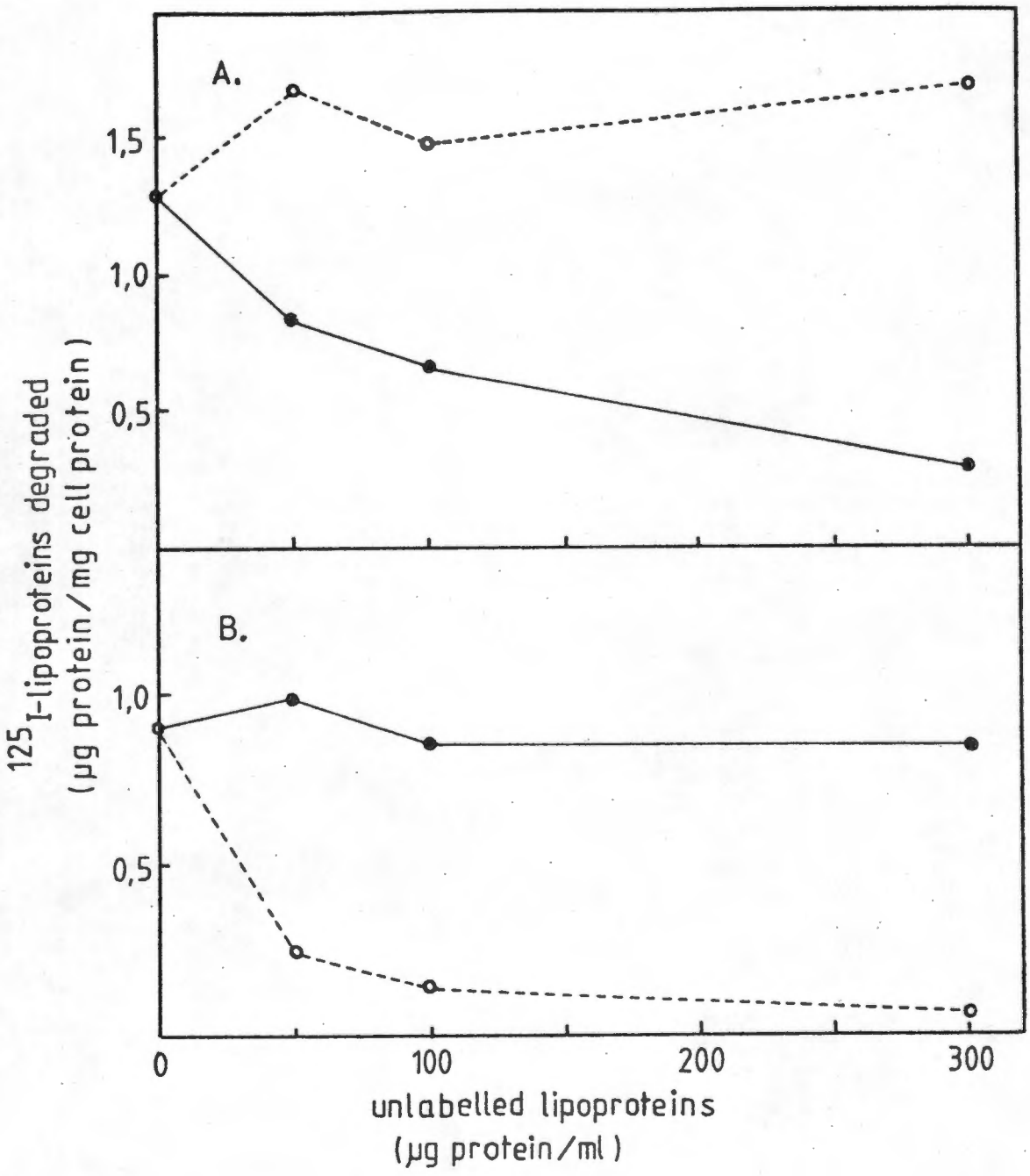

A.
B.
1,5
1,0
0,5
1,0
0,5
0
100
200
300
^{125}I-lipoproteins degraded
(µg protein/mg cell protein)
unlabelled lipoproteins
(µg protein/ml)

FIGURE 3.12

From the above results it was concluded that two distinct receptors exist on endothelial cells for LDL and AcLDL, and that these receptors had different affinities for their respective lipoprotein.

3.4. INHIBITORS OF LYSOSOMAL FUNCTION

Since the degradation of LDL is known to occur via the lysosomes in a variety of cell types, the dependence of the degradation of AcLDL was also investigated. This was done by incubating cells with ^{125}I-lipoproteins in the presence or absence of lysosomal inhibitors. The two inhibitors used were chloroquine and leupeptin. Chloroquine is a weak base which accumulates inside the lysosomes by being trapped within the organelles by protonation (157). This reduces the pH and hence the hydrolytic activities of the acidic lysosomes. Chloroquine also directly inhibits cathepsin B (158). Leupeptin, a modified tetrapeptide, isolated from actinomycetes (159) acts against thiol proteinases such as the cathepsins B, H and L. Cells (A_3Cl_2) were incubated for 4 h at 37^{o}C with ^{125}I-lipoproteins in the presence or absence of chloroquine or leupeptin, and the degradation rates of the ^{125}I-lipoproteins were measured (Table 3.15). The degradation rate of ^{125}I-LDL was inhibited 86-99% by chloroquine and 23-52% by leupeptin. Similarly, ^{125}I-AcLDL degradation was inhibited 90-98% by chloroquine and 8-24% by leupeptin. Thus, it could be concluded that both LDL and AcLDL

TABLE 3.15: INHIBITION OF ^{125}I-LIPOPROTEIN DEGRADATION

Endothelial cells were incubated with 2 ml of LPDS-medium containing 10 µg protein/ml of ^{125}I-lipoprotein in the presence or absence of chloroquine (70 µM) and leupeptin (100 µg/ml) at 37°C. After 4 h, the amounts of ^{125}I-lipoproteins degraded were determined as described previously (Methods 2.1.4). The cells used in Experiment 1 were a 12-day old culture which had not been up-regulated. Those used in Experiment 2 were an 8-day culture which had been up-regulated for 48 h in LPDS-medium. Data are the mean of duplicate dishes.

	High affinity ^{125}I-lipoprotein degradation (ng protein/mg cell protein)		% Inhibition of ^{125}I-lipoprotein degradation	
	LDL	AcLDL	LDL	AcLDL
Expt. 1: Control	218	2025	100	100
+ chloroquine	30	200	86	90
+ leupeptin	104	1849	52	8
Expt. 2: Control	1041	1018	100	100
+ chloroquine	13	21	99	98
+ leupeptin	807	774	23	24

degradation occurs via the lysosomes. Chloroquine was a much more powerful inhibitory agent, possibly due to its basic property which could perhaps inactivate the majority of the enzymes involved in lipoprotein degradation.

3.5. COMPARISON TO OTHER CELL TYPES

The amounts of LDL and AcLDL that were cell-associated and degraded in confluent endothelial cells (A_3Cl_2) were compared to the amounts processed by macrophages (J 774) and human skin fibroblasts (GM 0203 and SB).

All the cells were incubated with either ^{125}I-LDL or ^{125}I-AcLDL.(Table 3.16). When endothelial cells were compared to fibroblasts, the amout of cell-associated and degraded ^{125}I-LDL was found to be about 10-fold higher in fibroblasts after a 4 h incubation period. On the other hand, the amount of cell-associated ^{125}I-AcLDL in endothelial cells was about 3-6 times greater than that obtained with fibroblasts. In addition, the amount of ^{125}I-AcLDL degraded by endothelial cells was considerably more than that degraded by fibroblasts. In fact, the amount of ^{125}I-AcLDL degraded by fibroblasts was only 1-3% by comparison, even after a 24 h incubation period.

Macrophages, like endothelial cells, degraded far more ^{125}I-AcLDL than LDL, in confluent cultures. Although the

TABLE 3.16: COMPARISON OF ENDOTHELIAL CELL METABOLISM OF ^{125}I-LIPOPROTEINS TO OTHER CELL TYPES

Different cell types were incubated with 10 µg protein/ml of ^{125}I-LDL or ^{125}I-AcLDL at 37°C for 4 h. All had been pre-incubated with LPDS-medium for 48 h. The medium was analysed for degradation products as described in Methods 2.1.4 and the cells were washed extensively before being dissolved in 1 M NaOH at 37°C overnight for determination of the values obtained of cell-associated ^{125}I-lipoproteins. Data are the mean of duplicate dishes.

	Age of cultures (days)	Time of incubation (h)	Cell-associated ^{125}I-lipoproteins (ng protein/mg cell protein)		Degraded ^{125}I-lipoproteins (ng protein/mg cell protein)	
			LDL	AcLDL	LDL	AcLDL
Experiment 1: Endothelial cells (A_3Cl_2)	10	4	102	160	278	665
		24	79	127	2407	4830
Fibroblasts (GM0203)	7	4	1118	49	2634	15
		24	148	81	16732	52
Experiment 2: Endothelial cells (A_3Cl_2)	12	4	94	316	163	1664
		24	144	446	653	6663
Fibroblasts (SB)	12	4	914	53	1630	5
		24	881	134	7685	43
Macrophages (J774)	9	4	108	402	376	2838
		24	217	295	2482	6559

amount of cell-associated and degraded ^{125}I-LDL by macrophages was greater than that by endothelial cells, the amounts remained considerably lower than those obtained using fibroblasts, after either a 4 h or 24 h incubation period. In macrophages, the amount of cell-associated ^{125}I-AcLDL was greater than that associated with endothelial cells after a 4 h incubation period, but less after a 24 h incubation period. This same pattern applied to the degraded ^{125}I-AcLDL, where, after a 4 h incubation period, macrophages degraded almost twice the amount of ^{125}I-AcLDL degraded by endothelial cells. Yet after a 24 h incubation period, both endothelial cells and macrophages degraded similar amounts of ^{125}I-AcLDL. This non-linearity in the degradation of AcLDL by macrophages could possibly be explained if the added substrate had been depleted. However, upon calculation of the amount added to that degraded, this was not the case. The other possibility could have been that the cells were not favourably disposed to such a long incubation period with ^{125}I-lipoproteins. However, the catabolism of LDL by macrophages was linear from 4 h to 24 h, implying that the cells were not affected adversely by the long incubation with ^{125}I-lipoproteins. In addition, the appearance of the cells had remained unchanged during the 24 h. Therefore, it seems that there is no clear answer to clarify this phenomenon.

From the above results it could be concluded that while fibroblasts catabolize LDL in far greater quantities than either macrophages or endothelial cells, the catabolism of

AcLDL by fibroblasts is negligible. In contrast, macrophages and endothelial cells have receptors which can bind large quantities of AcLDL, as well as receptors which recognize LDL. The activities of the two different receptors are modulated according to age of cells in culture.

3.6. CHOLESTEROL METABOLISM

3.6.1. Determination of ACAT activity

Since LDL is able to stimulate the incorporation of ^{14}C-oleic acid into cholesteryl oleate in fibroblasts (160), the ability of both LDL and AcLDL to cause the same effect in endothelial cells was investigated. To control for variations in the esterification capacity of different cell types, the acyl-CoA cholesterol acyltransferase (ACAT) activities, induced by either LDL or AcLDL, were compared to those induced by 25-hydroxycholesterol. The effect of the latter does not depend on receptor number.

In order to measure the LDL induction of ACAT activity, the cells were up-regulated for 48 h in LPDS-medium (Table 3.17). In both confluent (Expt. 1) and sparse (Expt. 2) cultures, the ACAT activity was induced by LDL. The relative amount of LDL induction of ACAT was less in confluent cultures than in sparse cultures. This was not unexpected, since, as previously shown (Table 3.7), LDL receptor activity decreased with age of culture. The fact that induction by LDL occurred

TABLE 3.17: DETERMINATION OF ACAT ACTIVITY

The endothelial cell cultures were incubated in LPDS-medium or LPDS-medium containing either 200 µg/ml LDL or AcLDL or 5 µg/ml 25-hydroxycholesterol (25-OH-C) for 5 h. Following this incubation, ^{14}C-oleate (± 15000 dpm/nmol), complexed to bovine serum albumin, was added and the cells were incubated for a further 2 h. Cells were harvested, the lipids extracted with chloroform:methanol and radioactivity determined in the cholesteryl ester fraction after separation of lipids by thin layer chromatography (Methods 2.1.7). The cells used in Expt. 1 were confluent (Day 10), up-regulated cells, and in Expt. 2 they were sparse (Day 5), up-regulated cell cultures. All determinations were done in duplicate and ratios were determined using the means.

	dpm	Cholesteryl ^{14}C-oleate (nmoles/h/mg protein)	Cholesteryl ^{14}C-oleate formation	
			LDL-induced/25-hydroxy-cholesterol-induced	AcLDL-induced/25-hydroxy-cholesterol-induced
Experiment 1:				
LDL-induced	841	0.063		
AcLDL-induced	1763	0.132	0.38	0.80
25-OH-C-induced	2203	0.166		
Experiment 2:				
LDL-induced	2596	0.865		
AcLDL-induced	780	0.260	1.27	0.38
25-OH-C-induced	2044	0.681		

in confluent cultures is rather significant, since it has been shown that ACAT activity is regulated only by receptor-mediated LDL and not by adsorptive endocytosis (9). Thus, this is a further contradiction to the studies by Fielding et al (163) and Vlodavsky et al (66), in that confluent cultures do in fact bind, internalize and degrade LDL. The observations by these authors that confluent endothelial cells only bind, but do not internalize or degrade LDL is incompatible with these results.

The measurement of ACAT inducibility by AcLDL was obtained under the same conditions as for LDL. It was found that in up-regulated confluent (Expt. 1) and sparse (Expt. 2) cultures, AcLDL was indeed able to induce ACAT activity. The relative induction of ACAT was greater in confluent cultures than in sparse cultures. This is again consistent with the results obtained of AcLDL receptor activity (Table 3.7), which increased with age of culture.

3.6.2. Determination of cellular cholesterol

AcLDL and MDA-LDL (another negatively-modified LDL) have been shown to cause a massive accumulation of cholesteryl esters in macrophages (76,77,78,133). In addition, it had been shown by Stein and Stein (84) that incubation of endothelial cells with AcLDL caused an increase from 5.7 $\pm$ 1.4 in control cells to 14.6 $\pm$ 2.6 µg cholesteryl ester/mg cell protein. These results were difficult to interpret since details of the experiment were not given. Whether AcLDL

would cause a similar effect in the A_3Cl_2 strain of bovine aortic endothelial cells was investigated.

Endothelial cells (A_3Cl_2) and macrophages (J 774) were incubated with AcLDL, and the amount of cellular cholesterol in both cell types was determined. In the case of macrophages the increase in total cellular cholesterol was in the form of cholesteryl esters, while in endothelial cells the increase was in the form of free cholesterol. In Expt. 1 and Expt. 2 (Table 3.18) endothelial cells were incubated with 25 µg protein/ml of AcLDL or LDL for 72 h with medium changes every 24 h. LDL and AcLDL were added to LPDS-medium in Expt. 1 and to FCS-medium in Expt. 2. After incubation with the lipoproteins, the cells were harvested and the amounts of cellular cholesterol determined using a fluorimetric enzymatic assay (see Methods 2.1.8).

In confluent cultures, cells which had been exposed to LPDS-medium had considerably less cholesterol than those which had been exposed to FCS-medium. This would explain why the increase in total cholesterol in cells exposed to LDL in Expt. 1 (LPDS-medium) was almost double that in Expt. 2 (FCS-medium). However, the increase in total cholesterol upon exposure of the cells to AcLDL was similar in both cases. This was not unexpected as the AcLDL receptor has been found not to be very susceptible to regulation.

In Expt. 1 and Expt. 2, the increase in free cholesterol was greater upon exposure of the cells to AcLDL than to LDL when

TABLE 3.18: DETERMINATION OF CELLULAR CHOLESTEROL

Endothelial cells (A_3Cl_2) in Expt. 1 were incubated for 72 h with 25 µg protein/ml of LDL or AcLDL in LPDS-medium, with medium changes every 24 h. The medium on dishes which had been treated with lipid was also changed every 24 h. In Expt. 2, the cells were incubated for 72 h with 25 µg protein/ml of LDL or AcLDL in FCS-medium with medium changes every 24 h. Macrophages (J774) were treated similarly but were exposed to the various treatments for only 48 h. In all cases, 30 µM of chloroquine (CQ) was added during the last 24 h of the incubation. Subsequently, the cells were harvested in chloroform:methanol (40%:60%) and the lipids extracted and determined using a fluorimetric method (see Methods 2.1.8).

Each fluorimetric determination was done in duplicate. In Expt. 2 only single dishes were used, whereas in Expt. 1 duplicate dishes were used, of which an aliquot from each was determined in duplicate and an overall mean of four readings was obtained. The limit of the sensitivity of the cholesteryl ester determination was estimated to be less than 10% of the free cholesterol value.

* The concentration of AcLDL added to the dishes was 125 µg protein/ml.

TABLE 3.18

	Treatment	Total cholesterol (µg/mg cell protein)	Free cholesterol (µg/mg cell protein)	Cholesteryl ester (µg/mg cell protein)
Experiment 1:				
Endothelial cells confluent (Day 12)	No lipid	22.0	19.5	3.0
	LDL	41.0	36.5	4.5
	LDL + CQ	45.5	43.5	<3.0
	AcLDL	42.5	43.0	-
	AcLDL + CQ	56.0	56.2	-
	AcLDL*	40.0	37.5	<3.0
Experiment 2:				
Endothelial cells sparse (Day 5)	No lipid	43.0	42.0	<3.0
	LDL	47.0	43.5	3.5
	AcLDL	58.0	56.0	<3.0
	AcLDL + CQ	62.5	62.5	-
confluent (Day 11)	No lipid	37.5	37.5	-
	LDL	47.8	43.3	4.5
	AcLDL	54.5	52.5	<3.0
	AcLDL + CQ	65.8	59.5	6.3
Macrophages	No lipid	39.0	40.0	-
	AcLDL	95.0	38.0	57.0
	AcLDL + CQ	126.0	53.0	73.0

compared to those cells exposed to medium alone. For example, in sparse endothelial cultures, the amount of free cholesterol increased by about 14 µg/mg cell protein, from 42 to 56 µg/mg cell protein when cells were incubated with FCS-medium in the presence of AcLDL. In contrast, the addition of LDL to the cells under the same conditions resulted in only about a 1.5 µg/mg cell protein increase. In confluent cultures, the increase of free cholesterol upon addition of AcLDL to the cells was slightly more than in sparse cultures. Similarly, the addition of LDL to confluent cultures resulted in a greater amount of free cholesterol than in sparse cultures. In the confluent cultures in which LPDS-medium had been supplemented with LDL, the increase in free cholesterol was about 17 µg/mg cell protein from about 20 to 37 µg/mg cell protein, while those exposed to AcLDL under the same conditions resulted in an increase of free cholesterol of about 23 µg/mg cell protein. The addition of 125 µg protein/ml of AcLDL to the cells did not result in a further increase of cholesterol. In these endothelial cells, the increase in total cellular cholesterol was due to the increase of free cholesterol, while only a very small proportion existed as cholesteryl esters. The variation between duplicate cholesterol determinations were always less than 5%. Since the accuracy of the cholesteryl ester determination is dependent on the ratio of free cholesterol to cholesteryl ester, the limit of sensitivity of the cholesteryl ester determination was estimated to be less than 10% of the free cholesterol value. The cholesteryl ester content in endothelial cells exposed to AcLDL was less than 3 µg/mg cell protein, and that of cells exposed to LDL

was about 4 μg/mg cell protein.

Since it had been previously demonstrated that chloroquine inhibited LDL and AcLDL degradation (see Results 3.4), chloroquine was added to the medium containing either LDL or AcLDL, to investigate whether a further increase in cholesterol would occur. The increase in total cholesterol was far more marked with AcLDL (about 10-15 μg/mg cell protein) than LDL (about 4 μg/mg cell protein). However, the increase in total cholesterol was mainly due to free cholesterol.

On the other hand, macrophages incubated with AcLDL for 48 h resulted in more than twice the amount of total cholesterol obtained in macrophages exposed to FCS-medium alone (i.e. from 39-95 μg/mg cell protein). In this case, the amount of free cholesterol did not increase, and most of the cholesterol was detected as cholesteryl esters. Upon addition of chloroquine to the cells incubating with AcLDL, the total cellular cholesterol further increased to 126 μg/mg cell protein, and although the amount of free cholesterol increased by 13 μg/mg cell protein, the bulk of the cholesterol in the cells existed as cholesteryl esters (73 μg/mg cell protein).

In summary, it has been observed that AcLDL causes a greater cholesterol increase in endothelial cells, than does LDL. However, considering the rate of uptake of AcLDL by endothelial cells, the limited accumulation of cholesterol in

these cells is not incompatible with the AcLDL uptake data. For example, in confluent endothelial cells, the expected amount of cholesterol uptake via AcLDL in 72 h is approximately 32 μg/mg cell protein, assuming an internalization rate for AcLDL of 4 μg/mg protein/h (Figure 3.8). This phenomenon in endothelial cells is different to that in macrophages, where incubation with AcLDL results in a relatively large increase in total cholesterol content. Furthermore, the bulk of the increase in total cholesterol exists as cholesteryl esters, whereas in endothelial cells the cholesterol increase is due to the increase in free cholesterol.

4. DISCUSSION

Before analysing the results obtained in this manuscript, it is of relevance to examine the cell system employed, in the light of the different conditions under which endothelial cells have been maintained *in vitro*. The saturation cell density obtained in a confluent monolayer of bovine aortic endothelial cells (A_3Cl_2) grown on gelatin-coated Petri dishes was 0.64×10^5 cells/cm^2. This was consistent with the saturation cell density obtained by Gospodarowicz *et al* (38), which was 0.56×10^5 cells/cm^2. On the other hand, Schwartz (19), Duthu and Smith (41) and Davies (40) have shown endothelial cells to grow to a saturation cell density of 1.5×10^5 cells/cm^2. The bovine aortic endothelial cells used by Gospodarowicz *et al* (38) were isolated in a similar way to the cells obtained in this study, i.e. by mechanical scraping of the surface of the lumen. In addition, these cells were isolated and maintained in the presence of 100 ng/ml of fibroblast growth factor (FGF), resulting in a confluent monolayer of cells for a long period of time, with no evidence of "sprouters". According to these authors, upon removal of FGF, the cells continue their growth in a similar way to cells continually exposed to FGF. In contrast, Duthu and Smith (41), Schwartz (19) and Davies (40) prepared bovine aortic endothelial cells by dissociation of the intima with collagenase. Duthu and Smith (41), after isolation of the cells, maintained them in the presence of FGF. However, upon removal of FGF, the cells no longer

maintained a confluent monolayer and formed a multilayer. Schwartz (19) obtained cells by either a ^{3}H-thymidine selection or cloning. In both cases, the cells formed a cobblestone monolayer of small, round cells, which could be maintained in the absence of FGF. However, this monolayer eventually gave rise to "sprouters". Although the cells used in this study, when grown on gelatin-coated dishes, visually behave in a similar manner to those obtained by Schwartz (19), the saturation cell density was closer to that obtained by Gospodarowicz *et al* (38). Hence, there appears to be some variation in the morphology and growth characteristics of cultured bovine aortic endothelial cells, which in turn could possibly affect certain functional aspects of the cells. It should also be borne in mind that such cells grown *in vitro* need not reflect the true *in vivo* endothelial layer, although they do continue to provide a convenient model system for studies, which can form a basis for future *in vivo* studies.

A number of studies has previously identified a pathway in several different cell types by which low density lipoproteins (LDL) are taken up into the cells via a saturable, high-affinity receptor. Most of the studies reported have been carried out with cultured fibroblasts (8,9) and smooth muscle cells (115,116), and more recently with endothelial cells (21,28,66,135). After internalization, the lipoprotein apoprotein is degraded in the lysosomes to amino acids and the cholesteryl ester component is hydrolysed by a lysosomal

cholesterol esterase to free cholesterol, which can be re-esterified by the enzyme, acyl-CoA : cholesterol acyl transferase. This LDL pathway has been found to regulate the uptake, storage and synthesis of cholesterol and thus protect cells from an over-accumulation of sterols (9). In this study, the LDL receptor affinity for LDL in bovine aortic endothelial cells (A_3Cl_2), as measured from degradation data, was found to correspond to a k_δ value of about 20 µg protein/ml. This equilibrium dissociation constant value in bovine aortic endothelial cells was similar to the value obtained for endothelial cells from foetal bovine heart, which had been reported as 15 µg protein/ml (66), but was considerably higher than that found in rabbit arterial endothelial cells, where the receptor for LDL reached saturation at 5 µg protein/ml (135).

In addition, it was shown in this study that bovine aortic endothelial cells could catabolize AcLDL via a saturable high-affinity receptor. In the acetylation reaction, acetyl groups are attached to the lysine residues of LDL, neutralizing the positive charges and increasing the net negative charge of the protein. The ability to catabolize negatively-modified LDL is not shared by the majority of cultured cell types, such as fibroblasts, smooth muscle cells, lymphocytes or adrenal cells (78). However, a high-affinity receptor which recognizes negatively-modified LDL has been identified in mouse peritoneal macrophages (78,127,161) and in human monocyte-derived macrophages (76,77,133). The receptor affinity for AcLDL in bovine aortic endothelial cells was found to correspond to a

k_δ value of about 2 μg protein/ml, which was considerably greater than the receptor affinity for LDL. Stein and Stein (84), also using bovine aortic endothelial cells, reported that the degradation rates of AcLDL showed evidence of saturation at 10-20 μg protein/ml. By comparison, the macrophage AcLDL receptor on mouse peritoneal cells exhibited a half-maximal value of about 10-25 μg protein/ml (78,127,161). This receptor was found to be the same as that for MDA-LDL (78), and the degradation rates of these two negatively-modified LDL's were found to be similar. The receptor for MDA-LDL on human monocyte-derived macrophages had a half-maximal value of about 5 μg protein/ml (76). An interesting observation in these two types of macrophages is that, although mouse macrophages do not take up and degrade native LDL by a high-affinity receptor (127), human macrophages were shown to possess LDL receptors which exhibited a k_δ value for LDL of 10-25 μg protein/ml (77,162). Therefore, it appears that in human macrophages, as in bovine aortic endothelial cells, the receptor affinity for negatively-modified LDL is greater than that for LDL. Furthermore, these receptor affinities for LDL and AcLDL in endothelial cells were temperature-independent. This is contrary to the receptor affinity for LDL in human skin fibroblasts, where the receptor affinity at 4^oC is about twice that at 37^oC (114).

One of the problems in this study was the very large low-affinity (and non-saturable) binding of AcLDL to the endothelial cells, which complicated the experimental processing

of results. The approach often used, therefore, was to employ degradation data as a measure of receptor activity. In the case of LDL, the amount of non-saturable material was similar for binding, internalization and degradation. This implies that non-receptor bound LDL was internalized and degraded as effectively as receptor-bound material. However, in the case of AcLDL, there was a relatively large amount of non-receptor-bound material, compared to receptor-bound material. However, the material that was internalized and degraded was evidently taken up by the cells to a very large extent via receptors. Stein and Stein (90) have presented evidence for arterial transendothelial transport of plasma low-density lipoproteins via pinocytosis. More recently, Leake and Bowyer (164) have studied rates of pinocytosis by arterial endothelial cells, and concluded that a major function of vascular endothelial cells is to transfer substances between the plasma and interstitial fluid by diacytosis. Diacytosis is the transport process of intact substances, such as plasma lipoproteins, involving pinocytosis at one surface of the cell, followed by the movement of the pinosome to the opposite surface of the cell, and the exocytosis would probably become operational for LDL transport once the surface receptors are saturated. Since there is no obvious reason why this transport system should exclude the transport of AcLDL, it could be possible that a large fraction of the non-receptor-bound material is actually transported by this pathway. Since degradation of material transported by diacytosis is not expected, the assay used for analysis of

degradation products in the medium would not be able to detect any intact lipoprotein which had been transported by diacytosis. The reason why intracellular material is not detected may depend on the level of flux of the vesicles through the cell. Also, cells in culture may not exhibit diacytosis. However, the high level of non-receptor binding may reflect the situation *in vivo*, and thereby contribute to an absorptive endocytotic process. Since endothelial cells see the full complement of the plasma and thus, presumably, a higher concentration of lipoproteins than that present in the interstitial fluid, the large non-receptor binding component may play a more significant role than that in subendothelial cells.

A new finding was that cultured bovine aortic endothelial cells possess distinct receptors for LDL and AcLDL, of which the AcLDL receptors exhibit a higher affinity for their respective lipoprotein. The receptors for AcLDL did not recognize LDL, and conversely the receptors for LDL did not recognize AcLDL. Recently, the isolation of the AcLDL receptor from membranes of murine macrophage line P338D.1 was reported (134). Furthermore, it was observed that the incubation of the cells with either AcLDL or LDL caused the down-regulation of the LDL receptor, but neither the addition of LDL nor AcLDL down-regulated the AcLDL receptor. This phenomenon, whereby only LDL receptors were subject to regulation, was also reported for human monocyte-derived macrophages (135). Both LDL and MDA-LDL failed to down-

regulate the MDA-LDL receptor in these cells. The inability of the AcLDL receptor to respond to regulation could be an explanation for the eventual loading of the macrophages with cholesterol to form foam cells and atherosclerotic plaques.

Down-regulation of the LDL receptor was observed in both sparse and confluent cells. This contradicts the findings of Vlodavsky et al (66) that only LDL receptors of sparse endothelial cell cultures are susceptible to down-regulation. The relationship between LDL receptor activity in endothelial cells and cell density has been a matter of controversy for some time. Gospodarowicz and co-workers (21,28,66,163) have consistently reported that confluent, contact-inhibited endothelial cell monolayers bind, but do not internalize or degrade LDL. The block in lipoprotein protein uptake extends to the cholesterol uptake, thus preventing regulatory effects of LDL on cholesterol metabolism (163). By incubating confluent, contact-inhibited endothelial cells with cationized LDL, these authors observed that the cells were able to degrade the lipoprotein and exhibit the expected regulation of the activities of the enzymes required for sterol synthesis from acetate and the conversion of cholesterol to cholesteryl esters. From these studies, they concluded that the primary regulation of LDL catabolism in confluent, contact-inhibited cells was the inhibition of uptake of LDL as a result of the cell-cell contact (66). In sparse cultures, however, the uptake of LDL is primarily via the down-regulation of the receptor number. In contrast, Coetzee et al (67) have shown

that while endothelial cell receptor activity decreases with increasing cell density, confluent cultures nevertheless take up and degrade significant amounts of LDL. In agreement with these results are the findings reported here that in a confluent monolayer of cells, a significant amount of LDL was bound, internalized and degraded, although at a lower rate than in sparse cultures. Furthermore, the efficiency with which bound LDL is internalized (as indicated by the internalization index) remained unchanged for sparse and confluent cultures (see Results 3.3.2). This implies that the decrease in internalization and degradation was to a large extent, or completely, due to the observed decrease in cell surface receptors. This decrease was observed with increasing cell density. A factor that could give rise to these contradicting results is the different methods used in obtaining and culturing endothelial cells. Although the cells used in this study appear as a confluent monolayer, the fact that the monolayer eventually gave rise to "sprouters" could imply that the monolayer was perhaps not completely contact-inhibited, or that the measured amount of internalized and degraded material was due to the "sprouting". The latter possibility, however, appears unlikely, as cells were used at confluence before the development of "sprouters". The possibility exists that the cells might have appeared confluent, but were not completely contact-inhibited. However, given the similarity between cell densities obtained in confluent cultures used in this study and those reported by Gospodarowicz *et al* (38), and the morphological appearance of the two cell cultures, at

least partial confluence in the present cultures used here is suggested. It would therefore be expected that a block in endocytosis would be evident even in cultures not absolutely confluent or contact-inhibited. However, there was no indication of any such change as observed above, i.e. no change in the internalization index from sparse to confluent cultures. This is consistent with the studies of Coetzee et al (67), who reported that confluent cultures degrade significant amounts of LDL. These authors had used endothelial cells which had been a gift from Dr D. Gospodarowicz. The cells were foetal bovine heart endothelial cells, isolated and maintained in the presence of fibroblast growth factor (FGF). It was these cells, too, which had been used to look at the LDL receptor in confluent, contact-inhibited cells by Vlodavsky et al (66). It would be unusual for these cells to change in functional characteristics from one laboratory to another, when maintained under similar conditions. This thus leaves one with equivocal results, which cannot easily be explained.

The number of receptors for AcLDL also changed with cell density. In comparing the receptor activity of AcLDL to that of LDL, whereas the receptor activity of LDL decreased with confluence, that of AcLDL increased steadily with increasing age of culture. This latter phenomenon was also observed in human monocyte-derived macrophages (133), with the MDA-LDL receptor being fully expressed only in the second week of growth in culture. It would therefore appear that in confluent,

monolayered cultures, endothelial cells, to a large extent, shut down the mechanism of LDL uptake, thus protecting against an over-accumulation of cholesterol. Thus it is contradictory to this protection mechanism that confluent cells preferentially take up negatively-modified LDL. AcLDL has been shown to be recognized by the same receptor that binds negatively-modified LDL, namely MDA-LDL (78), which can be produced in vivo. This provides evidence indicating that it is possible to have negatively-modified LDL circulating in the bloodstream which can be taken up and degraded by endothelial cells as well as by macrophages.

Another question which arises is the possibility of injury to other areas by such a modified lipoprotein found in the circulatory system. Although it has been postulated that LDL can lead to injury of the endothelium (13), it has yet to be investigated whether negatively-modified LDL would have a similar effect. It is rather interesting that, although it was shown that AcLDL had hardly any (10-15% down-regulation) regulatory effect on the AcLDL receptor, it could regulate the LDL receptor, as well as induce the enzyme, acyl-CoA : cholesterol acyl transferase (ACAT) into esterifying cholesterol to cholesteryl esters. An area which needs further investigation is the possible regulatory effect of AcLDL on the synthesis of cholesterol, i.e. regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. It is possible that the 10-15% down-regulation of the AcLDL receptor by AcLDL may not represent the maximum down-regulation of this receptor.

This would be the case if the receptor for AcLDL had a longer half-life. It has been assumed that the half-life of the AcLDL receptor did not differ from that of the LDL receptor which is about 16-20 h. However, an experiment employing cycloheximide would be able to clarify this point.

In the atherosclerotic plaques, the cells which have been identified as being laden with cholesterol were macrophages and smooth muscle cells (10), and not endothelial cells. However, Stein and Stein (84) reported an increase in cholesteryl esters of about 9 µg/mg cell protein in endothelial cells upon incubation with AcLDL. In this study it was found that incubation of endothelial cells with AcLDL caused a larger increase in free cholesterol than did LDL, but the cholesteryl ester content remained relatively small (<3 to 6 µg/mg cell protein).

In the one experiment, macrophages were used for comparison, and in these cells there was a large accumulation of cholesteryl esters (57 µg/mg cell protein), which coincided with reports by Fogelman and coworkers (76,77,133) and Goldstein, Brown and coworkers (78,127,161).

On looking at the receptor-mediated pathway of AcLDL, it does not appear to differ considerably from the LDL receptor-mediated pathway. In this study, endothelial cells incubated with LDL or AcLDL reached a steady-state of lipoprotein binding and internalization at about 2 h, with an initial lag of

30 min before degradation products were formed. After internalization, both lipoproteins were delivered to the lysosomes, as suggested from the observation that the degradation of the lipoproteins was inhibited upon incubation with chloroquine (a lysosomal inhibitor). The ACAT activity is dependent on the delivery of cholesterol via saturable, high-affinity bound material, and was induced by both LDL and AcLDL binding to the cell surface. Although the delivery of cholesterol from AcLDL caused an induction in the ACAT activity and a down-regulation of the LDL receptor, neither this cholesterol nor that from LDL regulated the AcLDL receptor.

At this stage it is possible to postulate on the role of endothelial cells in the formation of the atherosclerotic plaques. It has been shown that endothelial cells, whether sparse or confluent, do not accumulate excessively large a amounts of cholesterol upon exposure to AcLDL. Thus, it was of interest that smooth muscle cells, which were incubated with medium that had been previously incubated with endothelial cells in the presence of AcLDL, accumulated a large amount of cholesteryl esters (85). It is attractive to postulate that in the process of diacytosis, the AcLDL becomes modified in such a way as to be recognized by smooth muscle cells. Although in cultured cells there is the possibility of retro-endocytosis (165) of the AcLDL particle back into the medium, whether the in vivo situation would retro-endocytose AcLDL back into the bloodstream or into the interstitial fluid, is still unknown. In comparing endothelial cells to macrophages,

the latter appear to possibly have a dual role in the formation of an atherosclerotic plaque, i.e. to alter negatively-modified LDL for uptake by smooth muscle cells (85,86), as well as to accumulate cholesteryl esters upon exposure to the negatively-modified LDL (76,77,78,127,133,161). In contrast, endothelial cells do not appear to accumulate cholesteryl esters markedly under any conditions, but may be involved in the modification of lipoproteins, thus resulting in the accumulation of cholesteryl esters in smooth muscle cells.

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