EFFECTS OF FATTY ACIDS UPON LDL CATABOLISM IN CULTURED CELLS

by

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ABSTRACT

The aim of these studies was to investigate how changes in the fatty acid composition of cellular phospholipids affect the receptor-mediated catabolism of low-density lipoprotein (LDL) and the formation of cholesteryl esters by fibroblasts.

Changes in the fatty acid composition were achieved by growing the fibroblasts in medium supplemented with different fatty acids. Under the appropriate conditions the proportion of linoleate could be increased almost 4-fold. This had little effect on the binding of LDL at either 4°C or 37°C but did result in a considerable increase in the ability of cells to internalise and degrade LDL by both the receptor-mediated and receptor-independent pathways. When palmitate or oleate gradually replaced linoleate in the fatty acid supplement, there was a strong correlation ($p<0.001$) between the rate of LDL degradation and the ratio of polyunsaturated:saturated fatty acids in the fibroblast phospholipids.

To examine how this enhanced LDL degradation affected the rate of cholesterol esterification, an assay was developed which allowed acyl-coenzyme A:cholesterol acyltransferase (ACAT) to be measured directly in isolated fibroblast membrane preparations. The enzyme activity was assayed both in freshly isolated membranes and in membranes that had been incubated with cholesterol-rich donor lipoprotein which resulted in an equilibration of cholesterol between membranes and donor. Without supplementary fatty acids, the addition of LDL to fibroblasts preincubated in lipoprotein-deficient serum led to an increase in membrane ACAT activity which reached a peak after 7 hours. However, after incubation with donor lipoprotein, each preparation had the same activity regardless of how long the fibroblasts had been incubated with LDL. Thus, under normal conditions, the increase in membrane ACAT activity can be explained entirely by an increase in the amount of cholesterol in the substrate pool. In contrast, ACAT activity, both before and after the 7-hour incubation with LDL, was higher in cells grown with linoleate than in cells grown in normal medium or with palmitate. The increase in activity produced by LDL was also greater and was associated with an increase in the activity observed after equilibration with donor. Also, incubating cells with free cholesterol produced a prolonged increase in ACAT activity and an increase in the activity observed after equilibration.

Thus, under conditions where there is an exceptionally high or prolonged cholesterol supply, fibroblasts can increase the amount of active enzyme on their membranes to prevent any, potentially damaging, accumulation of free cholesterol.

ABBREVIATIONS

ACAT	-	acyl-coenzyme A:cholesterol acyltransferase
acetyl-LDL	-	low-density lipoprotein covalently modified by acetylation
apoA-I	-	apoprotein A-I (similarly apoB, *etc.*)
CHD-LDL	-	low-density lipoprotein modified by the covalent linkage of cyclohexane di-one
EDTA	-	ethylenediaminetetra-acetic acid
FCS	-	foetal calf serum
FH	-	familial hypercholesterolaemia
HDL	-	high-density lipoprotein
HEPES	-	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
HMG-CoA reductase	-	3-hydroxy-3-methylglutaryl-coenzyme A reductase
IDL	-	intermediate-density lipoprotein
LCAT	-	lecithin:cholesterol acyltransferase
LDL	-	low-density lipoprotein
LPDS	-	lipoprotein-deficient serum
PBS	-	Dulbecco's phosphate-buffered saline
P/S ratio	-	ratio of polyunsaturated:saturated fatty acids
S.E.M.	-	standard error in the estimation of the mean
tissue culture medium A	-	Eagle's minimum essential medium with Hanks' salt solution, 20 mM HEPES, 10 mM $NaHCO_3$, 100 units/ml penicillin and 100 µg/ml streptomycin
tissue culture medium B	-	Eagle's minimum essential medium with Earle's salt solution, 20 mM Tricine, 25 mM $NaHCO_3$, 100 units/ml penicillin and 100 µg/ml streptomycin
Tricine	-	*N*-((trishydroxymethyl)methyl)glycine
Tris	-	tris(hydroxymethyl) methylamine
VLDL	-	very-low-density lipoprotein

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INTRODUCTION

1.1 Introduction

The main role of cholesterol in the body is to maintain the structure and function of cellular membranes. In particular, interactions between cholesterol and phospholipid can affect the fluidity and permeability of membranes (Demel and De Kruyff, 1976). Since the efficient functioning of cells is dependent upon these properties, the proportion of cholesterol in the membrane is important and needs to be closely controlled.

In mammals most lipids, including cholesterol, are transported around the body in lipoprotein complexes which have a core of non-polar lipid such as triglyceride and cholesteryl ester and a surface made up of protein and amphipathic lipids such as cholesterol and phospholipids. There are several types of these lipoprotein complexes, of differing functions, which vary in size, in the proportions of the different types of lipid and in their protein components (Soutar and Myant, 1979; Myant, 1982).

The metabolism of low-density lipoprotein (LDL) has been extensively studied, both *in vivo* and *in vitro*, because the plasma concentration of this lipoprotein has been positively correlated with an increased risk of atherosclerosis (Stamler, 1973). At a cellular level, much of the initial work on the catabolism of LDL and its effect upon cholesterol metabolism

has been carried out on cultured human fibroblasts (Goldstein and Brown, 1977). Fibroblasts can meet their cholesterol requirements either from *de novo* synthesis when there is no alternative source, or from plasma LDL which is bound by specific, high-affinity, cell-surface receptors, taken into the cell and degraded in the lysosomes releasing free cholesterol. Both the rate of cholesterol synthesis and the number of receptors expressed at the cell surface are under strict feedback regulation. Any cholesterol in excess of the fibroblasts' requirements is esterified with fatty acids by acyl-coenzyme A:cholesterol acyltransferase (ACAT) to form cholesteryl ester and stored, in this biologically inactive form, as lipid droplets within the cell (Goldstein and Brown, 1977).

A diet with a high content of polyunsaturated fat lowers the concentration of LDL in the plasma (Shepherd *et al.*, 1980) and may thus reduce the risk of atherosclerosis.

The fatty acid composition of fibroblast phospholipid can be modified by altering the proportions of individual fatty acids in the growth medium (Spector *et al.*, 1979). However, despite the use of cultured human fibroblasts in clarifying the processes involved in the LDL receptor pathway, very little work has been carried out using cultured cells to investigate the possible mechanisms by which fatty acids affect LDL metabolism.
After general discussions of membrane structure and function, lipoprotein metabolism *in vivo* and *in vitro* and fatty acid metabolism, the specific problems investigated in the work presented in this thesis, are introduced.

1.2 Membrane structure and function

Since it was first postulated by Singer and Nicolson (1972), the fluid mosaic model of membrane structure has provided both a feasible explanation of membrane organisation and a basis for understanding how this organisation is related to membrane function. In keeping with earlier models (Gorter and Grendel, 1925; Danielli and Davson, 1935), it was proposed that the bulk of the phospholipid is organised as a fluid bilayer with the hydrophilic portion of these molecules in contact with the aqueous phases on either side of the membrane. The non-polar fatty acyl chains are sequestered together away from contact with water, forming a hydrophobic region sandwiched between the two hydrophilic regions (Singer and Nicolson, 1972).

The alignment of cholesterol is similar, with the hydrophilic hydroxyl group in contact with the polar regions of the phospholipid and the non-polar sterol nucleus positioned within the hydrophobic core of the bilayer.

Proteins may be loosely associated with the surface of the bilayer by predominantly polar forces, or inserted into the lipid matrix, in which case there are interactions with both the polar and non-polar regions of the lipid. These amphipathic proteins may protrude from either the internal or external surface of the membrane or may pass through the entire bilayer with exposed hydrophilic regions projecting into the aqueous phases on both sides. Proteins display asymmetry between the internal and external faces of the bilayer. Every copy of a polypeptide

has the same orientation in the membrane. Thus, some proteins or parts of proteins are associated exclusively with the cytoplasmic surface of the membrane, while other proteins or parts of proteins are restricted to the external surface (Rothman and Lenard, 1977).

Phospholipids, which form the basic membrane structure, consist of a glycerol group esterified with fatty acids in both the 1 and 2 positions to form the hydrophobic portion of the molecule. The third position of the glycerol is joined by a phosphodiester linkage to one of a number of possible groups which form the polar region of the molecule. Different classes of phospholipid, determined by their polar head groups, have different functions in cell membranes. Some membrane-bound proteins have specific phospholipid requirements for complete activation (Sanderson, 1978). In mammals, a large proportion of phosphatidylinositol possesses an arachidonyl residue at position 2 and is involved in prostaglandin formation and transmembrane cation transport following stimulation of a variety of cells (Michell, 1975; Ross, 1981). Phospholipids, like proteins, are distributed asymmetrically between the two halves of the bilayer. In human erythrocytes phosphatidylserine and phosphatidylethanolamine are located predominantly in the inner half of the bilayer while phosphatidylcholine and sphingomyelin are found mainly in the outer half (Rothman and Lenard, 1977). The localisation of phosphatidylinositol is unclear but circumstantial evidence suggests that it is found mainly on the cytoplasmic face of the membrane (Michell, 1975). The reason

for the differences between the lipids in the two bilayers is not fully understood but one possible explanation is that a high proportion of anionically charged phospholipid on the cytoplasmic surface of the membrane may permit ionic interactions with membrane associated or peripheral membrane components (Nicolson, 1976). However, another possibility is that phosphatidylcholine and sphingomyelin, which have large head groups, may be excluded from the inner, concave membrane surface because their shape makes packing difficult. There is some evidence that curvature effects determines the asymmetric distribution of phospholipids in artificial vesicles (Bruni and Palatini, 1982).

Although the polar head groups are obviously of importance in determining phospholipid function, the work presented in this thesis, and therefore this discussion, will be more concerned with the properties of the fatty acyl residues. Of special interest are the metabolic changes which accompany the substitution of different fatty acids into membrane phospholipids.

In artificial membranes made from a single type of phospholipid, the molecules may exist in a close-packed "crystalline" state, or, at higher temperatures, in a less structured, more fluid "liquid-crystalline" state. Each type of phospholipid moves from one state to the other at a characteristic transition temperature which depends on the length and degree of saturation of the fatty acid chains, increasing as the chain either becomes longer or becomes more saturated. Chains with *trans*-unsaturated bonds cause phase transitions at a higher temperature than chains with *cis*-unsaturated

bonds in the equivalent position, although double bonds in naturally-occurring fatty acids tend to be in the *cis* orientation (Quinn, 1976). When artificial membranes, prepared from mixtures of two or more types of phospholipid, are exposed to temperatures in excess of the highest transition temperature of the constituent phospholipids, the molecules of the membrane are usually randomly distributed in the liquid-crystalline phase. On cooling the membrane, each phospholipid "freezes out" into exclusive crystalline domains as the temperature falls below its characteristic transition temperature while those phospholipids still above their own transition temperature remain randomly distributed in the liquid crystalline state.

When cholesterol is added to artificial membranes in increasing amounts, these phase transitions, measured by differential scanning calorimetry, become less marked, although still occurring at the same temperature. Eventually, when the molecular ratio of cholesterol:phospholipid reaches 1:1, no phase transitions are detectable (Ladbroke *et al.*, 1968). In plasma membranes of mammalian cells the normal ratio of cholesterol: phospholipid is approximately 1:1. However, in other membranes it is frequently much less and some phase transitions which take place at physiological temperatures may be of significance to the metabolism of the cell.

Addition of cholesterol causes liquid crystalline membranes to become more condensed. Interactions between cholesterol and the hydrocarbon chains of the phospholipid hinder the motional freedom of the chains and bring about a reduction in the cross-sectional area of the phospholipid. Below the transition

temperature, cholesterol prevents the ordering and crystallisation of phospholipids so that they remain in a more liquid state (Quinn, 1979). It is not clear whether cholesterol is distributed symmetrically between the two halves of the membrane. There is some evidence that there is more present in the external face than the internal face (Fisher, 1976). However, overall, phospholipid and cholesterol are present in plasma membranes in equimolar proportions and it is unlikely that either half of the bilayer would accommodate cholesterol in amounts greatly exceeding a 1:1 ratio with phospholipid (Demel and De Kruyff, 1976; Rothman and Lenard, 1977). Fluorescent probes inserted into membranes can be used to measure the mean microviscosity of artificial bilayers and cell membranes (Shinitzky and Barenholz, 1978). Increased microviscosity reflects an increase in lipid-lipid interaction and packing density. Thus, above the transition temperature, increasing the proportion of cholesterol in a membrane leads to an increase in microviscosity (Shinitzky and Inbar, 1976). Phospholipids with saturated fatty acid chains have a greater viscosity than those with unsaturated chains of the same length although this difference is less marked as the chain length is increased. The microviscosity of membranes also increases as the fatty acid chains of the phospholipid are lengthened (Lentz *et al.*, 1976).

While interactions between lipids can influence some of the physical characteristics of membranes, it is the range and specificity of proteins which gives rise to the enormous diversity of membrane properties. Once a protein has been synthesised and inserted into a membrane it will always remain

in the same orientation with respect to the membrane because of the difficulties in forcing the polar groups of an amphipathic protein through the hydrophobic region of the bilayer. Having proteins held in the correct alignment by the membrane not only ensures that, for example, transmembrane transport proteins and cell-surface receptors function correctly, but also assists in assembling and anchoring multi-protein complexes. Membranes can influence the properties of a protein in several ways. Membrane-bound subcellular organelles can provide specialised microenvironments for proteins such as lysosomal enzymes. The bilayer may provide a non-polar medium for the substrates or products of enzyme catalysed reactions. Alternatively, an enzyme may require specific membrane components as co-factors or substrate (Sanderson, 1978). However, even in situations where the bilayer appears to play a non-specific role, such as forming a matrix for membrane proteins, altering the lipid components can lead to significant changes in the properties of the protein. This may reflect changes in membrane fluidity or some related alterations in the spatial arrangement of the lipids producing an effect on the packing of membrane proteins. Thus, the degree of saturation of the fatty acids of membrane phospholipids can affect the lateral mobility and degree of exposure of membrane proteins and the activities of membrane-bound enzymes (Horowitz *et al.*, 1974; Solomonsen *et al.*, 1976; Shinitzky and Rivnay, 1977). The diversity and specificity of protein-protein interactions allow a wide range of precise modifications to membrane function to be achieved. Although integral membrane

proteins may move independently of each other by random diffusion in the plane of the membrane (Frye and Edinin, 1970), their movement is frequently affected by other proteins (Nicolson, 1976). These may also be embedded in the bilayer or they may be soluble and bind to the integral protein on either the cytoplasmic or the external surface of the membrane. One important example of this extramembrane influence is the cytoskeleton which is a complex system of actino-myosin microfilaments and tubulin microtubules. It is involved in processes which require the movement of sections of membrane in a coordinated manner, such as cell movement, mitosis, secretion, receptor movement and endocytosis (Nicolson, 1976).

One important group of membrane proteins are cell surface receptors, since, upon specifically binding extracellular ligand, they can undergo conformational changes which in turn initiate changes in the behaviour of the whole cell. The binding of ligands may bring about a regrouping of cell surface receptors and, in some cases, the receptors appear to get modified on the cytoplasmic surface of the plasma membrane, possibly by transglutaminase enzymes which cross-link glutamine residues on adjacent polypeptide chains, allowing the complex to become attached to the cytoskeletal apparatus (Pastan and Willingham, 1981). The binding of human chorionic gonadotropin to receptors on the external face of the plasma membrane of Leydig cells in the testis precipitates the release of cyclic AMP inside the cell. A conformational change in the receptor after binding its ligand seems to bring about an increase in the activity of an adjacent

molecule of adenyl cyclase. It is not clear whether the enzyme is permanently attached to the receptor or merely associates with it after the hormone has been bound (Catt *et al.*, 1979).

It can be seen that, far from being inert containers for cytoplasm, membranes play an active part in a variety of cell functions. Specific interactions between different membrane constituents lead to the formation of areas where some components are excluded while others are concentrated. This ensures that cell membranes are not homogeneous, but have a number of specialised regions, each responsible for its own aspect of cellular metabolism. Thus mammalian cells have not only a range of subcellular organelles, each with its own characteristic lipid and protein composition, but also areas of specialisation within a single organelle. This is most clearly demonstrated by the heterogeneity of the plasma membrane which has a number of highly differentiated regions (Quinn, 1976). Since fibroblasts, by no means the most metabolically active of cells, endocytose the equivalent of their own surface area every 50-100 minutes (Bretscher, 1981), replacement membrane must be produced at a rapid rate. This is mainly achieved by membrane recycling, and, to preserve the specialisation observed in membranes, this recycling must occur in a highly ordered manner. Membrane recycling is a vectoral process and as the membrane is moved from one organelle to the next its constituents are sorted so that those "resident" in the organelle remain behind while the "migrant" components are moved on to the next stage (Brown *et al.*, 1983*a*; Steinman *et al.*, 1983). The rapid turnover of membranes allows newly-synthesised

components to be inserted and old components to be removed so that the cell's response to its external environment may be constantly updated, for instance by increasing or decreasing the number of molecules of a specific receptor expressed on the cell surface. One of the regions where this sorting occurs is the Coated Pit which has recently excited much interest because of its role in receptor-mediated endocytosis. Coated pits are characterised by a large amount of a structural protein called Clathrin which is assembled into a mesh, around which the coated pit forms (Pearse, 1976). While receptors binding their ligands are concentrated in this region (Anderson *et al.*, 1977), other membrane components such as theta or H63 antigens are excluded (Bretscher, 1981). Neither could filipin-sterol complexes be detected in coated pits (Montesano *et al.*, 1979), suggesting that cholesterol is also excluded. Thus, membranes form a dynamic system in which highly specialised components are constantly being turned over, enabling the cell to respond rapidly to changes in its environment by altering the amounts of each component as required.

1.3 Lipoproteins and their metabolism *in vivo*

In order that lipids can be transported in plasma they are packaged, with protein, into water-soluble complexes called lipoproteins. These complexes consist of a core of non-polar lipid (cholesteryl esters and triglyceride) surrounded by a micellar shell of phospholipid, cholesterol and protein which is arranged like one face of a cell membrane. There are

several main classes of lipoprotein which differ in protein and lipid composition, in size and in density. It is these differences in hydrated density that are normally exploited to separate lipoproteins in bulk (Havel *et al.*, 1955; Chung *et al.*, 1980). Ultracentrifugation of plasma after its density has been adjusted to the appropriate value with potassium bromide results in lipoproteins floating to the surface and, by increasing the density of the plasma before each successive ultracentrifugation step, a series of lipoproteins of increasing density can be obtained. The major classes of lipoproteins found in human plasma are: chylomicrons and very-low-density lipoprotein (VLDL), both of which float at density 1.006 g/ml (the density of normal human plasma), intermediate-density lipoprotein (IDL, density 1.006-1.019 g/ml), low-density lipoprotein (LDL, density 1.019-1.063 g/ml) and high-density lipoprotein (HDL, density 1.063-1.21 g/ml). These are operational definitions of human lipoproteins and it cannot be assumed that lipoproteins from other species which have the same hydrated density necessarily have the same function.

There is continual exchange of material between different lipoproteins and between lipoproteins and cells. In addition lipoproteins may be modified through interactions with enzymes. Consequently lipoproteins do not have a fixed structure in which all the components are present in constant proportions and, frequently, the structure of a mature lipoprotein differs radically from that of the nascent particle from which it is derived (Soutar and Myant, 1979).

The original way of naming the proteins associated with lipoproteins has needed

modification as knowledge of the relationships between these particles has increased. Initially, when lipoproteins were divided only into HDL and LDL, the proteins associated with HDL were known as apoprotein A (HDL migrates with α globulins during agarose gel electrophoresis) and the proteins associated with LDL were known as apoprotein B (LDL migrates with β globulins). This system has had to be expanded as more apoproteins have been discovered. Five main classes, designated A to E, have now been identified. With subdivision of some classes, the human apoproteins now identified are: apoA-I, apoA-II, apoB100, apoB48, apoC-I, apoC-II, apoC-III, apoD and apoE. As might be expected, apoproteins play an important role in the recognition of lipoproteins by enzymes or cell surface receptors and thus determine the eventual fate of the particle (Soutar and Myant, 1979; Myant, 1983).

Dietary fat, taken up in the small intestine is secreted into the plasma as chylomicrons. These are large complexes, 10^3-10^4 Å in diameter, of which almost 90% of the dry weight is triglyceride. The liver secretes a similar particle, VLDL, into the plasma. VLDL particles are smaller than chylomicrons, with a diameter of 250-750 Å, but the major lipid (50-65% dry weight) is, again, triglyceride. Nascent chylomicrons and VLDL contain apoB, apoC-I, apoC-II and apoC-III with some apoE. VLDL secreted by the liver contains apoB100 while chylomicrons, secreted by the small intestine, contain apoB48. These apoproteins are so called because the molecular weight of apoB48 (approximately 264,000 Daltons) is 48% of the molecular

weight of apoB100 (approximately 549,000 Daltons) (Kane *et al.*, 1980). In the plasma, chylomicrons and VLDL, which are collectively known as the triglyceride-rich lipoproteins, first acquire more C apoproteins by non-enzymatic transfer from HDL. Lipoprotein lipase (E.C.3.1.1.34), an enzyme found on the lumenal surface of capillary endothelial cells, and activated by apoC-II, hydrolyses upto 90% of the triglyceride at the core of these lipoproteins to fatty acid, monoglyceride and some diglyceride. This leaves an excess of surface material, consisting of unesterified cholesterol, phospholipid and protein which is taken into HDL. The remnants of chylomicrons are metabolised rapidly and there is evidence in rats and dogs as well as some circumstantial evidence in humans that the removal of chylomicron remnants is mediated by an hepatic receptor for apoE (Soutar and Myant, 1979; Myant, 1981, 1982). The hydrolysis of the core triglyceride of VLDL leaves particles of IDL which are 250 Å in diameter and have greater proportions of cholesterol and protein than are found in VLDL. The main apoproteins are B and E, with some C-II and C-III.

Both VLDL and IDL may be degraded by the liver, but the majority is eventually converted to LDL by a process which is unclear. LDL is slightly smaller than IDL, with a diameter of 200-250 Å. The bulk of plasma cholesterol is found in the LDL fraction and 35-40% of the dry weight of the particle consists of cholesteryl esters and a further 7-10% consists of unesterified cholesterol. The vast majority if not all the protein associated with this lipoprotein is apoB100 (Soutar and Myant, 1979; Myant, 1982, 1983).

There is a strong positive correlation between the plasma concentration of LDL and risk of coronary heart disease (Stamler, 1973), which has led to considerable interest in the mechanisms by which this lipoprotein is removed from the plasma. In particular much attention has been focused upon the role of the LDL receptor, a cell surface protein discovered by Brown and Goldstein (1974). The LDL receptor, which will be discussed in more detail in Section 1.4 of this thesis, is specific for apoB and apoE and it is with LDL taken up by this receptor that most extrahepatic cells satisfy their cholesterol requirements (Anderson *et al.*, 1977; Goldstein and Brown, 1977, 1982; Mahley and Innerarity, 1983).

LDL receptors have been found in the livers of rats and rabbits (Kovanen *et al.*, 1979; Slater *et al.*, 1980) and more recently human liver membranes have been shown to bind the lipoprotein (Harders-Spengel *et al.*, 1982). In the liver, cholesterol may be converted to bile acids which are secreted into the intestine to facilitate the absorption of dietary fat. Only a proportion of the bile acids is subsequently reabsorbed; the rest is excreted from the body in the faeces and, since cholesterol cannot be broken down, it is by faecal excretion that most steroids are removed from the body. Although the liver clearly makes an important contribution to clearing LDL from the plasma, there is some disagreement over the exact proportion of LDL catabolised by this organ, ranging from 39% to 67% (Pittman *et al.*, 1979; Koelz *et al.*, 1982; Pittman *et al.*, 1982).

In addition to the LDL receptor, two alternative pathways exist for removing LDL from plasma. The first is via another receptor expressed by macrophage cells which recognises LDL which has become more electronegative than normal. The second process takes place independently of receptors and is probably receptor-independent adsorptive endocytosis (Miller *et al.*, 1977). The macrophage receptor, which does not recognise normal LDL, is known as the acetyl-LDL receptor since it was first shown to bind LDL which had been made strongly anionic by acetylation of the lysine residues of its apoB (Fraenkel-Conrat, 1975; Brown and Goldstein, 1983*b*). Since this modification to LDL is unlikely to occur *in vivo*, a physiological counterpart to acetylation has been sought. LDL incubated in the presence of cultured endothelial cells can be converted into a denser, more electronegative form which is recognised by the acetyl-LDL receptor (Hendrikson *et al.*, 1981). These changes seem to involve lipid peroxidation and degradation of LDL phospholipids (Steinbrecher *et al.*, 1984).

The receptor-independent pathway seems to be very important in removing LDL from the plasma of normal human adults. Estimates of the contribution of this pathway have been made by coupling cyclohexanedione (CHD) to the arginine residues (Mahley *et al.*, 1977, 1980) or 2-hydroxyacetaldehyde (HOET) to the lysine residues (Slater *et al.*, 1984) of apoB so that the treated LDL is no longer recognised by the LDL receptor. Patients with the homozygous form of familial hypercholesterolaemia (FH) lack functional receptors for LDL and consequently catabolise all their LDL independently of the apoB receptor pathway. In those

subjects LDL and CHD-LDL are catabolised at the same rate, showing that although CHD blocks the ability of LDL to bind to apoB receptors, it does not affect the metabolism of the lipoprotein in any other way. Comparison of the rates at which normal LDL and CHD-LDL are cleared from the plasma suggests that 60-70% of LDL metabolism may occur through the receptor-independent pathway (Shepherd *et al.*, 1979). However, more recently, comparing normal LDL and HOET-LDL, Slater *et al.* (1984) suggest that only 50% of LDL catabolism may occur through the receptor-independent pathway.

In contrast to LDL, which seems to be primarily concerned with supplying cholesterol to peripheral tissues, HDL seems to be involved in removing excess cholesterol from the extrahepatic tissues and returning it to the liver for removal from the body. HDL, in fact, contains several subfractions. Nascent HDL is secreted by the liver as bilayer discs 45 Å in thickness and 190 Å in diameter. It is composed of phospholipid, unesterified cholesterol, some apoA-I and apoA-II, although the main protein component is apoE. Nascent HDL is rapidly metabolised to mature HDL through the action of lecithin:cholesteryl acyltransferase (LCAT, E.C. 2.3.1.43). This plasma enzyme esterifies the free cholesterol of the nascent particle and the discs are converted to spheres as the cholesteryl esters form an apolar core to the particle. Consequently the surface layer of the HDL complex has a high phospholipid:cholesterol ratio which may be responsible for promoting the net transfer of cholesterol to the HDL surface from the plasma membranes of cells or other lipoproteins with

which the HDL has come into contact. This cholesterol is, in turn, esterified by LCAT (Glomset, 1968), helping to preserve the high phospholipid:cholesterol ratio at the particle surface. As HDL matures it also acquires triglyceride, apoC, additional apoA-I but sheds apoE. There are two types of mature HDL (HDL_2 and HDL_3) which seem to represent two stages in the maturation process, with the smaller HDL_3 particle (50-100 Å diameter) being converted to the larger HDL_2 particle (70-120 Å diameter) which is relatively richer in lipids (Soutar and Myant, 1979; Myant, 1981, 1982, 1983). The ease with which apoproteins are exchanged between triglyceride-rich lipoproteins and HDL has led to the suggestion that another function of HDL is to act as a reservoir for apoproteins, preserving them from hydrolysis when the rest of the lipoprotein complex is catabolised and supplying apoproteins, as required, to newly-synthesised particles (Myant, 1981).

Lipids may also be transferred between lipoproteins. When human plasma is incubated *in vitro* HDL cholesteryl esters may be transferred to VLDL and replaced by an equimolar transfer of triglycerides from VLDL to HDL. This exchange seems to be promoted by a transfer protein found in human plasma, possibly apoD, which, presumably, facilitates the passage of non-polar lipid across the polar shell of the lipoprotein particle (Chajek and Fielding, 1978).

There is some evidence, in rats, that the liver is the major site of HDL catabolism (Rachmilewitz *et al.*, 1972), although the eventual fate of HDL particles in humans has yet to be conclusively demonstrated.

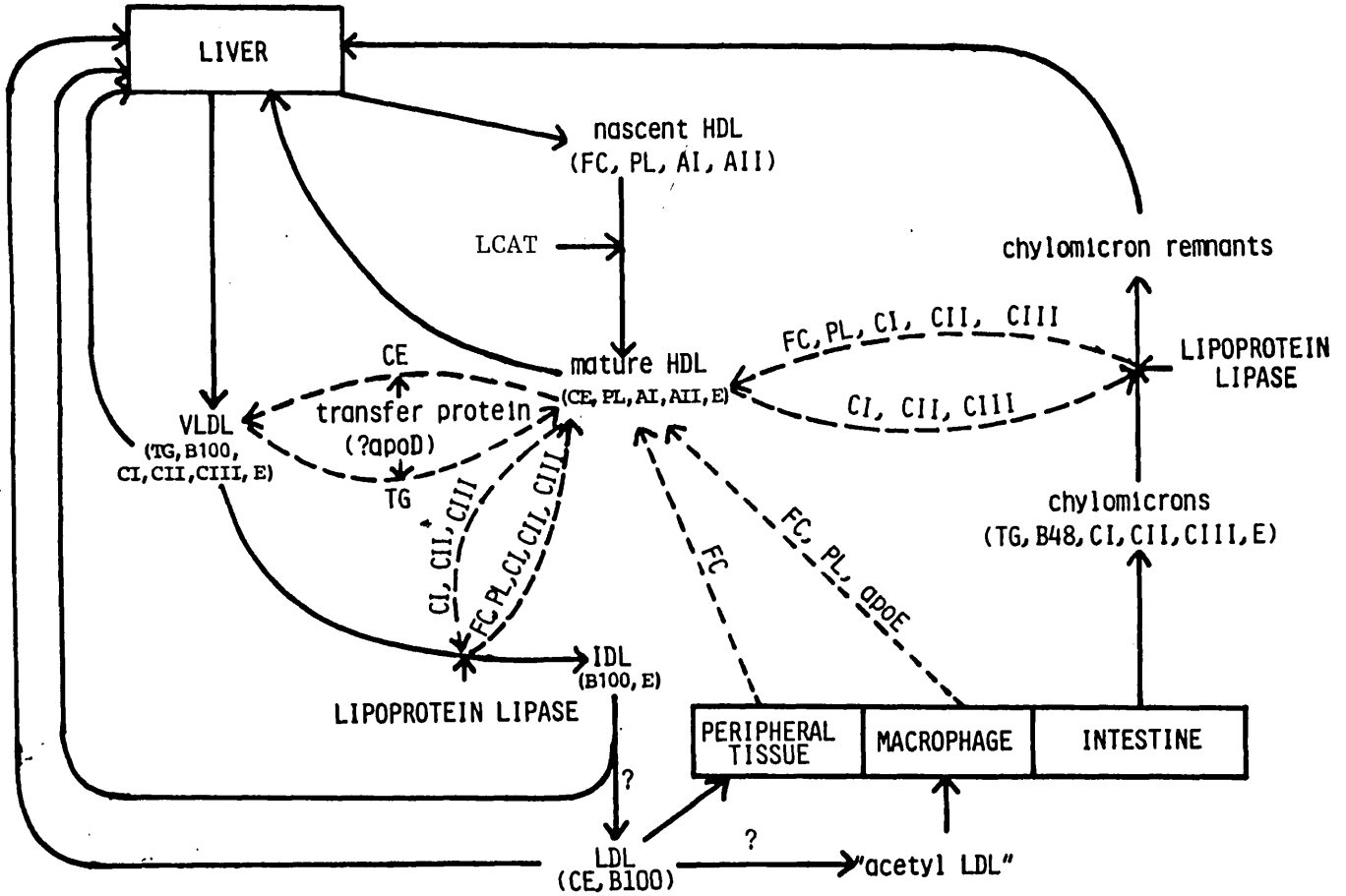


Figure 1.1 Lipoprotein metabolism *in vivo*

A flow chart showing the interactions between the various lipoproteins and the exchange of their lipid and protein components which takes place *in vivo*.
CE, cholesterol ester; FC, free cholesterol (unesterified); PL, phospholipid; TG, triglyceride; AI, AII, B48, B100, CI, CIII, D, E, apoproteins; LCAT, lecithin:cholesterol acyltransferase.
"Acetyl LDL" refers to the putative particle derived from LDL which, if it exists, is degraded via the macrophage acetyl LDL receptor.

Thus it can be seen that individual lipoproteins do not exist as isolated entities but are interrelated not only by the conversion of one particle type to another but also by the continuous exchange of components between these particle types (Fig. 1.1). However, most lipoproteins do exist as particles with clearly defined properties rather than as a spectrum of every possible particle between two extremes, and it seems probable that lipoproteins represent the most stable energy states for given combinations of lipid and protein.

One of the most serious consequences of a breakdown in lipoprotein metabolism is atherosclerosis. Although there are several risk factors which increase the probability of an individual developing this disease, atherosclerosis rarely ensues unless plasma cholesterol concentrations are above 160 mg/dl.

It has been proposed by Ross (1981) that a series of events, eventually leading to atherosclerosis, is initiated by damage to and removal of arterial endothelial cells allowing blood platelets to attach to the artery wall at the damaged sites. Growth factors released by these platelets promote the migration of smooth-muscle cells and macrophages into the intimal layer of the artery. The smooth-muscle cells may proliferate and both smooth-muscle cells and macrophages accumulate large quantities of cholesteryl esters and are converted into the foam cells characteristic of atherosclerotic lesions. As the lesion progressively expands into the lumen of the artery, the restricted blood flow in the coronary artery may lead to myocardial infarction (Ross, 1981).

The accumulation of cholesteryl esters in macrophages and the conversion of these cells to foam cells has led to considerable interest in lipoprotein metabolism by macrophages (Brown and Goldstein, 1983*b*). *In vitro* macrophages take up lipoproteins by several specific receptors. In the lysosomes hydrolysis of esterified cholesterol releases free cholesterol which may be used to meet the cells' cholesterol requirements. Excess cholesterol is re-esterified by ACAT for storage as cholesteryl ester droplets in the cytoplasm. Foam cells owe their morphology to this bulk storage of lipid. Some cholesteryl ester enters a futile cycle in which it is hydrolysed by cholesteryl esterase, an extra-lysosomal enzyme, and the free cholesterol is re-esterified by ACAT (Brown *et al.*, 1979, 1980*a*; McGookey and Anderson, 1983). Atherosclerotic lesions may regress if the store of cholesteryl esters can be removed from the cell and, *in vitro*, free cholesterol may be secreted by the cell in the presence of a suitable acceptor such as HDL (Brown and Goldstein, 1983*b*) or phospholipid dispersions (Yau-Young *et al.*, 1982; Glick *et al.*, 1983).

Overloading of macrophages with cholesterol stimulates the synthesis and secretion of disc-like complexes similar to nascent HDL, but containing only phospholipid and apoE. These complexes lack cholesterol, either free or esterified, and are not directly responsible for removing cholesterol from the cell. However, they might transfer apoE to HDL which has taken up cholesterol from the cell, forming a new particle which can be rapidly removed from the circulating by the liver, enabling

surplus cholesterol to be removed from the body (Brown and Goldstein, 1983*b*). This hypothesis is supported by the observation that cholesterol feeding in dogs, rabbits and swine leads to the production of HDL_C, a lipoprotein rich in apoE and cholesteryl esters (Mahley, 1979). However, the origin of this particle has yet to be demonstrated.

Understanding of the process by which atherosclerosis occurs requires further research into both the mechanisms by which cholesteryl esters accumulate in foam cells and the mechanisms by which they are removed. Decreasing the plasma cholesterol concentration results in a lower risk of coronary heart disease, especially in the 10% of people whose plasma cholesterol concentrations are the highest in the population and who are therefore most at risk (Concensus Conference, 1985). Such a decrease can be achieved, at least in part, by diets rich in polyunsaturated fatty acids (Kinsell *et al.*, 1952; Keys *et al.*, 1965).

Several possible mechanisms exist to explain this effect on plasma cholesterol concentrations:

(a) decreased hepatic cholesterol synthesis;

(b) decreased absorption of cholesterol in the intestine;

(c) increased excretion of bile steroids in the faeces; or,

(d) a shift of cholesterol distribution between the plasma and the tissues.

Hepatic cholesterol synthesis appears to be unaltered by the degree of saturation of the fat in the diet (Grundy and Ahrens, 1970), although LDL production as measured by rate of LDL apoB synthesis is decreased (Cortese *et al.*, 1983). Evidence of any

effect of polyunsaturated fatty acid on the absorption of cholesterol in the intestine is contradictory (Paul *et al.*, 1980). However, polyunsaturated fatty acid certainly increases the rate of excretion of bile steroids (Grundy, 1975; Paul *et al.*, 1980; Myant, 1981) and shifts the distribution of cholesterol between the plasma and tissue compartments (Spritz and Mishkel, 1969; Grundy and Ahrens, 1970), possibly by altering the rate of uptake and degradation of lipoproteins by cells. An increase in lipoprotein catabolism by the liver may in turn be responsible for the increased excretion of bile steroids leading to a net loss of cholesterol from the body. Uptake of lipoproteins by tissues may be enhanced either by changes in the properties of the lipoproteins, affecting their interactions with enzymes or receptors, or by changes in the cells affecting their ability to bind, internalise and degrade the lipoprotein particle.

The effects of diets containing a high proportion of polyunsaturated fatty acid are most noticeable on LDL and VLDL, the concentrations of both being reduced, with linoleate replacing palmitate, stearate and oleate in the lipoprotein triglyceride, cholesteryl ester and phospholipid (Spritz and Mishkel, 1969; Shepherd *et al.*, 1980). There are conflicting explanations for this observation. Shepherd *et al.* (1980) found that the fractional catabolic rate of LDL (the proportion of the total intravascular LDL-apoB pool catabolised per day) is increased without any alteration in the synthetic rate. However, Cortese *et al.* (1983) have found that subjects on diets rich in

polyunsaturated fatty acid have lower rates of synthesis of VLDL-apoB and LDL-apoB than subjects on a diet rich in saturated fatty acids. In this study there was no change in the fractional catabolic rates of either VLDL or LDL.

Although there are reports that plasma HDL was unaltered by diets rich in polyunsaturated fatty acids (Spritz and Mishkel, 1969), other workers have found that these diets lowered the plasma HDL concentration (Schaeffer *et al.*, 1981). Any change seems to be caused by a decreased rate of synthesis rather than any effect on the catabolic rate (Shepherd *et al.*, 1978). Despite these *in vivo* observations, the mechanisms responsible for the hypocholesterolaemic effect of polyunsaturated fatty acids are barely understood at a cellular level.

1.4 LDL metabolism *in vitro*

Normal cultured fibroblasts can satisfy their cholesterol requirements by the uptake of serum LDL from the growth medium. The early research of Goldstein and Brown (1977) has shown that LDL is bound to the cell surface by a specific, high-affinity receptor, rapidly taken into the cell and degraded in the lysosomes. The apoB component of the LDL is broken down into its constituent amino acids and the cholesteryl esters are hydrolysed to release free cholesterol. The receptor does not undergo lysosomal degradation and is returned to the cell surface. The binding of LDL to the receptor requires a divalent cation such as Ca^{++} and is inhibited by excess EDTA (Goldstein *et al.*, 1983*b*).

Cells react to an increased requirement for cholesterol by increasing both the number of receptors expressed on their surface and the rate at which cholesterol is synthesised *de novo*. The rate of cholesterol synthesis is raised by increasing the activity of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA reductase, E.C.1.1.1.34), the enzyme chiefly responsible for controlling the rate of cholesterol biosynthesis.

Faced with the opposite situation, cells containing excessively high amounts of cholesterol respond by lowering the number of receptors expressed at their surface, decreasing the activity of HMG-CoA reductase, and esterifying the excess cholesterol with fatty acids through the action of ACAT. Since cholesteryl esters are completely non-polar, and thus cannot be incorporated into membranes, they form lipid droplets which are a convenient, biologically inactive store for cholesterol.

Much of our understanding of the way in which LDL is processed by cells comes from studies carried out on fibroblasts from patients with FH, an inborn error of metabolism in which cells are unable to produce functional receptors for LDL. The disease is inherited as an autosomal, dominant trait in which the effects are more pronounced in the homozygote than in the heterozygote. The clinical manifestations of the disease are an increased plasma LDL concentration, xanthomas of the skin and tendons, and atheromas leading to premature heart disease. Death by myocardial infarction before the age of 20 years is common in homozygous patients. The average plasma LDL concentration in normal subjects under 20 years of age is

approximately 110 mg cholesterol/dl. In heterozygous FH subjects in the same age group, this figure rises to 240 mg cholesterol/dl and in homozygotes it is over 620 mg cholesterol/dl (Kwiterovich *et al.*, 1974; Goldstein and Brown, 1983*a*; Myant, 1983). The plasma concentrations of other lipoproteins also differ from normal, the total amount of HDL being decreased, while the IDL concentration is increased. Although there are no detectable differences between the apoB of normal and FH subjects, FH LDL contains more cholesterol (both free and esterified), less triglyceride and there are changes in the proportions of the different phospholipid classes present (Myant, 1983).

There are several abnormal alleles at the gene locus coding for the LDL receptor and these may be divided into three classes on the basis of their gene product. These are (i) receptor-negative, where there is no detectable binding of LDL; (ii) receptor-defective, where LDL binding activity is detectable but considerably less than normal; and (iii) internalisation-defective, where LDL is bound normally by receptors which are unable to transport the bound lipoprotein into the cell to catabolise it (Goldstein and Brown, 1983*a*). More is now understood at a molecular level about how these mutant alleles lead to mistakes in translation and post-translational processing which in turn causes a lack of functional LDL receptors (Tolleshaug *et al.*, 1983).

Cultured fibroblasts of homozygous FH subjects obtain little or no cholesterol from extracellular LDL since they are unable to express the normal number of functional binding sites whatever the concentration of the lipoprotein in the medium. Hence most of the cholesterol requirement of these cells has to be met by endogenous synthesis. Fibroblasts from heterozygous FH patients express approximately half the number of receptors of normal cells under comparable culture conditions (Goldstein and Brown, 1977). More recently the clathrin-rich, coated pits have been implicated in the internalisation of a range of cell-surface receptors and their ligands (Goldstein *et al.*, 1979; Willingham *et al.*, 1981). It has been known for 20 years that coated pits were involved in the uptake of extracellular particles (Roth and Porter, 1964) although it is only recently that their importance in receptor-mediated endocytosis was first demonstrated (Goldstein *et al.*, 1979). Fibroblasts from one particular homozygous, internalisation-defective FH patient could bind normal amounts of LDL at their surface but were unable to take it into the cell to degrade it. The LDL receptors in these cells remained randomly distributed about the surface while, in cells from normal subjects, the coated pits, which represent only 2% of the total plasma membrane surface area contain 50-80% of the LDL receptors.

Coated pits have subsequently been shown in several cell types mediating in the internalisation of a variety of receptors ranging from hormones and growth factors to lysosomal enzymes (Gorden *et al.*, 1978; Maxfield *et al.*, 1978; Gonzalez-Nonega, 1980). The Semliki Forest Virus also gains entry into cells

via the coated pit pathway (Helenius *et al.*, 1980). An unusual feature of the LDL receptor is that it seems to migrate into the coated pits even in the absence of LDL, whereas receptors for other ligands remain randomly distributed over the entire cell surface, only congregating in coated pits when their ligand is bound. In these cases it would appear that ligand binding produces a conformational change in the receptor which allows it to be translocated into the coated pit. There are some indications that cross-linking of the receptors may be involved, in a process similar to patching and capping of antibodies on lymphocytes (Catt *et al.*, 1979; Pastan and Willingham, 1981). After being concentrated in coated pits, the receptors are internalised in coated vesicles formed by invaginations of the coated pits. Internalisation of receptors for several different ligands can occur simultaneously in a single coated pit. The internalisation of receptors for one ligand does not influence the internalisation of receptors for any other ligand (Pastin and Willingham, 1981; Willingham *et al.*, 1981; Ciechanover *et al.*, 1983). The coated vesicles rapidly lose their clathrin to become receptosomes, the clathrin probably being returned to the surface to become reincorporated into coated pits (Goldstein *et al.*, 1979). The pH inside the receptosome decreases, leading to the dissociation of LDL from its receptor, allowing the receptor to be returned to the cell surface (Brown *et al.*, 1983*a*). A substantial proportion, perhaps as much as half of the LDL taken up by cells, may be released back, unaffected, into the medium

by a process termed retro-endocytosis (Aulinskas *et al.*, 1981). The rest of the LDL is retained in vesicles which fuse with lysosomes, resulting in hydrolysis of the lipoprotein: the apoprotein is degraded into its constituent amino acids and the cholesteryl esters are hydrolysed to release unesterified cholesterol (Goldstein and Brown, 1977).

The exact fate of receptors and their ligands varies. Lysosomal enzymes, taken up in an identical way to LDL, enter the lysosomes but are obviously not degraded, while insulin enters the lysosome still bound to its receptor which is degraded along with the hormone (Goldstein *et al.*, 1979). An interesting adaptation of this pathway is shown by transferrin. When complexed with iron this protein binds to the transferrin receptor and is internalised. As the pH decreases, the iron dissociates from the protein and is retained by the cell, while the transferrin, still bound to its receptor, is returned to the cell surface. At the cell surface the return to normal pH causes the transferrin (no longer complexed with iron) to dissociate from its receptor (Brown *et al.*, 1983*a*).

Once LDL has been internalised and degraded, the fibroblasts use the unesterified cholesterol released by the process in the synthesis of new membranes. An increase in the amount of unesterified cholesterol in the cell brings about a decrease in both the number of LDL receptors expressed at the cell surface and the rate of cholesterol synthesis by the cell. Cholesterol in excess of the fibroblast's requirements is re-esterified with fatty acids, usually palmitic or oleic acid, by ACAT (Goldstein and Brown, 1977).

A number of factors besides an exogenous source of cholesterol can influence the number of LDL receptors expressed at the cell surface. Certain oxygenated sterols bring about a decrease in receptor number while a variety of hormones such as insulin and triiodothyronine, in fibroblasts and plasma-derived growth factor in cultured smooth-muscle cells, cause an increase (Chait *et al.*, 1978, 1979, 1980; Myant, 1981). Binding of LDL is also affected by the degree of confluency, and thus the rate of mitosis, of cultured fibroblasts and by cholesterol efflux from the cells (Kruth *et al.*, 1979; Oram *et al.*, 1981).

Control over the expression of the LDL receptor ensures that, on the one hand, the cholesterol requirements of the cell can be met, while, on the other hand, the amount of the sterol in the cell is not allowed to rise to levels where membrane functions become affected. The vectoral cycle of LDL receptors from the cell surface, through the coated pits, coated vesicles and receptosomes back to the cell surface, offers several potential sites for controlling receptor expression, and much recent research has concentrated on how this control is effected. Using the techniques of molecular biology, the structure and control of the LDL receptor have been studied in great detail, most notably by Brown, Goldstein and their coworkers. Their use of monoclonal antibodies to the receptor (Beisiegel *et al.*, 1981) has allowed the biosynthesis to be examined. The receptor is synthesised as a polypeptide precursor with an apparent molecular weight of 120,000 Daltons on SDS polyacrylamide gel electrophoresis. This is processed in the Golgi apparatus to

a molecule which has an apparent molecular weight of 160,000 Daltons (Tolleshaug *et al.*, 1982). Although there is a slight increase in weight due to the addition of polysaccharide chains by *N*- and *O*-glycosylation (Cummings *et al.*, 1983) most of this change seems to be artefactual, possibly resulting from a conformational change which alters the migration of the receptor in the gel (Goldstein and Brown, 1984). The radiation inactivation technique gives an estimate of approximately 110,000 Daltons for the receptor's molecular weight (Innerarity *et al.*, 1981). In contrast to the orientation of other receptors that internalise their ligands via coated pits, the LDL receptor has its NH_2-terminus on the external surface of the plasma membrane (Schneider *et al.*, 1983). The nucleotide sequence of a 2.8 Kb partial *c*DNA coding for the COOH-terminal 25% of bovine LDL receptor has been determined. This predicts a 50 amino-acid cytoplasmic domain at the COOH-terminus, followed by a membrane-spanning region of 27 hydrophobic amino acids and an externally situated, 42 amino-acid domain, rich in serine and threonine residues which appears to be the site of *O*-linked glycosylation (Russell *et al.*, 1984). This *c*DNA hybridises to an *m*RNA of 5.5 Kb which is about nine times more abundant in the adrenal gland than in the liver and is suppressed in cultured cells grown in the presence of cholesterol (Russell *et al.*, 1983).

Similar techniques have led to an understanding of the control over endogenous cholesterol synthesis. This occurs mainly by the regulation of HMG-CoA reductase, an enzyme found in the smooth endoplasmic reticular fraction of the microsomal

pellet and which catalyses the conversion of HMG-CoA to mevalonic acid (Durr and Rudney, 1960; Mitropoulos *et al.*, 1978). Mevalonic acid, besides being a key intermediate in cholesterol synthesis, is a precursor of several other compounds including one, possibly isopentenyl adenine, which is required for DNA synthesis and thus cell division (Brown and Goldstein, 1980; Quesney-Huneeus *et al.*, 1983). HMG-CoA reductase is under the coordinate control of a sterol, possibly cholesterol, and at least one other product of mevalonic acid metabolism (Brown and Goldstein, 1980*b*). There is some evidence that LDL cholesterol exerts its effect by controlling the production of *m*RNA coding for the enzyme (Chin *et al.*, 1982). However, HMG-CoA reductase can exist in either an inactive (phosphorylated) or an active (dephosphorylated) form and the rapid changes in enzyme activity initiated by hormones may be brought about by an interconversion of these two forms (Gibson and Ingebritsen, 1978). There is some evidence that exceptionally large amounts of cholesterol in membranes can promote the breakdown of the smooth endoplasmic reticulum containing HMG-CoA reductase (Orci *et al.*, 1984; Goldstein and Brown, 1984). The cholesterol biosynthetic pathway can also be controlled at a later stage, where the enzyme squalene synthetase catalyses the "head-to-head" condensation of two molecules of farnesyl pyrophosphate to form squalene. Farnesyl pyrophosphate is the last compound common to all three of the cholesterol, dolichol and ubiquinone synthetic pathways. Although it is known that LDL uptake by fibroblasts can bring about a 90% reduction in the activity of squalene synthetase,

little is known about the mechanism by which this reduction is achieved (Brown and Goldstein, 1980*b*).

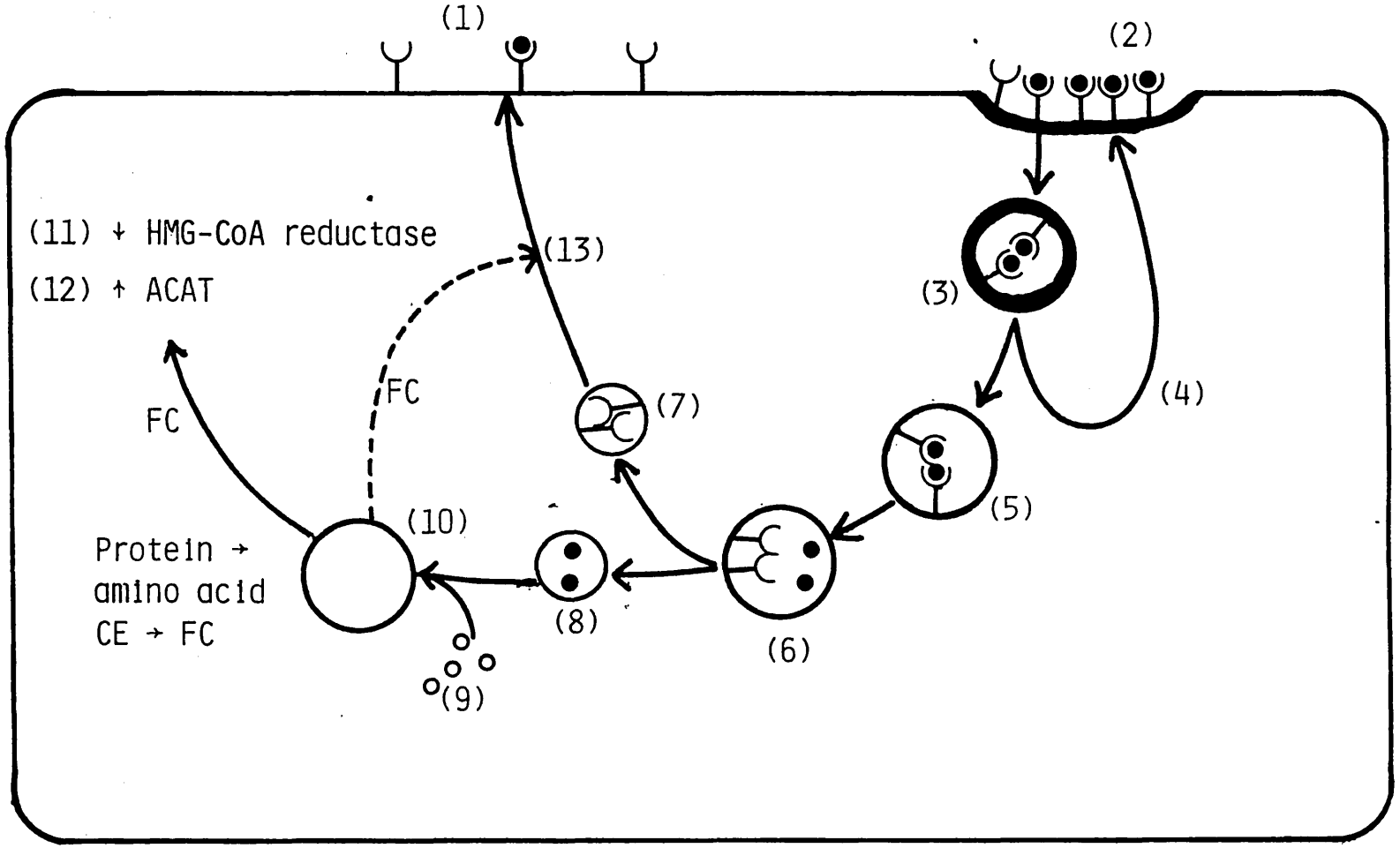
ACAT, the enzyme catalysing the esterification of cholesterol with fatty acids, is a membrane-bound enzyme found in the rough endoplasmic reticular fraction of the microsomal pellet (Balasubramaniam *et al.*, 1978). While the bulk of cholesteryl esters in lipoproteins consist of cholesteryl linoleate, in cells the cholesterol released by LDL degradation is re-esterified with more saturated fatty acids such as palmitate and oleate (Goldstein and Brown, 1977). The early work on cholesteryl ester formation in fibroblasts, when either changes in the mass or the net incorporation of radioactive oleate into cholesteryl ester were measured, indicated that as the amount of cholesterol in the cell increased as a result of LDL degradation, the rate of its esterification also increased (Goldstein *et al.*, 1974*a*; Brown *et al.*, 1975*a*). More recently, as its importance in the formation of foam cells found in atherosclerotic lesions was realised, ACAT activity has been measured directly in subcellular microsomal preparations (Bretscher and Chobaniam, 1974; Hashimoto *et al.*, 1974; Bretscher and Chan, 1980; Severson and Fletcher, 1981). The observed activity of ACAT can be altered by a range of factors. The addition of cholesterol to the culture medium, either dissolved in ethanol or in cholesterol-phospholipid dispersions, brings about an increase in ACAT activity (Argoblast, 1976; Goldstein and Brown, 1977). The addition of mevalonic acid to the culture medium also produces an increase in enzyme

activity, presumably by increasing the amount of cholesterol in the cell (Drevon *et al.*, 1980). However, oxygenated sterols such as 7-ketocholesterol and 25-hydroxycholesterol bring about increased ACAT activity even in circumstances where cellular cholesterol becomes depleted (Brown *et al.*, 1975*b*). Since the stimulatory effects of mevalonic acid and 25-hydroxycholesterol are additive it appears that two separate mechanisms are involved in these controls over ACAT activity (Drevon *et al.*, 1980). Other steroids, such as progesterone, bring about a decrease in ACAT activity (Goldstein *et al.*, 1978). Progesterone, 7-ketocholesterol and 25-hydroxycholesterol all produce their effects on ACAT activity whether they are added to the culture medium in which the cells are grown, or directly to the microsomes on which the ACAT activity is measured (Goldstein *et al.*, 1978; Drevon *et al.*, 1980). ACAT activity in rat-liver microsomes also seems to be enhanced by the presence of a lipid carrier protein (Gavey *et al.*, 1981, 1982; Poorthuis and Wirtz, 1982). Attempts to raise the cholesterol content of microsomal membranes are not always successful. However, when the amount of cholesterol has been increased, for instance by transfer during incubation of the microsomes with cholesterol-rich donor particles, ACAT activity also rises (Hashimoto and Dayton, 1979). Reconstitution of ACAT enzyme into artificial membranes also indicates that the enzyme's activity increases as the amount of cholesterol in the membrane increases (Doolittle and Chang, 1982*a*; Suckling *et al.*, 1982). ACAT activity is also affected by the phospholipid head groups of the membranes (Doolittle and Chang, 1982*b*). Phosphorylation

Figure 1.2 LDL catabolism in cultured fibroblasts

Schematic representation of LDL catabolism by cultured fibroblasts.

(1) Receptors, randomly inserted into the membrane, some binding LDL, migrate to the coated pit (2), where the membrane becomes invaginated, forming a coated vesicle (3). The clathrin returns to the cell surface where it is reincorporated into the coated pit (4). The pH of the vesicles containing the receptors and LDL (5) decreases causing the ligand to dissociate from the receptor (6). The receptors are returned to the surface (7) while the vesicles containing LDL (8) fuse with lysosomes (9) leading to the degradation of the lipoprotein particle (10). The cholesteryl esters (CE) are hydrolysed to unesterified (free) cholesterol (FC) which suppresses HMG-CoA reductase activity (11), increases ACAT activity (12) and decreases the number of LDL receptors expressed at the cell surface (13).


(1)
(2)
(11) ↓ HMG-CoA reductase
(12) ↑ ACAT
(13)
(3)
FC
FC
(7)
(4)
(5)
(10)
Protein →
amino acid
CE → FC
(8)
(6)
(9)

and dephosphorylation can regulate the ACAT activity of microsomes in a reciprocal fashion to HMG-CoA reductase, *i.e.* ACAT becomes activated by phosphorylation and inhibited by dephosphorylation (Suckling *et al.*, 1983*a*,*b*). Microsomes prepared from a range of tissues from animals fed on diets rich in polyunsaturated fatty acid have higher ACAT activity than those from animals fed on diets supplemented with saturated fatty acid (Mitropoulos *et al.*, 1980; Spector *et al.*, 1980; Field and Salome, 1982). The coordinated fashion in which ACAT, HMG-CoA reductase and LDL receptor expression are regulated allows the cell to react flexibly to changes in its environment. Overall they represent an efficient method of stabilising the level of unesterified cholesterol inside the cell within certain limits, thereby preserving the integrity of the cell membranes (Fig. 1.2). However, the price to be paid for this system can be the accumulation of cholesteryl esters in the foam cells of atherosclerotic lesions, and understanding the processes by which these lesions form and regress represents one of the main problems now facing medical science.

1.5 Metabolism of fatty acids and phospholipids

A discussion of how LDL catabolism is affected by fatty acids, or, more specifically, by fatty acids incorporated into phospholipids, must include a brief review of fatty acid and phospholipid metabolism in cells. Much of the early work in this field was carried out by Kennedy and coworkers and has been reviewed by him (Kennedy, 1962). Phospholipids are important

constituents of membranes and can be synthesised by most cells from more basic components. As mentioned before, phospholipid consists of glycerol esterified with fatty acids in the 1 and 2 positions and joined to a polar group by a phosphodiester linkage in the 3 position. Under normal circumstances the fatty acid in the 1 position is saturated while that in the 2 position is unsaturated. There is some variety in the polar groups with the most common being choline, ethanolamine, serine or inositol. The glycerol moiety of the phospholipid is derived from glycerol-3-phosphate. This may be synthesised either from dihydroxyacetone phosphate (an intermediate in glycolysis) through the action of glycerol-2-phosphate dehydrogenase (E.C. 1.1.95.5), or through the phosphorylation of glycerol by glycerol kinase (E.C. 2.7.1.30). Glycerol-3-phosphate is then acylated with two molecules of fatty acyl coenzyme A to yield 1,2-diacylglycerophosphate (phosphatidic acid). The acylation reactions occur preferentially with 16- or 18-carbon saturated or unsaturated fatty acyl coenzyme A. Phosphatidic acid is the simplest form of phospholipid and, although it is present in cells only in small amounts, it is a key intermediate in the synthesis of both more complex phospholipids and triglycerides. In the formation of either class of lipid the next stage is usually the removal of the phosphate group by the enzyme phosphatidate phosphatase (E.C. 3.1.3.4), giving a diacyl glycerol molecule (Kennedy, 1962).

Before the polar head groups can be added to the diacylglycerol to form a phospholipid they must be activated by coupling to cytidine nucleotide carriers. Ethanolamine is phosphorylated to form

phosphoethanolamine which, in turn, reacts with cytidine triphosphate to give cytodine diphosphoethanolamine. It is this activated form of phosphoethanolamine which reacts with diacylglycerol to yield phosphatidylethanolamine. The enzymes catalysing these reactions, ethanolamine kinase (E.C. 2.7.1.82), ethanolamine phosphate cytidylyltransferase (E.C. 2.7.7.14) and ethanolamine phosphotransferase (E.C. 2.7.8.1) are all tightly bound to the smooth endoplasmic reticulum (Kennedy, 1962; Crane *et al.*, 1982). Choline may be incorporated into phospholipids by an analogous series of reactions, with phosphocholine and cytidine diphosphocholine as intermediates. However, phosphatidylcholine may also be formed by the three-fold methylation of the amino group of phosphatidyl-ethanolamine. The reactions are catalysed by phosphatidyl-ethanolamine methyltransferase (E.C. 2.1.1.17) and each methyl group is donated by a molecule of 5-adenosyl methionine. Phosphatidyl serine is formed from phosphatidylethanolamine by the substitution of serine for ethanolamine. The serine of phosphatidyl serine may undergo decarboxylation to give rise to phosphatidyl ethanolamine. In the formation of phosphatidyl inositol it is the diacyl glycerol which is activated in the form of cytidine diphospho diacyl glycerol. The reaction with inositol produces phosphatidyl inositol and cytidine monophosphate (Kennedy, 1962).

Even after the synthesis of the complete phospholipid, alternative fatty acids may be substituted into the molecule. Firstly, the fatty acyl group in the 2 position is cleaved by

phosphatidase A (E.C. 3.1.1.4), leaving 1-acyl lysophosphatide. This is re-esterified in a reaction with a replacement fatty acyl coenzyme A to form a new phospholipid molecule (Lands *et al.*, 1982). 1,2-Diacylglycerophosphate may be produced by the esterification of 1-acylglycerophosphate with 16- and 18-carbon fatty acids both saturated and unsaturated. However, when 1-acylglycerophosphorylcholine is the acceptor, unsaturated fatty acids are preferred to saturated fatty acids of equivalent chain length in the formation of phosphatidylcholine (Hill and Lands, 1968). This explains why mammalian tissue phospholipids contain a much higher proportion of polyunsaturated fatty acid in the 2 position than is found in triglycerides despite their common precursor (1,2-diacylglycerol). Pulse labelling experiments indicate that polyunsaturated fatty acids are first incorporated into the phosphatidylcholine fraction before appearing in the phosphatidyl serine or phosphatidyl-ethanolamine fractions (Aeberhard *et al.*, 1978).

Variations in either the fatty acid composition or in the polar group can significantly affect the physical and biochemical properties of the phospholipid. It is clear that these properties can be modified to suit the requirements of the cell, although how control is exerted over the phospholipid composition is less clear. The probability of a particular fatty acid being incorporated into phospholipid appears to depend upon the affinity of the acyl-transferase enzyme for it, although this can be overcome by increasing the proportion of a particular fatty acid in the fatty acyl coenzyme A pool (Lands *et al.*, 1982; Spector *et al.*, 1979).

Table 1.1

The common names and structure

of the most frequently occurring fatty acids

All double bonds are in the *cis* configuration

16:0 Palmitic acid	$CH_3(CH_2)_{14}COOH$
16:1 Palmitoleic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
18:0 Stearic acid	$CH_3(CH_2)_{16}COOH$
18:1 Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
18:2 Linoleic acid	$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOH$
18:3 Linolenic acid	$CH_3(CH_2CH=CH)_3(CH_2)_7COOH$
20:4 Arachidonic acid	$CH_3(CH_2)_3(CH_2CH=CH)_4(CH_2)_3COOH$

Like cholesteryl esters, and in contrast with phospholipids, the main function of triglycerides appears to be lipid storage. Triglycerides are formed from 1,2-diacylglycerol and fatty acyl coenzyme A in a reaction catalysed by diacylglycerol acyltransferase. In keeping with their storage function, triglycerides seem to be less discriminating than phospholipid over the fatty acids which are incorporated into them (Spector *et al.*, 1979).

Most fatty acids in animal tissues consist of a carboxylic group and an unbranched hydrocarbon chain which may be saturated (no double bonds) or show various degrees of unsaturation (one or more double bonds). Fatty acids may be referred to, in shorthand, by the number of carbon atoms and the number of double bonds in the molecule. Thus, palmitic acid, which consists of 16 carbon atoms with no double bonds, would be referred to as 16:0, while arachidonic acid, with 20 carbon atoms and 4 double bonds would be labelled 20:4. Some of the more common fatty acids are shown in Table 1.1. All of these fatty acids have an even number of carbon atoms, and fatty acids with an odd number of carbon atoms are rare. This is because fatty acids are synthesised from 2-carbon units by the multifunctional enzyme fatty acid synthase. Wakil, whose early work led to an understanding of fatty acid synthesis, has recently reviewed the subject (Wakil *et al.*, 1983). Although palmitic acid is the usual end-product of this enzyme, the fatty acid chain can be lengthened by the further addition of 2-carbon units. The elongation may take place either in the mitochondria, where the chain length is increased by successive condensations with acetyl CoA, or in the endoplasmic reticulum, where the 2-carbon unit is supplied by malonyl CoA (Wakil *et al.*, 1983). The monoenic fatty acids palmitoleate (16:1) and

oleate (18:1) may be formed by the insertion of *cis* double bonds in the Δ^9 position of palmitate and stearate respectively (Δ^9 indicates that the double bond is between the 9th and 10th carbon atoms of the chain in which the 1st carbon atom is that forming the carboxylic acid; in an alternative system of nomenclature ω9 indicates that the double bond is between the 9th and 10th carbon atoms of the chain but in this case the 1st carbon atom is taken as that forming the methyl group). The double bond is introduced by a specific monooxygenase system located in the endoplasmic reticulum (Lehninger, 1975). Palmitoleic, oleic, linoleic (18:2) and linolenic (18:3) acids can undergo further elongation and desaturation and are the precursors of four families of long-chain polyenoic acids shown in Fig. 1.3. Although mammals can synthesise both palmitoleic and oleic acids, they are unable to make linoleic or linolenic acids, which must be obtained from the diet and are therefore referred to as essential fatty acids. Some of these essential fatty acids and their derivatives serve as precursors for prostaglandins, a group of pharmacologically active hormone-like substances (Lehninger, 1975). In addition to their roles in phospholipids and as precursors to prostaglandins, fatty acids are one of the most efficient ways of storing chemical energy inside a cell, yielding, on oxidation, 9 Kcal g^{-1} compared with about 4 Kcal g^{-1} from glycogen and starch. This energy is released by a process known as β oxidative degradation which, like fatty acid synthesis, proceeds by 2-carbon units.

Figure 1.3

Four families of polyenoic fatty acids

All four fatty acids have a double bond in the Δ^9 position but, because of differing chain length and additional double bonds, the double bond furthest from the carboxyl group is a different distance from the methyl end in each case. Since mammalian cells are unable to insert double bonds into chains further from the carboxyl group than the Δ^9 position this gives rise to the four different families shown below and also means that, while palmitoleic acid and oleic acid can be synthesised by the cells, linoleic acid and linolenic acid must be obtained from the diet.

Palmitoleic ($\omega7$, Δ^9)

$$CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH=CH-CH_2-CH_2-(CH_2)_5COOH$$

Oleic ($\omega9$, Δ^9)

$$CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH=CH-(CH_2)_7COOH$$

Linoleic ($\omega6$, Δ^9, Δ^{12})

$$CH_3-CH_2-CH_2-CH_2-CH_2-CH=CH-CH_2-CH=CH-(CH_2)_7COOH$$

Linolenic ($\omega3$, Δ^9, Δ^{12}, Δ^{15})

$$CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-(CH_2)_7COOH$$

Although fatty acids can be modified by elongation or desaturation within the cell, the proportions of fatty acids incorporated into more complex lipids reflect, to a certain extent, the proportions in which they are available to the cell (Spector *et al.*, 1979; Lands *et al.*, 1982). This permits the fatty acid composition of cellular lipid to be modified quite easily, allowing the study of the effects of a range of different fatty acids on various aspects of cell metabolism.

1.6 The aims of the work presented in this thesis

Elevated levels of LDL in plasma are associated with an increased risk of coronary heart disease (Stamler, 1973) and subjects on a diet rich in polyunsaturated fatty acids have a higher fractional catabolic rate and lower plasma concentration of LDL than normal (Shepherd *et al.*, 1980).

Although much of the work elucidating the receptor-mediated pathway for LDL degradation was carried out using cultured human fibroblasts (Goldstein and Brown, 1977), there has been little research into the effects of fatty acids on LDL metabolism in these cells. As has been mentioned, the fatty acid composition of membrane phospholipids in cultured human fibroblasts can be easily modified by the addition of fatty acids to the growth medium and modifications to the membrane composition of a variety of mammalian cells may be accompanied by changes in a number of membrane functions (Spector *et al.*, 1979).

This raised the possibility that the LDL receptor or some of the membrane-bound enzymes involved in cellular cholesterol metabolism may be influenced by their lipid environment. Indeed the activity of the enzyme ACAT has been shown to be sensitive to changes in the membrane lipid composition (Brenneman *et al.*, 1977; Mitropoulos *et al.*, 1980; Spector *et al.*, 1980; Field and Salome, 1982).

To obtain a standard method, achieving maximum changes in the fatty acid composition of phospholipids (as measured by gas-liquid chromatography) it was first necessary to investigate such variables as the concentration of exogenous fatty acid and time of growth in fatty acid.

Binding, internalisation and degradation of [^{125}I]LDL were measured by the methods of Goldstein and coworkers (1974*b*, 1976). After characterising the assay using fibroblasts grown in medium without supplements it was possible to examine the effect of growing fibroblasts in the presence of additional fatty acids upon the number of LDL receptors expressed at the cell surface and the rate at which LDL was degraded.

ACAT activity in fibroblasts has been measured only indirectly by assaying the incorporation into cholesteryl esters of [^{14}C]oleic acid added to the growth medium. Values obtained by this method represent the net accumulation of labelled cholesteryl oleate resulting from different rates of formation and hydrolysis of cholesteryl ester. This assay is obviously unsuitable for measuring the rate of cholesteryl ester formation in cells already growing in the presence of large concentrations of fatty acid which would dilute out the radioactive substrate.

Therefore it became necessary to modify an existing ACAT assay (Goodman *et al.*, 1963) and to characterise it for use with fibroblast membrane preparations. Incorporation of [^{14}C]oleate into cholesteryl esters by fibroblasts grown in medium without fatty acid supplements was also measured to allow a more direct comparison with other published results.

Changes in ACAT activity could be brought about either by changes in the property of the enzyme or by changes in the supply of cholesterol as substrate. When membrane preparations were incubated with cholesterol-rich donor particles, redistribution and equilibration of cholesterol occurred (Hashimoto and Dayton, 1979), allowing the amount of cholesterol available to the enzyme as substrate to be controlled, thereby permitting investigation of the effect of cholesterol supply on ACAT activity. A method was set up and characterised for equilibrating the cholesterol of LDL from a homozygous FH patient with that of membrane preparations from fibroblasts. This method was used to examine the mechanisms underlying the changes in ACAT activity observed in membrane preparations from cells grown in the presence of different fatty acids.

This thesis presents an investigation into the way in which LDL catabolism by cultured human fibroblasts is affected by alterations in the fatty acid composition of membrane phospholipid. In addition, the effects of changing the supply of substrate cholesterol and the membrane phospholipid composition on ACAT activity have been investigated.

Chapter 2

METHODS AND MATERIALS

2.1 Preparation and maintenance of fibroblast cultures

Tissue culture techniques were adopted from the methods described by Paul (1975). All cultures were grown at 37°C in an atmosphere of air/CO_2 (19:1 v/v) in a humidified incubator. Two modifications of Eagle's minimum essential medium were used for the growth of fibroblasts (Eagle, 1959). Cells grown in closed flasks were cultured in Eagle's minimum essential medium with Hanks' salt solution (Hanks and Wallace, 1949), 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES), 10 mM $NaHCO_3$, 100 units/ml penicillin and 100 µg/ml streptomycin (tissue culture medium A). The medium was adjusted to pH 7.4 and was supplemented with foetal calf serum (FCS) (10% v/v). On the other hand, the medium which was used for cells grown in tissue culture dishes was in equilibrium with the CO_2 atmosphere of the incubator and contained a higher concentration of bicarbonate buffer than tissue culture medium A. It consisted of Eagle's minimum essential medium with Earle's salt solution (Earle, 1943), 20 mM *N*-((trishydroxymethyl)methyl)glycine (Tricine), 25 mM $NaHCO_3$, 100 units/ml penicillin and 100 µg/ml streptomycin (tissue culture medium B). The medium was adjusted to pH 7.4 and was supplemented

with either FCS (10% v/v) or lipoprotein-deficient serum (LPDS) to give a final concentration of 2.5 mg protein/ml.

Dulbecco's phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (pH 7.4) (Dulbecco and Vogt, 1954).

To start a fibroblast culture a skin biopsy, taken under aseptic conditions from the upper arm of the subject, was placed in a sterile, 60 mm, glass Petri dish containing 5 ml of tissue culture medium A supplemented with FCS. With the aid of a dissecting microscope the underlying tissue was teased away with a scalpel and the skin was cut into pieces of approximately 0.5 mm by 0.5 mm. It was usually possible to obtain about 100 of these explants from each biopsy. The explants were transferred to 60 mm plastic tissue culture dishes containing 3 ml of tissue culture medium B supplemented with FCS (approximately 10 explants in each dish). The explants were weighted down with a glass cover slip which kept them in position at the bottom of the dish and the dishes were placed at 37°C. After 10 days, during which time the dishes were left undisturbed, fibroblasts and epithelial cells could be seen growing out from approximately one-third of the explants. After washing the monolayers of cells with 3 ml of Puck's saline A (pH 7.4) (Puck *et al.*, 1958) and incubating at 37°C for 5 min with 3 ml of Puck's saline A (pH 8.5) containing trypsin (0.5 g/l) and ethylenediaminetetra-acetic acid (EDTA) (0.2 g/l) the fibroblasts became detached from the dish and suspended in the solution. The trypsin/EDTA solution from all the dishes was pooled and centrifuged at 200 x *g* for 5 min

at 4°C in a Mistral 6L centrifuge. The pellet of fibroblasts was resuspended in 5 ml of tissue culture medium A supplemented with FCS, placed in a 25 cm^2 tissue culture flask and placed at 37°C. The fibroblasts were allowed to grow and divide in the flask until a layer of cells completely covered the growth area of the flask. At this stage the fibroblasts were spindle-shaped and arranged themselves into confluent sheets of cells which were again detached from the flask by incubation in the trypsin/EDTA solution and centrifuged as before. The cell pellet was resuspended in 15 ml of tissue culture medium A supplemented with FCS, seeded in an 80 cm^2 tissue culture flask and incubated at 37°C. One of these flasks in which the fibroblasts had grown and divided to form a confluent sheet contained 1-2 x 10^6 cells. By resuspending the cells once a week and subculturing each pellet into two fresh flasks it was possible to increase the number of flasks.

Stocks of fibroblasts were also stored, frozen, in liquid nitrogen. Cell pellets obtained from single 80 cm^2 tissue culture flasks by the usual subculturing procedures were resuspended in 1 ml of medium supplemented with FCS (10% v/v) containing dimethyl sulphoxide (10% v/v). The suspension was transferred to a 2 ml plastic cryotube and cooled to approximately -100°C over 24 h and then placed in liquid nitrogen at -196°C.

For experiments, fibroblasts which had undergone 5-15 passages through the trypsin/EDTA resuspension procedures were used. A pellet of cells obtained by routine subculturing methods was resuspended in tissue culture medium B supplemented with FCS.

Fibroblasts were then seeded at a density of 5 x 10^4/dish into 60 mm plastic tissue culture dishes containing 3 ml of the medium. The cells were refed with 3 ml of fresh medium after 3 days, and after 5 days the monolayers of fibroblasts were washed with 3 ml of Puck's saline A and refed with 2 ml of tissue culture medium B supplemented with LPDS. Experiments were normally performed 24 h later when the fibroblasts were in the late logarithmic growth phase and each dish contained approximately 300 μg of cell protein.

The fibroblasts were harvested after 5 washes with 3 ml of either PBS or 150 mM NaCl/50 mM Tris(hydroxymethyl)-methylamine (Tris)/HCl (pH 7.4 at 4°C) by scraping them from the dishes with a rubber policeman into 1 ml of the same buffer. The buffer used for washing and harvesting was kept on ice throughout the procedure. The cell suspensions, either from a single dish or combined from two or more dishes, were centrifuged at 200 x *g* for 5 min at 4°C in a Mistral 6L centrifuge and the supernatant discarded. If the pellets were not to be used immediately they were stored in liquid nitrogen until required (Brown and Goldstein, 1974; Goldstein *et al.*, 1974*a*).

Eagle's minimum essential medium with either Hanks' or Earle's salts and Puck's saline A were prepared from 10 x concentrated stock solutions, which, together with the buffers, antibiotics, FCS and trypsin/EDTA solution, were obtained from Gibco Europe, Paisley, Scotland, U.K. The media and Puck's saline A were made up with sterile water purchased from Travenol Laboratories, Thetford, Norfolk, U.K. Tissue culture flasks

(25 cm^2 and 80 cm^2), and 2 ml cryotubes were also purchased from Gibco Europe and 60 x 15 mm Contur tissue culture dishes were from the Lux Scientific Corporation, 1000 Oaks, CA, U.S.A. PBS was prepared using AnalaR grade reagents from BDH Chemicals, Poole, Dorset, U.K. Tris was obtained from Sigma Chemical Corporation, St. Louis, MO, U.S.A. and dimethyl sulphoxide (spectrophotometric grade) was from Aldrich Chemical Company, Milwaukee, WI, U.S.A.

2.2 Preparation of low-density lipoprotein and lipoprotein-deficient serum

Blood taken from normal subjects, to which disodium EDTA (100 mg/l) had been added as an anticoagulant, was centrifuged at 1250 x *g* for 20 min at 4°C in a Mistral 6L centrifuge to obtain plasma. The first 200 ml of plasma removed during plasma exchange were obtained from homozygous FH patients (Thompson *et al.*, 1975). LDL and LPDS were prepared from plasma from either source by a combination of the methods of Havel *et al.* (1955) and Chung *et al.* (1980). The plasma was centrifuged at 50,000 rev/min for 18 h at 4°C using a 60ti rotor in a Beckman L8-70 preparative ultracentrifuge. The chylomicrons and VLDL, which floated to the top of the sample were discarded and the density of the infranatant solution was adjusted to 1.30 g/ml by adding the appropriate amount of solid KBr. This was calculated from the equation

$$M = \frac{V\ (d_f - d_i)}{1 - 0.312\ (d_f)}$$

where M is the required mass of KBr (g), V is the volume of the solution (ml) and d_f and d_i are the final and initial densities of the solution (g/ml) (Radding and Steinberg, 1960). 40 ml Polyallomer Quickseal tubes (Beckman) were prepared for centrifugation in a vertical rotor by layering 15 ml of plasma (*d* 1.30 g/ml) under 25 ml 150 mM NaCl (*d* 1.006 g/ml). The density gradient which was formed by centrifugation at 50,000 rev/min for 2½ h at 10°C using a Vti50 rotor with slow acceleration concentrated the LDL into a narrow band approximately three-quarters of the way up the tube. Both the band containing LDL, and the infranatant were collected from the tubes. The solution containing LDL (*d*≅1.050 g/ml) had its density adjusted to 1.063 g/ml and was recentrifuged at 50,000 rev/min for 24 h at 4°C using a 60ti rotor. The LDL, which floated to the top of the tube, was collected, dialysed extensively against PBS, sterilised by filtration through a 0.22 μm Millex filter and stored at 4°C. The density of the infranatant solution from the centrifugation in the Vti50 rotor was adjusted to 1.21 g/ml by adding solid KBr and the solution centrifuged at 50,000 rev/min for 40 h at 4°C in a 60ti rotor. The infranatant layer after this spin was collected, dialysed extensively against PBS and then treated with thrombin (20 units/ml) for 15 min at room temperature. The resulting clot was broken with applicator sticks and removed by filtration through Whatman No.54 paper. This LPDS was sterilised by filtration through a 0.22 μm Millex filter. After assaying the protein concentration the serum was diluted to 50 mg of protein/ml with sterile PBS and stored at 4°C.

Plasma from homozygous FH patients undergoing plasma exchange was kindly supplied by Dr. G.R. Thompson.

Potassium bromide (AnalaR grade reagent) was purchased from BDH Chemicals, dried in an oven at 120°C for 16 h and stored in a desiccator. Thrombin was from Parke-Davis and Company, Detroit, MN, U.S.A. The L8-70 preparative ultracentrifuge, 60ti and Vti50 rotors, 40 ml Polyallomer Quick-Seal tubes and tube sealing device were obtained from Beckman Instruments, Palo Alto, CA, U.S.A. Millex filters were from the Millipore Corporation, Bedford, MA, U.S.A.

2.3 Assays of protein and lipid

2.3.1 Protein assay

Protein concentrations were normally measured by the method of Lowry *et al.* (1951). However, a number of common reagents interfere with this assay and, if any of these were present in the samples, it was necessary to modify the procedure by first precipitating the protein with trichloroacetic acid as described by Bensadoun and Weinstein (1976). The amount of protein in the precipitate could then be assayed by the usual method of Lowry *et al.* (1951). The samples to be assayed generally contained 20-50 µg of protein. If they were to be precipitated they were made up to 3 ml with water.
A range of standards containing 0-100 µg of bovine serum

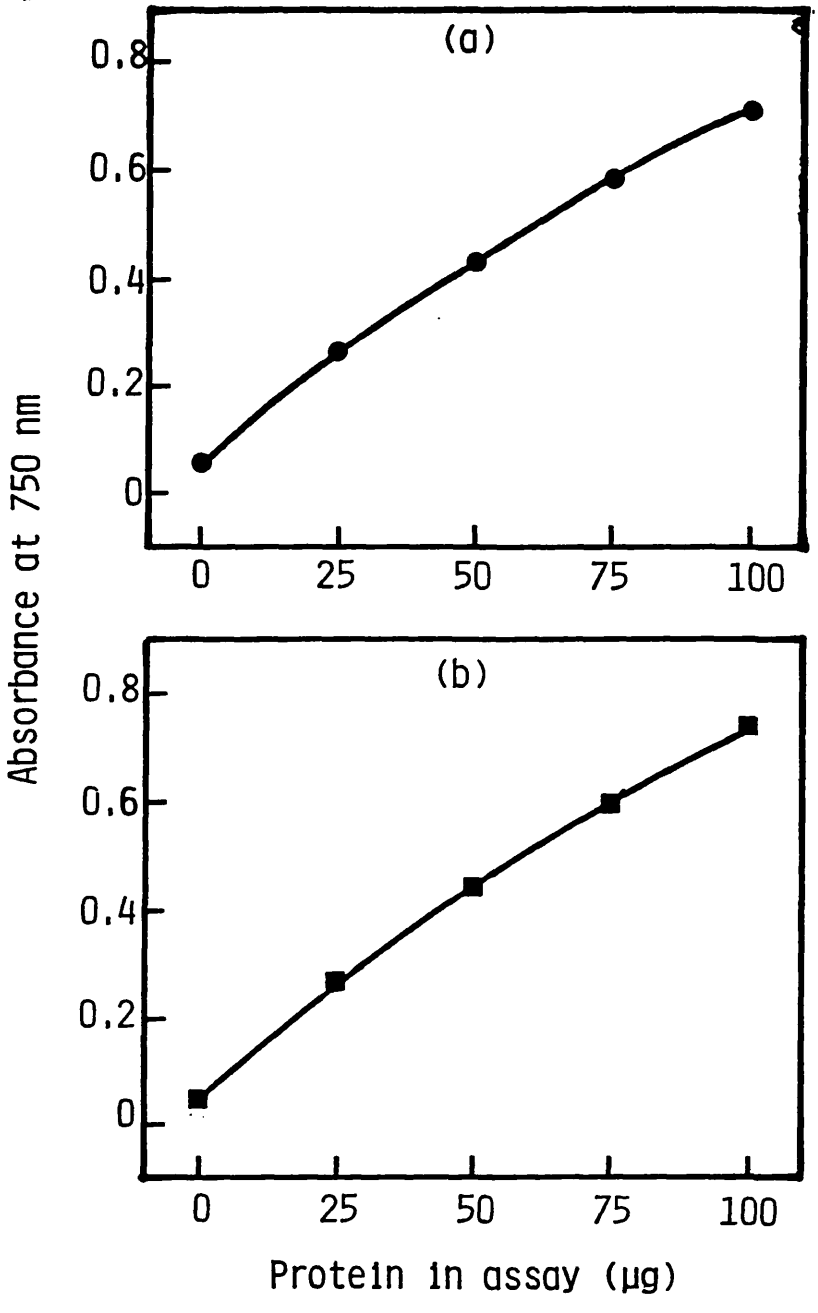


Figure 2.1 Protein determination by the methods of Lowry *et al.* and Bensadoun and Weinstein

The values for absorbance at 750 nm were compared for samples of bovine serum albumin assayed by the methods of Lowry *et al.* (a) and Bensadoun and Weinstein (b) as described in the text. Each value is the mean of at least 14 separate measurements. Bars depicting the standard error in the estimation of the mean were omitted since the values were too small for them to be distinguished from the appropriate points. Using the Student's *t*-test, no significant difference could be found between values obtained from the assay of Lowry *et al.* and the corresponding values obtained from the assay of Bensadoun and Weinstein.

albumin and any reagents present in the samples were also made up to 3 ml with water. 25 µl of sodium deoxycholate (20 mg/ml) were added to both standards and samples, the tubes shaken and allowed to stand for 15 min at room temperature. 1 ml of trichloroacetic acid (240 mg/ml) was then added, the tubes shaken, centrifuged at 1000 x *g* for 30 min at 4°C in a Mistral 6L centrifuge, and the supernatant removed by aspiration. The normal protein assay of Lowry *et al.* was then performed as follows.

The pellets were resuspended in 2 ml of 100 mM NaOH containing Na_2CO_3 (20 mg/ml), $CuSO_4$ (100 µg/ml) and potassium sodium tartrate (200 µg/ml). After 10 min, 200 µl of Folin Ciocalteu's phenol reagent (diluted to 1 N with water) were added and the tubes vortexed. The colour was allowed to develop for 30 min before the absorbance was measured at 750 nm in a spectrophotometer. If it was unnecessary to precipitate the protein, the samples and the standards were prepared in a small volume (≤200 µl) and the protein assayed as described. For comparison the curves obtained with and without the protein precipitation step are shown in Fig. 2.1. There was no significant difference between values obtained from the assay of Lowry *et al.* (1951) and the corresponding values obtained from the assay of Bensadoun and Weinstein (1976).

2.3.2 Fatty acid concentration

The concentration of free fatty acid in samples was measured by the method of Novak (1965) as follows:

Dole's extraction medium (Dole, 1956) consisted of propan-2-ol/heptane/1N sulphuric acid (40/10/1 v/v). Saturated solutions of K_2SO_4 and Na_2SO_4 were prepared by adding excess sulphate to boiling water, allowing the solution to cool and storing, over excess crystals at 37°C. Glacial acetic acid (final concentration 8 ml/l) and $Co(NO_3)_2$ (final concentration 60 mg/ml) were added to the saturated K_2SO_4 and 10 volumes of this solution were mixed with 1.35 volumes of triethanolamine and 7 volumes of saturated Na_2SO_4 to form the cobalt reagent. The stock indicator solution consisting of α-nitroso-β-naphthol (4 mg/ml) in 96% ethanol was diluted 12.5 fold with 100% ethanol before use. Fresh preparations of cobalt reagent and the indicator working solution were made before each assay.

Samples containing approximately 250 pmol of free fatty acid were made up to a volume of 3.5 ml with water and 2.5 ml of Dole's extraction medium and 3 ml of heptane were added. A range of standards containing 0-500 pmol of palmitic acid were made up to 3 ml in heptane and 2.5 ml of Dole's extraction medium and 3.5 ml of water were added. The tubes were shaken vigorously for 2 min and centrifuged at 300 x *g* for 15 min at room temperature in a Mistral 6L centrifuge. 600 µl of the upper phase, into which the fatty acids had been extracted, were removed and 800 µl of chloroform/heptane (5:1 v/v) and

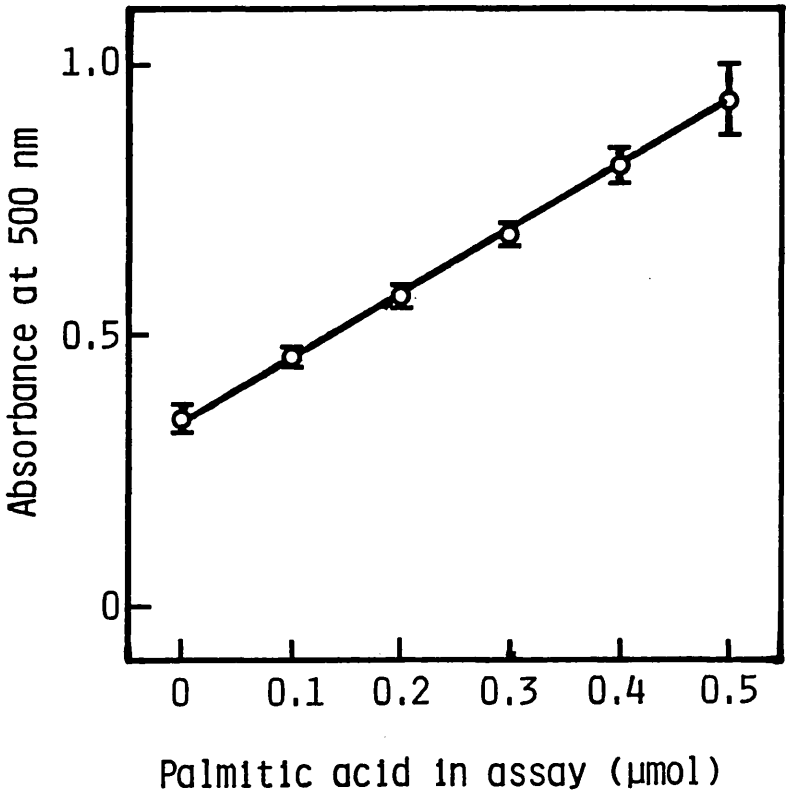


Figure 2.2 Assay of free fatty acid concentration

The absorbance at 500 nm was measured in samples containing 0-500 nmol of palmitic acid, assayed by the method of Novak (1965) as described in the text. Each point is the mean of 6 separate measurements with bars indicating the S.E.M. at that point. The regression line calculated from these data was:
absorbance = 1.169 (amount of palmitate (µmol)) + 0.339
(r = 0.9995).

1 ml of cobalt reagent were added. Cobalt forms salts with free fatty acids which give a colour reaction with α-nitroso-β-naphthol. After the tubes had been shaken for 3 min they were centrifuged at 300 x *g* for 15 min at room temperature in a Mistral 6L centrifuge. 800 µl of the upper phase, which contained the cobalt salt of the fatty acid, were added to 1 ml of the indicator solution and, after the tubes had been allowed to stand for 30 min at room temperature, the absorbance was measured at 500 nm using a spectrophotometer. The absorbance varies linearly with fatty acid concentration within the range assayed (Fig. 2.2).

2.3.3 Cholesterol concentration

Cholesterol concentrations were determined by a photofluorometric method (Heider and Boyett, 1978).

A stock solution of 100 mM potassium phosphate buffer (KPB) was prepared by mixing 100 mM KH_2PO_4 with 100 mM K_2HPO_4 to give a solution of pH 7.0. A reaction mixture of 50 mM KPB containing parahydroxyphenylacetic acid (150 µg/ml), sodium deoxycholate (6 mM), horse radish peroxidase (12 units/ml) and cholesterol oxidase (4 µg/ml) was prepared. Cell extracts were prepared by homogenisation as described in Chapter 5 (Section 2). 20 µl of propan-2-ol were added to samples containing 0.5-1.0 µg cholesterol in a small volume of saline (approx. 10 µl). A range of standards containing 0-1.5 µg of cholesterol were prepared, each in a total of 20 µl of propan-2-ol. 400 µl of the reaction mixture were added to each of the tubes which were then shaken

and incubated at 37°C for 20 min and 800 µl of NaOH (0.5 N) were added.

Cholesterol oxidase catalyses the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide reacts with parahydroxyphenylacetic acid in the presence of horse radish peroxidase to give a fluorescent product with an excitation wavelength of 325 nm and an emission wavelength of 415 nm. Using a spectrophotofluorometer it was found that the standard curve was linear within the range of values measured, the value of the blank being 40% of the value of the sample containing 1.5 µg cholesterol.

2.3.4 Serum phospholipid

Serum phospholipids were assayed using Marinetti's modification of the method of Bartlett (Marinetti, 1962; Bartlett, 1959). In this method the amount of inorganic phosphate, produced by the oxidation of phospholipid, is measured.

ANS reagent, which was freshly prepared for every assay consisted of 0.5 g 1-amino-2-naphthol-4-sulphonic acid made up to 200 ml with sodium bisulphite (15 mg/ml) to which were added 5 ml sodium sulphite (20 mg/ml). This was mixed and filtered through Whatman 54 filter paper before use. A range of standards containing 0-10 µg of inorganic phosphorus (0-44 µg KH_2PO_4) and samples containing approximately 125 µg of phospholipid (equivalent to 5 µg of phosphorus) were prepared in 100 µl water.

The samples were digested in 1 ml perchloric acid at 180°C until they were colourless (2-3 h). After cooling, 7 ml water, 500 µl ammonium molybdate (25 mg/ml) and 200 µl ANS reagent were added. After shaking the tubes were placed in a bath of boiling water for 10 min and, when they had cooled, the absorbance was measured at 810 nm in a spectrophotometer. The standard curve was linear within the range used, the blank having an absorbance of 0.054 while the standard containing 10 µg of phosphorus had an absorbance of 0.95.

2.3.5 Membrane phospholipid

The phospholipid content of cell membrane preparations was assayed by the photofluorometric method of Jouanel *et al.* (1980).

A solution was made of 30 µmol 1,6-diphenyl-1,3,5-hexatriene in 10 ml of freshly redistilled tetrahydrofuran. 200 µl of this solution was made up to 60 ml in water with vigorous stirring to form the reaction mixture. A range of standards containing 0-12 nmol of phospholipid were prepared from serum with a known phospholipid concentration. Samples containing approximately 6 nmol of phospholipid and the standards were made up to a total volume of 50 µl with 150 mM NaCl and 3 ml of the reaction mixture was added to each tube. The tubes were shaken and incubated at 40°C for 40 min in darkness. The samples were excited at 365 nm and the fluorescent emission at 460 nm was measured using a spectrophotofluorometer. The standard curve

was linear within the range used with the value of the blank being approximately 15% of the value of the sample containing 12 nmol phospholipid.

This method was also used to determine the phospholipid content of LPDS since this was dialysed against PBS during its preparation and therefore the total phosphorus in the sample greatly exceeded that present in phospholipid, rendering the method of Marinetti (1962) inappropriate.

2.3.6 Triglyceride concentration

The triglyceride content of samples was assayed using a kit obtained from Dow Chemical Company, IN, U.S.A.

Addition of the reaction solution initiates a series of steps, eventually producing NADH which in turn is used to reduce iodonitrotetrazolium violet (INT) to produce NAD + formazan (Stavropoulos and Crouch, 1974). The entire process is summarised below:

$$\text{Triglycerides} \xrightarrow{\text{Lipases}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-1-phosphate + ADP}$$

$$\text{Glycerol-1-phosphate + NAD} \xrightarrow{\text{Glycerol-1-phosphate dehydrogenase}} \text{Dihydroxyacetone phosphate + NADH}$$

$$\text{NADH + INT} \xrightarrow{\text{Diaphorase}} \text{Formazan + NAD}$$

The reaction medium contains $MgCl_2$ (300 µg/ml), adenosine-5'-triphosphate (ATP) (1.8 mg/ml), nicotinamide adenine dinucleotide (NAD) (7.2 mg/ml), 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyl-tetrazolium chloride (INT) (250 µg/ml), diaphorase (microbial origin) (5.52 U/ml), glycerol kinase (microbial origin (0.92 U/ml), glycerol-1-phosphate dehydrogenase (animal muscle) (13.8 U/ml) and 2 lipases (microbial origin) (total 100 U/ml).

500 µl of reaction medium was added to samples containing approximately 10 µg of triglyceride in 20 µl of water and to standards containing 0-4.16 µg of glycerol (equivalent to 0-40 µg of triglyceride) also in 20 µl of water. The tubes were shaken and incubated at 37°C for 10 min before the reaction was stopped by adding 1 ml HCl (30 mM). The tubes were shaken and allowed to stand for 5 min at room temperature before measuring the absorbance at 500 nm. The standard curve was linear within the range used. The adsorbance of the blank was approximately 0.1 while the adsorbance of the sample containing the equivalent of 40 µg of triglyceride was 0.77.

2.3.7 Materials

Bovine serum albumin (fraction V), palmitic acid, *p*-hydroxyphenylacetic acid, horse radish peroxidase (type II) and 1,6-diphenyl-1,3,5-hexatriene were purchased from Sigma Chemical Company. Cholesterol oxidase (from *Norcardia erythropolis*) and cholesterol standards in propan-2-ol were

obtained from BCL, Lewes, Sussex, U.K. Heptane and sulphuric acid (1.84 g/ml) were from May and Baker, Dagenham, Essex, U.K. and tetrahydrofuran was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. All the reagents and standards required for the measurement of triglyceride were supplied in a kit obtained from Dow Chemical Company.

All other reagents were purchased from BDH Chemicals. Sodium bisulphite (Na_2HSO_3), which does not exist as a solid, was prepared as a solution by dissolving sodium metabisulphite ($Na_2S_2O_5$) in water. Propan-2-ol was of spectroscopic grade. All other chemicals were either of AnalaR grade or of the highest purity otherwise available.

The spectrophotometer was the SP6-500 UV model from Pye-Unicam, Cambridge, U.K. and the Aminco-Bowman spectrophotofluorometer was from American Instrument Company, Silver Spring, MD, U.S.A.

2.4 Statistics

The data generated by the experiments reported in this thesis were analysed by standard statistical methods (Hayslett, 1971). For comparisons between different conditions to be statistically valid, measurements under each condition must be made in several different experiments. The results from such a series of experiments may then be expressed as the mean ± standard error in the estimation of the mean (S.E.M.).

Although these figures offer a useful guide as to whether or not differences exist between various conditions, it is more desirable to statistically test the probability that such differences are not merely brought about by chance. In the Student's t-test, the mean ($\bar{x}_1$) and S.E.M. (SEM_1) of n_1 observations under one condition can be compared with the mean ($\bar{x}_2$) and S.E.M. (SEM_2) of n_2 observations under another condition. The variance (S^2) is first calculated from the equation:

$$S^2 = \frac{[(n_1-1)(n_1)(SEM_1)^2] + [(n_2-1)(n_2)(SEM_2)^2]}{(n_1-1) + (n_2-1)}$$

The t value may then be obtained from the equation:

$$t_{(n_1+n_2-2)} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{S^2 \left(\frac{n_1+n_2}{n_1 n_2}\right)}}$$

The value (n_1+n_2-2) gives the number of degrees of freedom (v). Using a table of percentage points of the t-distribution (Lindley and Miller, 1953) it is possible to find the probability that both samples came from a single population, *i.e.* that there was no difference between the samples. If this probability ≤ 0.05, it is assumed that there is a statistically significant difference between the two samples. The paired t-test is a variation of this method in which comparisons are made between individual observed values under different conditions. The differences (z) between q pairs of observations under conditions a and b may be represented as:

$$z_1 = a_1 - b_1; \quad z_2 = a_2 - b_2 \quad \ldots\ldots \quad z_q = a_q - b_q$$

The variance may be calculated from the equation:

$$S^2 = \frac{\Sigma(z^2) - \frac{(\Sigma z)^2}{q}}{(q-1)}$$

The t value with $(q-1)$ degrees of freedom may be calculated from the equation:

$$t_{(q-1)} = \frac{\bar{z}\sqrt{q}}{\sqrt{S^2}}$$

In this case the probability that $\bar{z} = 0$ has been tested and, as before, if this probability ≤ 0.05, it is assumed that there is a statistically significant difference between the two samples. Occasionally the ratio of values observed under two different conditions within the same experiment may be consistently repeated in a series of experiments. However, the absolute values measured from one experiment to the next may vary so that neither the Student's t-test nor the paired t-test indicate any difference between the two conditions. In this situation it is necessary to compare the ratios of each pair of observations within the same experiment. This is done by carrying out a paired t-test on the logarithms of the data.

In order to draw the best straight line through a set of data plotted on a graph, the regression line is calculated. The general equation for any straight line is $y = bx + a$, where b and a are constant values and y and x are variables. b may be calculated from the equation:

$$b = \frac{\Sigma(xy) - \frac{(\Sigma x)(\Sigma y)}{n}}{\Sigma(x^2) - \frac{(\Sigma x)^2}{n}}$$

By substituting this value and the mean values of x and y ($\bar{x}$ and $\bar{y}$), a can be calculated from the equation for a straight line ($\bar{y} = b\bar{x} + a$).

The correlation coefficient (r) gives an indication of whether there is a linear relationship between two parameters and how close that relationship is. It may be calculated from the formula:

$$r = \frac{\Sigma(xy) - \frac{(\Sigma x)(\Sigma y)}{n}}{\sqrt{[\Sigma(x\ ) - \frac{(\Sigma x)^2}{n}][\Sigma(y^2) - \frac{(\Sigma y)^2}{n}]}}$$

This value may be substituted into the equation:

$$t_{(n-2)} = \frac{r}{\sqrt{1-r^2}} \times \sqrt{n-2}$$

This gives a t value with $(n-2)$ degrees of freedom, which may be used to find the probability that $r=0$ (no correlation). As before, if the probability ≤ 0.05 it is assumed that there is a statistically significant correlation between the two sets of data.

Chapter 3

INCORPORATION OF FATTY ACIDS INTO PHOSPHOLIPIDS

3.1 Introduction

One of the aims of this work was to investigate the effects of modifying the fatty acid composition of fibroblast phospholipids upon the ability of the cells to catabolise LDL. The fatty acid composition of fibroblast phospholipids may be altered by supplementing the cell growth medium with fatty acid (Spector *et al.*, 1979) and the preliminary experiments, presented in this chapter, were designed to discover the conditions that produced the optimum modifications to phospholipid fatty acids using this method.

The concentration of the fatty acid added to the medium is critical. Sufficient additional fatty acid must be present to minimise the effects of the fatty acids already present in the FCS, while high concentrations of fatty acids, particularly polyunsaturated fatty acids, have toxic effects upon the cells (Mulligan *et al.*, 1977; Spector *et al.*, 1979). The extent to which the time of exposure to the supplementary fatty acids affected the degree of modification of cellular phospholipid was also examined.

In order for fibroblasts to express LDL receptors they need to be incubated in medium containing LPDS for 24-48 h (Goldstein and Brown, 1977) and it was obviously important to show that this change from medium containing FCS into medium containing LPDS did not also effect a change in the fatty acid composition of cellular phospholipid.

Since membrane functions can be affected by changes in membrane fluidity, which can be influenced by the degree of saturation of the phospholipid fatty acids, the incorporation of three different fatty acids into fibroblast phospholipids was investigated. The first was a saturated fatty acid (palmitate, 16:0), the second a monounsaturated fatty acid (oleate, 18:1) and the third, a polyunsaturated fatty acid (linoleate, 18:2). For comparison, the fatty acid composition of cells grown in medium without a fatty acid supplement was also analysed.

3.2 Methods and materials

In order to supplement the fibroblast growth medium with fatty acids, the sodium salt of the fatty acid was prepared. In a typical preparation, 50 µmol of the fatty acid were dissolved in 3.36 ml of 0.015 N NaOH to give a concentration of 14.9 mM fatty acid. It was usually necessary to warm the solutions to 45°C in order to dissolve the soap. 672 µl of this solution, containing 10 µmol of the fatty acid sodium salt, were added,

dropwise, with continuous stirring to either 20 ml of FCS or 10 ml of LPDS. These solutions were sterilised by filtration through a 0.22 μm Millex filter and diluted with culture medium B; 10-fold in the case of FCS, 20-fold in the case of LPDS. This gave a final concentration of fatty acid sodium salt added to the medium of 50 μM which was the concentration normally added to fibroblast cultures. Obviously the concentration could be varied by changing the amount of the fatty acid sodium salt complexed with the serum.

Palmitic acid, oleic acid and linoleic acid were all purchased from Sigma Chemical Company. Because of the ease with which linoleic acid could be oxidised, all procedures involving any of the fatty acids, or their sodium salts, were carried out under nitrogen gas. Linoleic acid was purchased in small ampoules which were discarded after a single use. All fatty acid solutions were freshly prepared for each experiment.

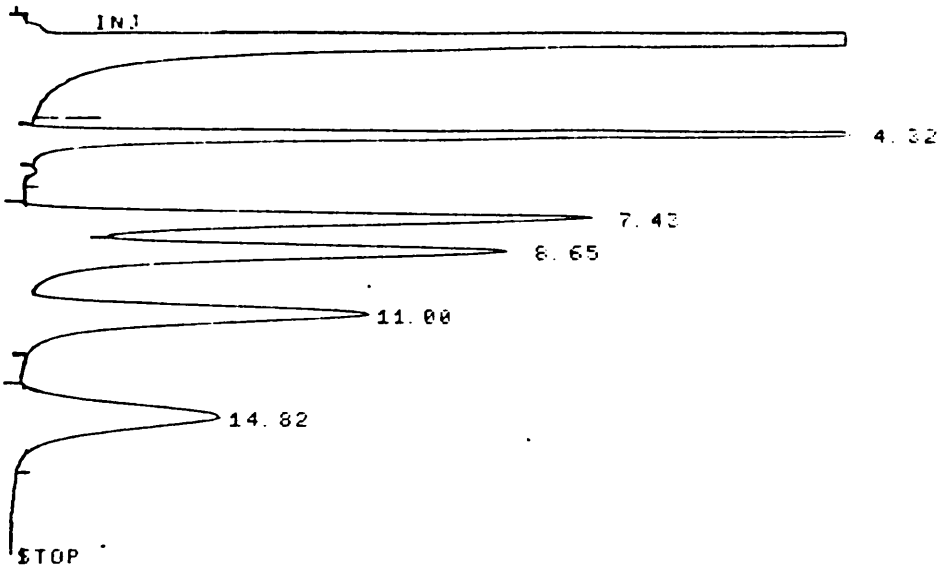
When fibroblasts were to be grown in medium supplemented with fatty acids the method described in Chapter 2 (Section 2.1) was modified slightly unless otherwise stated. The cells were seeded at 5 x 10^4 fibroblasts/60 mm dish in 3 ml medium containing FCS as described. They were incubated at 37°C for 24 h to allow the fibroblasts to become attached to the dish and then the medium was replaced with fresh medium containing FCS and supplemented with fatty acids. The cells were refed after a further 3 days again with fatty acid-supplemented medium. After another 2 days the cells were

washed with Puck's saline A, as described, and refed with medium containing LPDS supplemented with fatty acids. Thus the fibroblasts were grown in dishes for 1 day longer than usual but were in medium supplemented with fatty acids for the normal experimental period.

The fatty acid composition of lipid samples was analysed by gas-liquid chromatography. For the analysis of the fatty acid composition of cellular phospholipid, fibroblasts were washed and harvested in 150 mM NaCl/50 mM Tris-HCl and centrifuged. The pellets, each of cells pooled from 3 dishes, were stored in liquid nitrogen for up to 14 days until required. The first step was to extract total lipid by the method of Folch *et al.* (1957). The pellet was resuspended in 200 µl H_2O, 4 ml chloroform-methanol (2:1 v/v) were added and the tube shaken vigorously. 800 µl H_2O were added, the contents of the tube mixed and the two phases separated by centrifugation for 20 min at 300 x *g* in a Mistral 6L centrifuge. The upper phase was discarded and the lower phase containing the cellular lipid was dried under nitrogen. The lipids were then separated by thin-layer chromatography on silica gel G using heptane-diethyl ether-acetic acid (85:20:2 v/v) as the developing solvent. Under this system phospholipids remained at the origin while the R_f values of other lipids were cholesterol 0.08, free fatty acid 0.17, triglycerides 0.38 and cholesteryl esters 0.65. The phospholipid band was eluted with chloroform-methanol (2:1 v/v) and dried under nitrogen. The methyl esters of the fatty acids were then formed by hydrolysis and reductive methylation,

as described by Morrison and Smith (1964). The dried sample was dissolved in 500 µl of 14% BF_3 in methanol, the tube flushed with nitrogen and closed tightly with a teflon-lined screwcap. After incubating for 5 min at 95°C, 500 µl of H_2O were added and the methyl esters of the fatty acids were extracted in 2 ml hexane. Serum lipids were prepared in the same way. However, triglycerides require extensive incubation at 95°C with BF_3 in methanol for their fatty acids to become esterified and, to avoid this harsh treatment, samples containing triglycerides were hydrolysed first at a lower temperature. Ethanolic KOH was prepared by mixing 3 volumes of ethanol with 1 volume of aqueous KOH (200 mg/ml). Samples containing triglyceride were dissolved in 500 µl of this solution and incubated for 1 h at 60°C. The fatty acids were extracted in heptane, dried under nitrogen, methylated and extracted in the same way as those from phospholipids.

The fatty acid methyl esters were separated by gas-liquid chromatography on a Pye-Unicam, Series 104 machine with flame ionisation detector. The siliconised glass column (1.5 m x 0.4 mm internal diameter) was packed with 5% ethylene glycol succinate polyester on 100-120 mesh Supelcoport through which argon carrier gas flowed at a rate of 5 ml/min. The column oven temperature was 180°C while the injector and detector were maintained at 200°C. The relative amounts of fatty acids were calculated by a Hewlett-Packard 3380A Integrator. A typical trace can be seen in Fig. 3.1.



NO CAL => AREA %

RT	TYPE	AREA	
4.32		198890	22.62
7.43		185135	21.06
8.65	M	199029	22.64
11.00	M	161697	18.39
14.82		134416	15.29

Figure 3.1 Printout of fatty acid separation by gas-liquid chromatography

Fibroblast phospholipids were extracted and the fatty acid component reductively methylated as described in Section 3.2 of the text. The phospholipid fatty acids of fibroblasts from three 60 mm tissue culture dishes were dissolved in 25 µl of ethyl acetate and 2 µl were analysed by gas-liquid chromatography as described in Section 3.2 of the text. The Figure shows the printout of fatty acids in a standard solution containing equimolar amounts of palmitate (retention time 4.32 min), stearate (7.43 min), oleate (8.65 min), linoleate (11.00 min) and linoleate (14.82 min). The area of each peak was calculated by a Hewlett-Packard 3380A Integrator which also calculated the size of each peak as a percentage of total. When the relative amount of fatty acids in samples was calculated, allowance was made for the deviation from the expected 20% in each case observed in the standard.

Commercially-prepared, plastic-backed, thin-layer chromatography plates with a 0.25 mm layer of silica gel G were obtained from Camlab, Cambridge, U.K. 5% ethylene glycol succinate polyester on 100-120 mesh Supelcoport was purchased from Chromatography Services, Hoylake, Merseside, U.K. Tris and 14% BF_3 in methanol were from Sigma Chemical Company. 14% BF_3 in methanol was bought in 5 ml vials which were discarded after a single use. Methanol and ethanol were obtained from James Borrough, London, U.K., heptane, chloroform and diethylether from May and Baker, and KOH, NaCl and acetic acid were from BDH Chemicals.

3.3 Results

A comparison of the protein and lipid content of FCS and human LPDS revealed that, as would be expected, the concentrations of cholesterol, phospholipid and triglyceride, all associated with lipoproteins, are greatly decreased in LPDS (Table 3.1). Although the concentration of free fatty acid in LPDS is twice that of FCS (Table 3.1), since LPDS is diluted 20-fold while FCS is diluted only 10-fold, the final concentrations in the culture medium are approximately the same, between 10-15 μM.

Analysis, by gas-liquid chromatography, of the fatty acid composition of both FCS and LPDS (without the addition of supplementary fatty acids) shows that 16- and 18-carbon fatty acids predominate in both sera (Table 3.2). For the most part,

Table 3.1

Protein and lipid concentrations in FCS and LPDS

The assays were carried out by the methods described in Section 2.3. The figures are the mean ± S.E.M. of the number of assays (given in parentheses).

	Foetal calf serum	*Lipoprotein-deficient serum*
Protein	41±1.5 (3) mg/ml	50 mg/ml[1]
Free fatty acid	131.2±9.3 (15) μM	288±55 (11) μM
Triglyceride	774±28 (8) μg/ml	128±8 (6) μg/ml
Phospholipid[2]	3.7±0.1 (8) mg/ml	Not detectable
Cholesterol	354±18 (8) μg/ml	Not detectable[3]

[1] LPDS was diluted before use to 50 mg protein/ml.

[2] The phospholipid content of FCS was assayed by the method of Marinetti (1962) but, because LPDS was dialysed against PBS, the phospholipid content of this serum was measured by the method of Jouanel *et al.* (1980) using FCS as a standard.

[3] The cholesterol concentration of LPDS was less than 30 μg/ml since this was the minimum concentration which could be measured by this assay.

The figures in this Table are the concentration present in the serum. In the final medium, FCS is diluted 10-fold and LPDS is diluted 20-fold.

Table 3.2

The fatty acid composition of total lipid and free fatty acid in foetal calf serum and lipoprotein-deficient serum

Total lipid was extracted from FCS and LPDS. Part of this extract was separated by thin-layer chromatography and the free fatty acids were eluted from the silica gel. The fatty acid composition of both free fatty acids and total lipid were then analysed by gas-liquid chromatography. The results are the mean ± S.E.M. of several measurements; the number of analyses is given in parentheses after each condition.

	Total Lipid		*Free Fatty Acids*	
	FCS (5)	*LPDS (8)*	*FCS (3)*	*LPDS (7)*
16.0	26.8±1.6	28.3±1.6	29.2±0.5	36.1±1.7
16:1	5.2±1.3	4.1±0.4	0.2±0.1	4.9±0.8
18:0	15.9±2.4	10.8±0.8	16.3±0.5	12.4±0.8
18:1	30.0±2.4	24.6±1.7	8.8±1.7	17.5±1.7
18:2	13.3±3.0	28.0±3.6	40.9±1.6	23.0±3.2
18:3	0.2±0.1	0.2±0.1	-	-
20:4	5.2±1.5	2.0±0.6	-	-
Others	3.6±0.4	2.0±1.0	4.6±0.4	6.1±1.1

the relative amounts of fatty acid in total lipid are reflected by the proportions in the free fatty acid fraction. However, linoleate, which forms the major component of the free fatty acid fraction in FCS, forms only 10% of the fatty acid in the total lipids, while the proportion of oleate changes in a reciprocal manner.

Analysis of cell membrane phospholipids from fibroblasts grown in medium containing serum but no additional fatty acids indicates the fatty acid composition of these lipids is similar to that of the free fatty acid composition of the serum. Once again, 16- and 18-carbon fatty acids predominate (Table 3.3) although linoleate, which forms over 20% of serum free fatty acid, forms less than 4% of the fatty acid composition of cell membrane phospholipids,while the proportions of more saturated fatty acids are greater in cell membrane phospholipids than in the growth medium.

The sodium salt of a fatty acid, complexed with the albumin present in the serum, proved to be a more effective method of raising the proportion of that fatty acid in cellular phospholipids than supplementing normal medium, either with free fatty acids dissolved in ethanol or with the sodium salt of the fatty acid complexed with exogenous bovine serum albumin (data not shown).

Initially it was necessary to decide upon the optimum concentration of fatty acid to add to the growth medium. This concentration represents a compromise between achieving the largest possible modification of the fatty acids in cellular phospholipids

Table 3.3

A comparison of the fatty acid composition of phospholipids of fibroblasts grown in medium containing FCS either without supplement or supplemented with 50 μM fatty acid

Cells were seeded in medium containing FCS (10% v/v) and refed after 1 and 4 days with medium containing FCS and supplemented as described. After 7 days the cells from triplicate dishes were pooled for the analysis of their phospholipid fatty acids. The results are presented as the mean ± S.E.M. of the number of experiments (given in parentheses after each condition). Differences which are significantly different ($p<0.05$; Student's t-test) are listed in the text.

	No additions (11)	*Palmitate (9)*	*Oleate (13)*	*Linoleate (11)*
16:0	29.5±1.1	31.6±1.5	26.0±1.4	25.2±1.2
16:1	7.6±0.4	5.7±0.4	6.4±0.6	5.4±0.4
18:0	20.2±1.2	22.3±0.9	20.6±1.5	22.1±1.1
18:1	23.9±1.4	21.2±1.2	31.9±2.3	20.6±1.6
18:2	3.6±0.6	2.5±0.3	2.8±0.4	14.5±2.1
18:3	0.8±0.2	0.3±0.1	0.4±0.1	0.7±0.1
20:4	2.8±0.9	4.2±1.1	3.8±0.9	4.1±0.7
Saturated fatty acid	50.2±2.0	54.0±1.1	46.8±2.6	47.3±2.0
Monounsaturated fatty acid	32.0±1.4	26.9±1.3	37.4±2.6	26.1±1.8
Polyunsaturated fatty acid	6.8±1.1	5.8±1.2	5.6±1.0	18.3±1.6
P/S ratio	0.143±0.026	0.111±0.023	0.131±0.027	0.400±0.044

and exposing the cells to toxic levels of fatty acid.

An increase in the amount of palmitate added to the medium is accompanied by a slight, gradual increase in the proportion of palmitate in the cellular phospholipid and a similar decrease in the proportion of oleate (Fig. 3.2(a)). Even at a concentration of 100 μm palmitate did not affect the proportion of linoleate in the phospholipid.

As the amount of oleate in the medium increases to 75 μM the proportion of this fatty acid in the cellular phospholipids almost doubles, mainly at the expense of palmitate although there is also a slight fall in the proportion of linoleate (Fig. 3.2(b)). There is no further change in the phospholipid fatty acids as the concentration of oleate rises to 100 μM.

100 μm linoleate proved to be toxic to the fibroblasts. However, when the medium contained only 25 μM linoleate, the proportion of this fatty acid in cellular phospholipid underwent a 4-fold increase at the expense of palmitate (Fig. 3.2(c)). As the concentration of the supplement was raised to 75 μM the further slight increase in the amount of linoleate in the cell phospholipid was accompanied by a rise in the proportion of palmitate and a decrease in oleate.

The medium was supplemented with fatty acids at a concentration of 50 μM in all further experiments. This meant that the total free fatty acid concentration in medium containing 10% FCS would be over 60 μM of which approximately 80% would be specifically determined. Similarly, the total free fatty acid concentration in medium containing 5% LPDS would be almost 65 μM

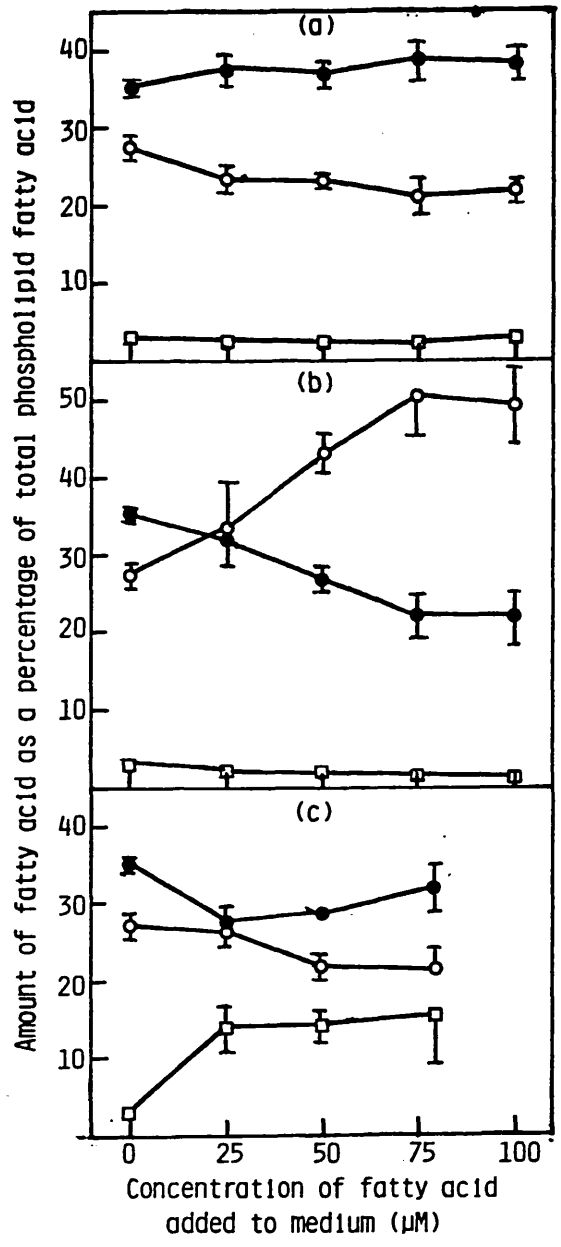


Figure 3.2 Variation in phospholipid fatty acid composition with the concentration of fatty acid added to the medium

Fibroblasts were grown for 24 h in medium containing foetal calf serum (10% v/v). The cells were refed after 1 and 4 days with medium containing foetal calf serum and supplemented with the indicated concentration of (a) palmitate, (b) oleate, or (c) linoleate. After 6 days in the presence of fatty acids, cells from triplicate dishes were pooled for the analysis of their phospholipid fatty acids. The Figure shows the variation with concentration of fatty acid in the medium of palmitate (●), oleate (○) and linoleate (□) in the cell phospholipids. The amounts are expressed as a percentage of total phospholipid fatty acid($n = 3$).

of which between 75 and 80% would be specifically determined.

This concentration of fatty acid supplement had no toxic effects upon the growth rate of cell, as indicated by the amount of protein per dish after different periods of exposure to the fatty acid (Fig. 3.3).

When fibroblasts were grown in medium without supplementary fatty acids, the relative proportions of fatty acids in the membrane phospholipids barely changed in the course of 6 days (Fig. 3.4).

In medium supplemented with 50 μM palmitate, the proportion of this fatty acid in cellular phospholipids increased slightly for 3 days before returning towards its original level (Fig. 3.5). A small rise and fall in the proportion of stearate coincided with the changes in palmitate (data not shown). These changes were compensated for by reciprocal changes in the amount of oleate. The proportions of the other major fatty acids changed little over the entire 6 days.

In contrast, dramatic changes in the fatty acids of cellular phospholipid occurred in the presence of 50 μM oleate (Fig. 3.6). The amount of oleate in the membrane lipids rose by 75% over the first 48 h, mainly at the expense of palmitate. These changes were maintained for at least a further 4 days. Total monounsaturated fatty acid gradually increased, replacing saturated fatty acids until by day 6 the same amounts of each class of fatty acid were present.

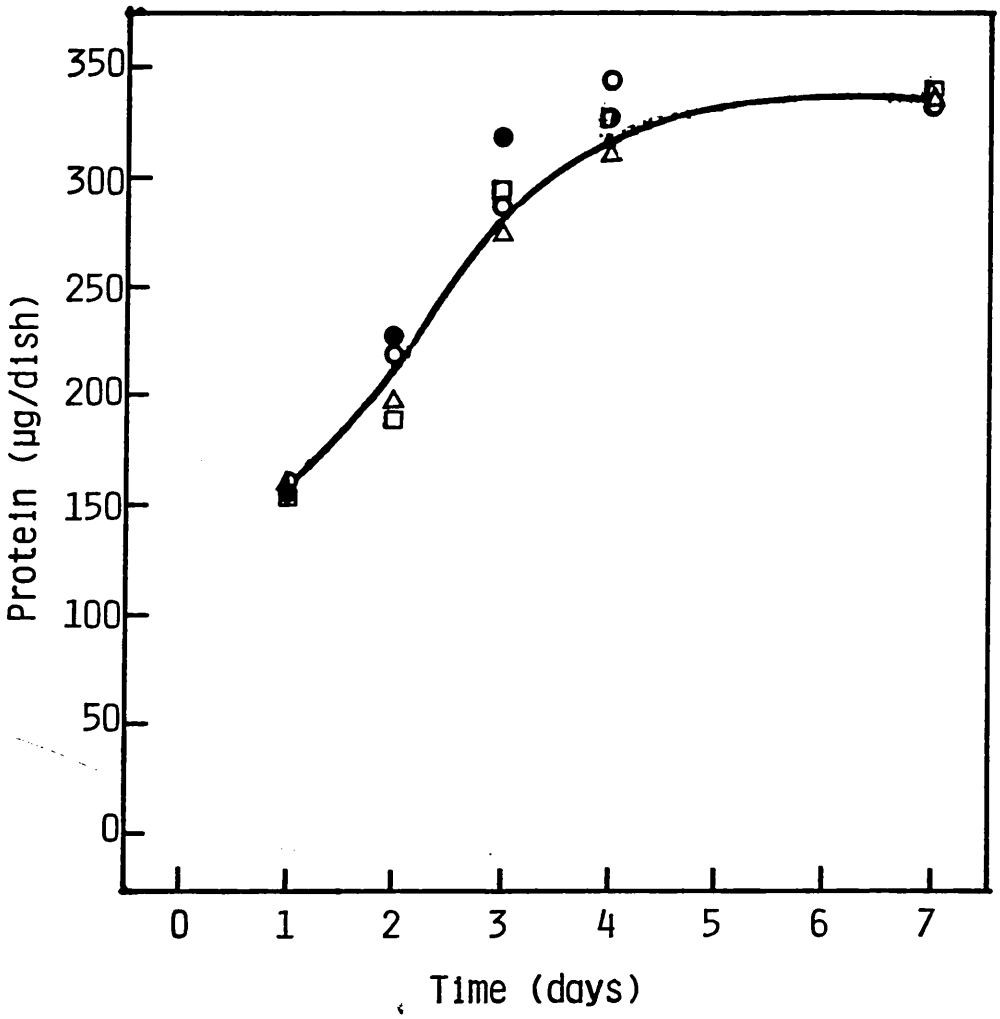


Figure 3.3 Effect of fatty acids on cell growth

Cells were seeded in medium containing foetal calf serum (10% v/v) with no supplementary fatty acids (△), or with 50 µM palmitate (●), 50 µM oleate (○) or 50 µM linoleate (□). After the indicated times dishes were washed with 3 x 3 ml PBS, the monolayers were dissolved in 2 ml NaOH (0.1 N) and the protein content of the dish determined. Each point represents the mean protein content of duplicate dishes.

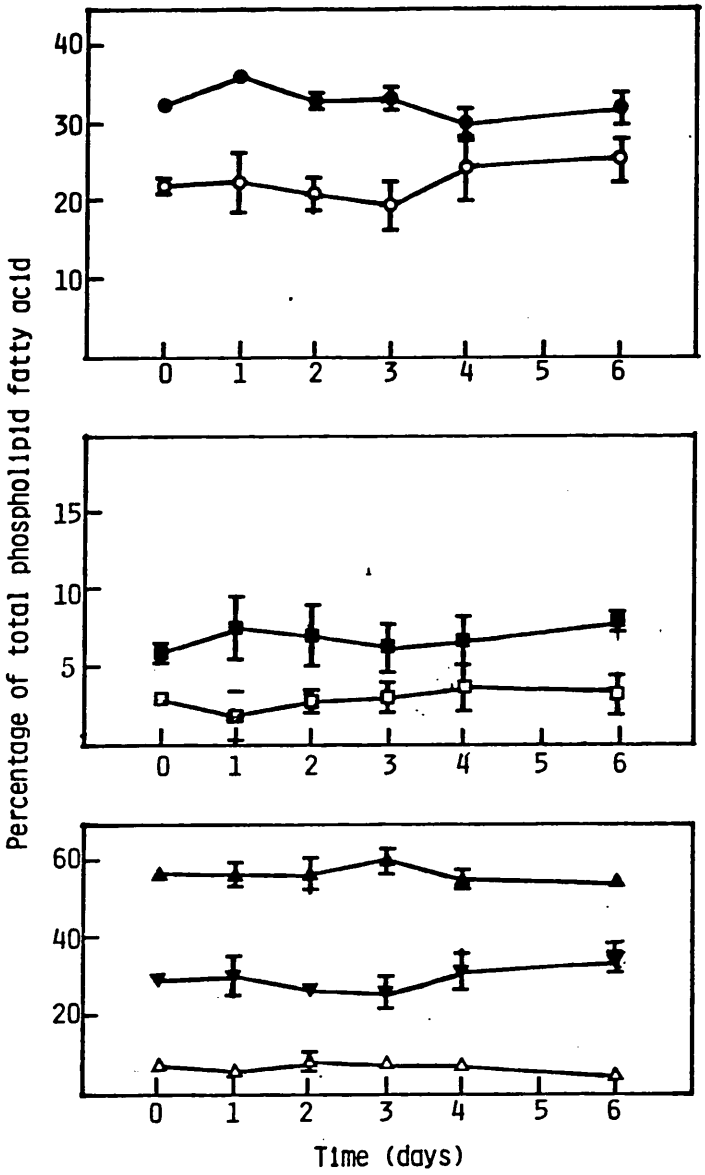


Figure 3.4 Variation in phospholipid fatty acids with time of growth in medium containing foetal calf serum without fatty acid supplement

Fibroblasts were seeded in medium containing foetal calf serum (10% v/v) and refed with the same medium after 1 and 4 days. At various times after the first refeeding (day 0) until up to 6 days later, the cells from triplicate dishes were pooled for analysis of their phospholipid fatty acids. The Figure shows the variation, with time, in the amounts of palmitate (●), oleate (○), palmitoleate (■) and linoleate (□) as well as total saturated (▲), monounsaturated (▼) and polyunsaturated (△) fatty acids. These amounts, expressed as a percentage of total phospholipid fatty acid, are shown as the mean and S.E.M. of 4 separate experiments.

Figure 3.5 Variation in phospholipid fatty acids with time of growth in medium containing 50 μM palmitate

Fibroblasts were grown for 24 h in medium containing foetal calf serum (10% v/v). The cells were refed after 1 and 4 days with medium containing foetal calf serum and supplemented with 50 μM palmitic acid. At various times after the addition of fatty acids to the growth medium (on day 0) until up to 6 days later, the cells from triplicate dishes were pooled for analysis of their phospholipid fatty acids. The Figure shows the variation, with time, in the amounts of palmitate (●), oleate (○), palmitoleate (■) and linoleate (□) as well as total saturated (▲), monounsaturated (▼) and polyunsaturated (△) fatty acids. These amounts, expressed as a percentage of total phospholipid fatty acid, are shown as the mean and S.E.M. from 4 separate experiments.

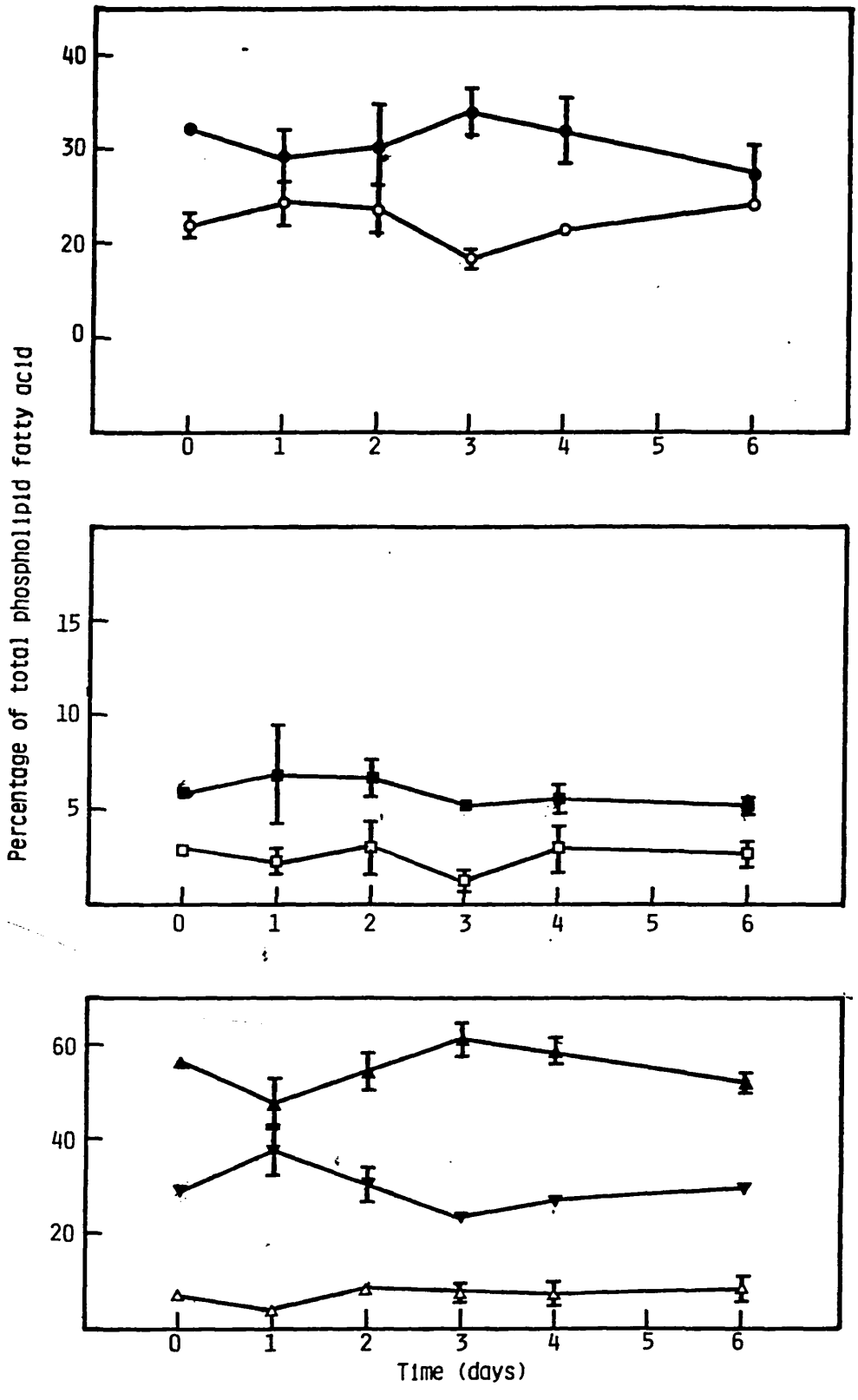
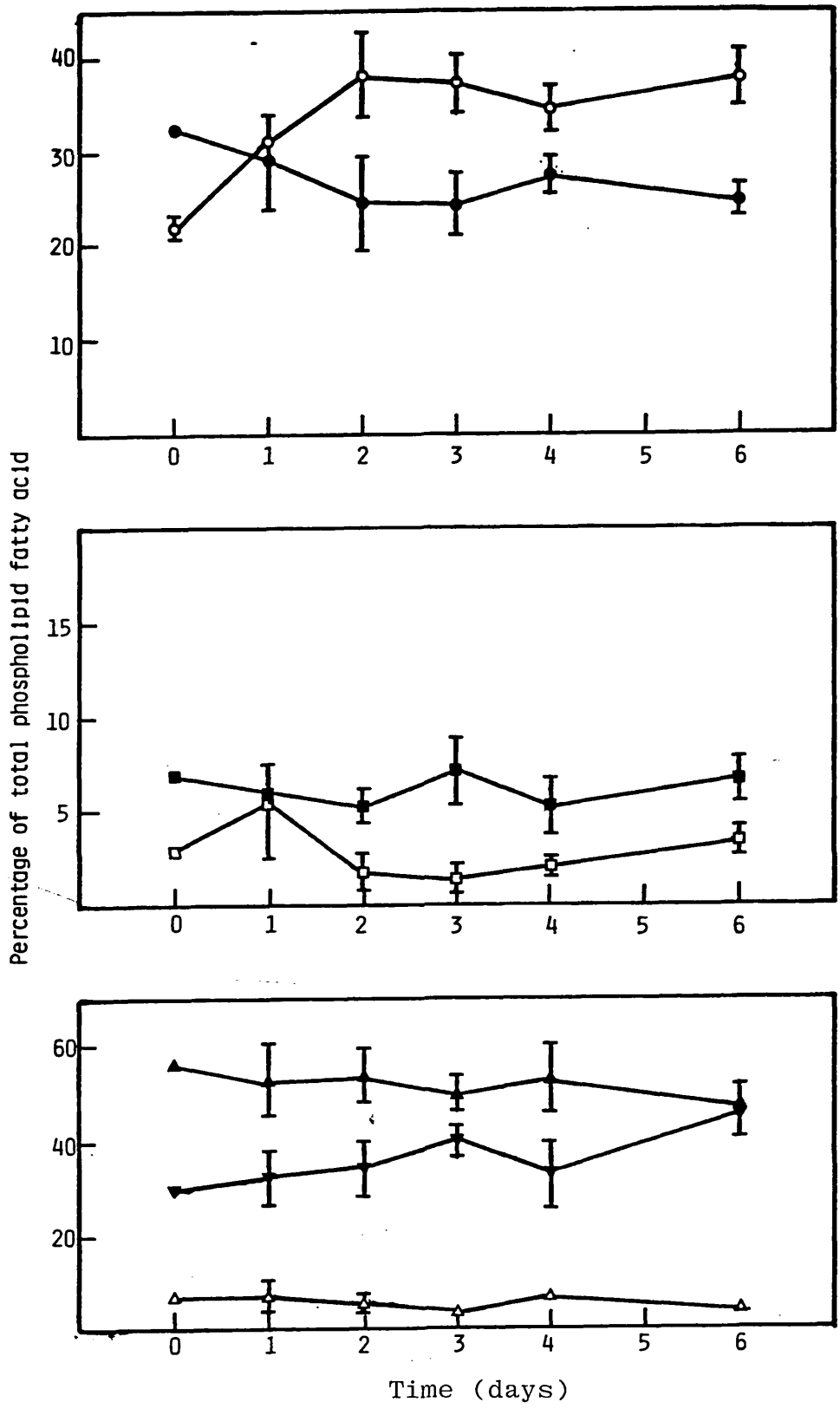

Percentage of total phospholipid fatty acid
40
30
20
0
15
10
5
60
40
20
0 1 2 3 4 5 6
Time (days)

Figure 3.6 Variation in phospholipid fatty acids with time of growth in medium containing 50 μM oleate

Fibroblasts were grown for 24 h in medium containing foetal calf serum (10% v/v). The cells were refed after 1 and 4 days with medium containing foetal calf serum and supplemented with 50 μM oleic acid. At various times after the addition of fatty acids to the growth medium (on day 0) until up to 6 days later, the cells from triplicate dishes were pooled for analysis of their phospholipid fatty acids. The Figure shows the variation with time in the amounts of palmitate (●), oleate (O), palmitoleate (■) and linoleate (□) as well as total saturated (▲), monounsaturated (▼) and polyunsaturated (Δ) fatty acids. These amounts, expressed as a percentage of total phospholipid fatty acid, are shown as the mean and S.E.M. from 4 separate experiments.


Percentage of total phospholipid fatty acid
40
30
20
10
0
1
2
3
4
5
6
15
10
5
60
40
20
Time (days)

Changes in the presence of 50 μM linoleate occurred even more rapidly (Fig. 3.7). After only 24 h, the proportion of this fatty acid had increased 3-fold in the membrane phospholipids. The increase continued at a more gentle pace, and after 6 days was about 5-fold higher than at the start. There was a slight fall in the amount of oleate and an even bigger fall in the amount of palmitate.

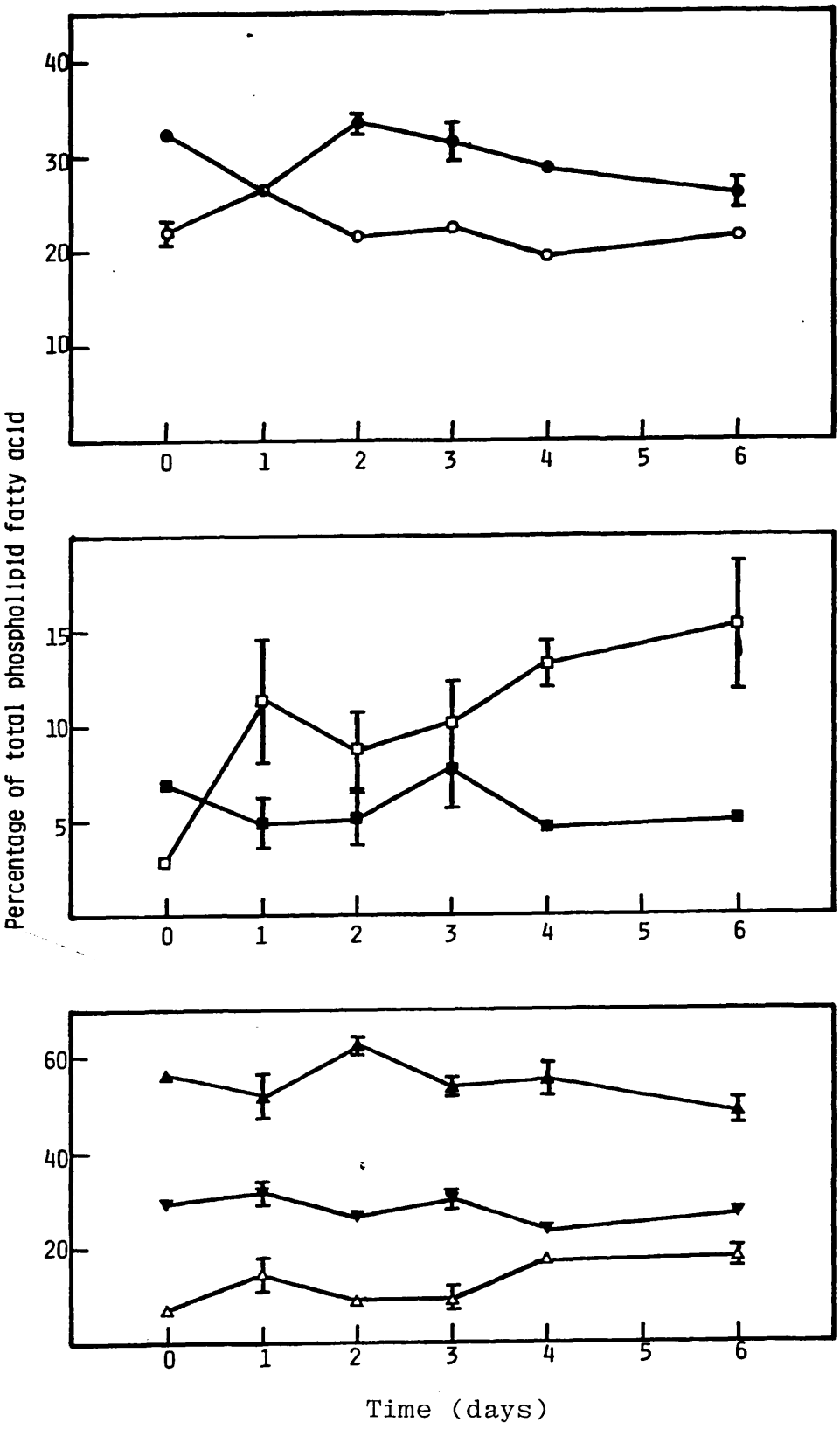
The mean and S.E.M. of the relative amounts of seven major fatty acids in cell membrane phospholipids were calculated from the results of a series of experiments in which fibroblasts were grown for 6 days in the presence of the 50 μM fatty acid supplement. The statistical significance of these figures was tested by comparison with the relative amounts of the same fatty acids in the phospholipid of fibroblasts grown for 6 days in medium without a fatty acid supplement (Table 3.3).

When cells were grown in the presence of palmitate there were slight but not significant increases in the proportion of palmitate (16:0) and stearate (18:0) accompanied by reciprocal decreases in palmitoleate (16:1) and oleate (18:1). These changes produced a significant decrease in the total proportion of monounsaturated fatty acids ($p<0.02$) but did not affect either the total proportion of saturated fatty acid or the ratio of polyunsaturated fatty acid to saturated fatty acid (P/S). This ratio has been used as an indicator of change in membrane fatty acid composition.

When cells were grown with oleate there were slight decreases in the proportions of both palmitate and palmitoleate

Figure 3.7 Variation in phospholipid fatty acids with time of growth in medium containing 50 μM linoleate

Fibroblasts were grown for 24 h in medium containing foetal calf serum (10% v/v). The cells were refed after 1 and 4 days with medium containing foetal calf serum and supplemented with 50 μM linoleic acid. At various times after the fatty acids were added to the growth medium (day 0), until up to 6 days later, the cells from triplicate dishes were pooled for analysis of their phospholipid fatty acids. The Figure shows the variation with time in the amounts of palmitate (●), oleate (○), palmitoleate (■) and linoleate (□) as well as total saturated (▲), monounsaturated (▼) and polyunsaturated (△) fatty acids. These amounts, expressed as a percentage of total phospholipid fatty acid are shown as the mean and S.E.M. from 4 separate experiments.


Percentage of total phospholipid fatty acid
40
30
20
10
15
10
5
60
40
20
0
1
2
3
4
5
6
Time (days)

accompanied by a significant 33% increase in the proportion of oleate ($p<0.02$) when compared with cells grown in medium without supplementary fatty acids. However, these changes failed to affect the total proportions of saturated or monounsaturated fatty acids or the P/S ratio.

Neither oleate nor palmitate added to the growth medium altered the relative amounts of linoleate (18:2), linolenate (18:3), arachidonate (20:4) or total polyunsaturated fatty acids found in cells grown in medium without a fatty acid supplement.

The most spectacular changes in the phospholipid fatty acid composition of fibroblasts occurred when they were grown in medium supplemented with linoleate. Compared with cells grown in medium without a fatty acid supplement, the proportion of linoleate itself underwent a highly significant 4-fold increase ($p<0.001$), compensated for by a slight decrease in oleate and significant decreases in palmitate ($p<0.02$) and palmitoleate ($p<0.001$). This produced a significant decrease in total monounsaturated fatty acids ($p<0.02$) and significant increases in both total polyunsaturated fatty acid ($p<0.001$) and the P/S ratio ($p<0.001$).

As would be expected, when the fatty acid compositions of phospholipids from cells grown with different fatty acids are compared directly instead of with that of cells grown without a fatty acid supplement, the contrasts are even more pronounced.

The phospholipids of cells grown in palmitate contain significantly more palmitate than those of cells grown in oleate ($p<0.02$) or linoleate ($p<0.01$), while cells grown in oleate have

significantly more of that fatty acid in their phospholipids than cells grown in palmitate ($p<0.002$) or linoleate ($p<0.001$). The phospholipids of cells grown with linoleate contain more linoleate than those of cells grown in palmitate ($p<0.001$) or oleate ($p<0.001$). They also contain more linolenate than those of cells grown with palmitate ($p<0.02$) or oleate ($p<0.05$). Predictably, these changes mean that cells grown with palmitate have a greater proportion of saturated fatty acids in their phospholipid than cells grown with oleate ($p<0.05$) or linoleate (<0.02), cells grown with oleate have a greater proportion of monounsaturated fatty acids in their phospholipid than cells grown with palmitate ($p<0.01$) or linoleate ($p<0.01$) while cells grown with linoleate have a greater proportion of polyunsaturated fatty acids in their phospholipids than cells grown with palmitate ($p<0.001$) or oleate ($p<0.001$). Although there is no significant difference between the P/S ratios of the phospholipids from cells grown with palmitate or oleate, the P/S ratio of phospholipids from cells grown with linoleate is significantly greater than that of phospholipids from cells grown in palmitate ($p<0.001$) or oleate ($p<0.001$).

After 6 days growth in medium containing 10% FCS supplemented with 50 μM fatty acid, a further 24 h in medium containing 5% LPDS and the same fatty acid supplement failed to produce any further significant changes in the fatty acid composition of membrane phospholipids (Table 3.4).

The maximum incorporation of fatty acids into cellular phospholipid could be expected to take place at times of fresh

Table 3.4

A comparison of the composition of phospholipid fatty acids of fibroblasts grown in FCS or LPDS supplemented with different fatty acids

Cells were seeded in medium containing FCS and refed after 1 and 4 days with medium containing FCS and supplemented with fatty acids as described. After 7 days the cells were washed with Puck's saline A and refed with medium containing either FCS or LPDS and supplemented as before. After a further 24 h the cells from triplicate dishes were pooled for the analysis of their phospholipid fatty acids. The results are presented as the mean ± S.E.M. of several experiments; the number of experiments is given in parentheses after each condition. There are no significant differences (paired *t*-test) between values for cells grown in FCS and the equivalent values for those grown in LPDS.

	No Additions		*50 μM Palmitate*		*50 μM Oleate*		*50 μM Linoleate*	
	FCS (3)	*LPDS (5)*	*FCS (3)*	*LPDS (5)*	*FCS (6)*	*LPDS (7)*	*FCS (5)*	*LPDS (3)*
16:0	29.5±3.2	29.8±1.7	27.4±2.9	33.6±1.2	27.4±1.9	24.9±2.0	26.0±1.4	21.3±1.1
16:1	7.0±1.0	8.5±0.5	5.2±0.4	6.4±0.6	6.2±1.0	6.5±0.8	5.0±0.3	4.3±0.4
18:0	23.7±1.5	18.4±1.9	25.3±1.0	21.4±1.4	22.1±3.2	18.1±1.1	22.7±1.5	19.3±2.1
18:1	23.3±4.1	24.0±1.9	24.3±0.2	20.2±1.6	34.9±2.7	29.2±3.4	21.6±1.7	17.0±3.6
18:2	3.3±1.0	4.2±1.1	2.8±0.6	2.2±0.5	2.8±0.6	2.8±0.7	15.2±3.4	16.0±5.1
18:3	0.6±0.3	1.0±0.2	0.5±0.3	0.3±0.1	0.6±0.1	0.4±0.1	0.6±0.1	0.8±0.1
20:4	2.7±1.9	3.0±1.7	5.3±2.2	2.9±1.6	3.1±0.2	4.1±1.4	6.2±0.5	4.6±0.8

membrane synthesis. This obviously occurs when cells are dividing regularly. In order to test the effect of cell division on the incorporation of fatty acids into phospholipids, fibroblasts were seeded at various densities from 1.7×10^4 to 5.6×10^5 cells/60 mm dish. After 6 days in the presence of either 50 μM oleate or 50 μM linoleate those cells seeded at the lowest densities were growing exponentially while those cells seeded at the highest densities were confluent and scarcely dividing at all. Exogenous oleate seemed to be most effectively incorporated into membrane phospholipids by fibroblasts which had been seeded at high densities (Fig. 3.8(a)). The increase in the proportion of oleate with density of seeding was accompanied by a small increase in the proportion of linoleate and large decreases in the proportions of the saturated fatty acids palmitate and stearate.

In complete contrast, exogenous linoleate was most effectively incorporated into membrane phospholipids by fibroblasts seeded at lower densities (Fig. 3.8(b)). The maximum incorporation was achieved by cells seeded at 5×10^4 per dish, yielding about 260 μg protein/dish on day 6. Compared with this maximum, cells seeded at lower densities had slightly less linoleate and slightly more oleate in their phospholipids, while at higher densities there is less linoleate but more stearate and palmitate.

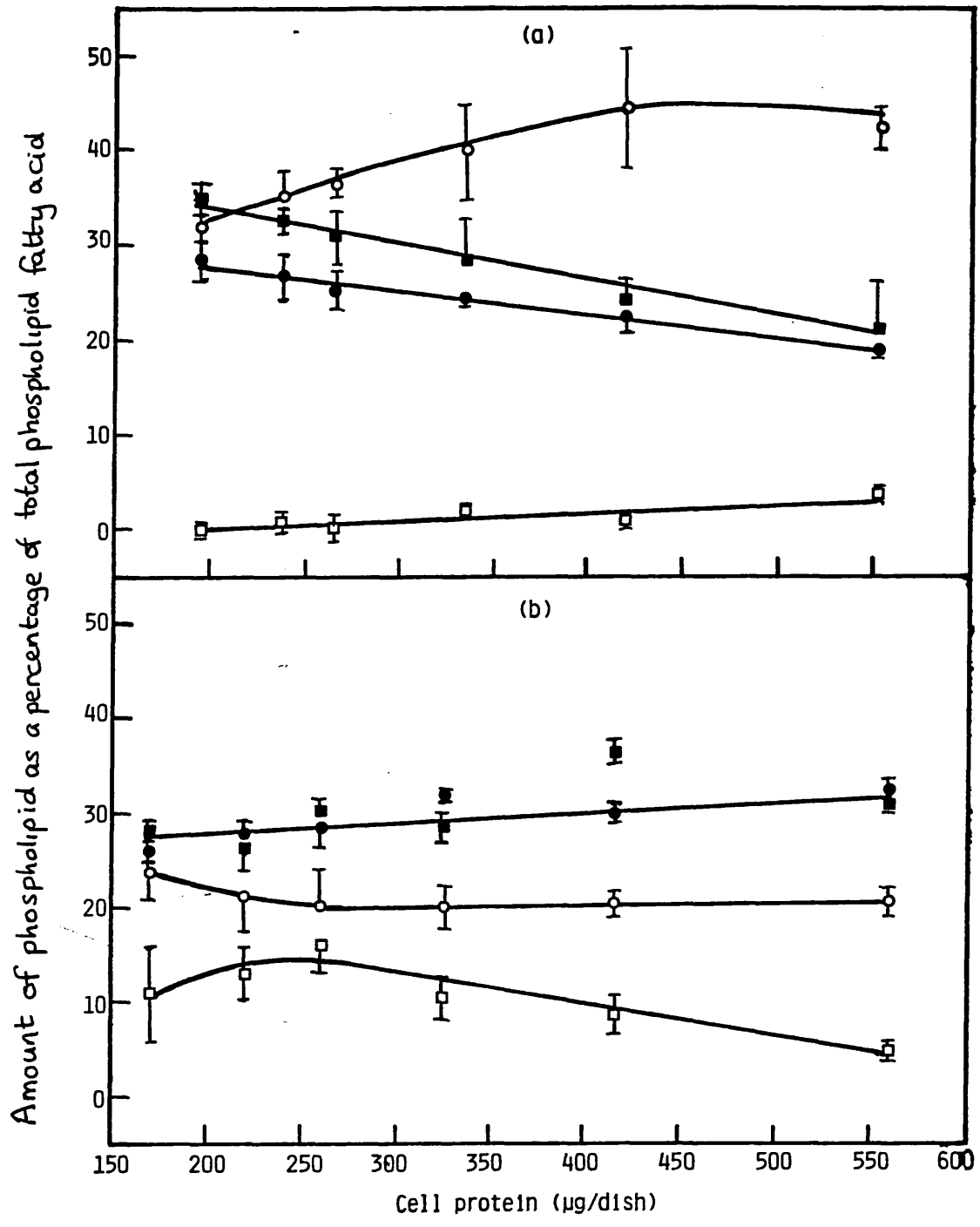


Figure 3.8 Effect of density of seeding upon the incorporation of fatty acid into fibroblast phospholipids

Fibroblasts were seeded at 17, 35, 70, 140, 280 and 560 thousand cells/dish and grown as described in the legend to Fig. 3.2, in the presence of either 50 µM oleate (a) or 50 µM linoleate (b). After 6 days in the presence of fatty acids the cells from triplicate dishes were pooled for analysis of their phospholipid fatty acid, while the cells from a fourth, identically treated dish were dissolved in 2 ml NaOH (0.1 N) for measurement of their protein content. The Figure shows the variation of palmitate (●), stearate (■), oleate (○) and linoleate (□) with the final protein concentration in the dish($n=2$).

3.4 Discussion

These results confirm the findings of Spector *et al.* (1979) that the fatty acid composition of fibroblast phospholipids may be influenced by the fatty acids present in the growth medium. Thus palmitate, which represents 30% of the fatty acid in serum lipid, also represents 30% of phospholipid fatty acids in fibroblasts. Similarly oleate, which forms 25% of the fatty acid in serum lipid also represents 25% of fibroblast phospholipid fatty acids. However, despite representing 20% of the fatty acid in serum lipid, linoleate only represents 3% of the cellular phospholipid fatty acid. Thus all fatty acids are not incorporated into phospholipids at the same rate. This is because the acyltransferase enzyme does not have the same affinity for the coenzyme A esters of all fatty acids (Lands *et al.*, 1982). The relative amounts of each fatty acid in fibroblast phospholipids seem to be controlled within certain limits. Thus the proportion of palmitate, which is the major fatty acid in the phospholipids of cells grown in medium without supplementary fatty acid, barely increases, even when the fatty acid is present in the growth medium at a concentration of 100 μM. The possibility that stearate may be formed by the elongation of palmitate is suggested by the observation of a slight increase in the proportion of stearate in cells grown in the presence of supplementary palmitate, although this increase is not statistically significant. Stearate may, in turn, be desaturated to form oleate since, over a period of 6 days, the initial increases in the proportions of palmitate and

stearate, at the expense of oleate, were reversed as the relative amounts of all three fatty acids tended towards their original levels. Although the proportion of palmitate in phospholipids cannot be raised above 40%, it can drop substantially to accommodate an increase in other fatty acids which might be added to the medium.

The normal proportion of oleate in cell phospholipids is such that it can be increased or decreased depending upon the circumstances. In the presence of high concentrations of supplementary oleate it can form 50% of the phospholipid fatty acid while, in the presence of high concentrations of other fatty acids, it may form only 20%. However, in this respect, it appears that the proportion of palmitate is more likely to decrease to accommodate increases in the amount of other fatty acids.

In contrast, the proportions of some fatty acids, such as linoleate, are normally low and do not decrease significantly when fibroblasts are grown in the presence of other fatty acids. However, the proportion of linoleate can be considerably increased if it is the predominant fatty acid in the medium. This increase in linoleate is accompanied by decreases in the amounts of other fatty acids, mainly palmitate and oleate.

These findings raise the possibility that the composition of a certain proportion of the fatty acids in phospholipids may be resistant to change while the remainder can be varied in response to the composition of the fatty acids in the growth medium. It is, perhaps, not surprising, given the importance

of membranes in the biochemical and physiological processes within cells, that certain constraints are placed on the variation of their physical characteristics.

It might be expected that any enrichment of the cellular phospholipid with fatty acids from the growth medium would be especially noticeable when the cells underwent a prolonged period with a comparatively high rate of membrane turnover. When the medium is supplemented with linoleate this fatty acid does seem to be more effectively incorporated into the phospholipids of cells seeded at lower densities than of cells seeded at higher densities. However, the reverse appears to be the case when the medium is supplemented with oleate, when more of this fatty acid is incorporated by cells seeded at higher densities. This again suggests that with changing circumstances the membrane may be required to display different physical properties and consequently require different fatty acids to be incorporated into the phospholipid.

By modifying the relative amounts of saturated, monounsaturated and polyunsaturated fatty acids in membrane phospholipids, the arrangement of the phospholipid within the bilayer can be altered, bringing about changes in many membrane functions (Horowitz *et al.*, 1974; Houslay *et al.*, 1976; Solomonsen *et al.*, 1976; Shinitzky and Rivnay, 1977; Muller and Shinitzky, 1979; Schroeder, 1981). Growing fibroblasts in medium supplemented with a high concentration of the required fatty acid offers a simple and convenient method for effecting these modifications.

From these experiments, a standard procedure emerged for the incorporation of fatty acids into membrane phospholipid. After being seeded at 5×10^4 cells/60 mm dish in 3 ml of medium containing FCS (10% v/v) the cells were refed after 1 and 4 days with the same medium containing a 50 μM fatty acid supplement. After 6 days in the presence of fatty acids, the cells were refed with medium containing LPDS and the same fatty acid supplement. After a further 24 hours the experiments could be performed. Since 24-hour growth in medium with LPDS brought about no further changes in the fatty acid composition of fibroblast phospholipids, this method could be used to examine the effects of fatty acids on various aspects of LDL metabolism.

Chapter 4

CATABOLISM OF LOW-DENSITY LIPOPROTEIN BY FIBROBLASTS

4.1 Introduction

Receptor-mediated endocytosis is the means by which cells are able to extract specific molecules from their environment and internalise them. It is responsible for the uptake of a range of ligands including hormones, growth factors, lysosomal enzymes and LDL (Brown *et al.*, 1983*a*). Because of the probable role of LDL in the build-up of atherosclerotic lesions (Goldstein and Brown, 1977), its receptor has been extensively studied at both cellular and molecular levels. These studies have led, not only to a clearer understanding of lipoprotein metabolism, but also to a general understanding of the fundamental mechanisms of receptor-mediated endocytosis.

Diets rich in polyunsaturated fatty acid lower the plasma concentration of cholesterol (Kinsell *et al.*, 1952; Keys *et al.*, 1965) and, in particular, the concentration of LDL (Spritz and Mishkel, 1969; Shepherd *et al.*, 1980). The decrease, which is brought about, at least in part, by an increased rate of LDL catabolism, is accompanied by changes in the lipid composition of the LDL particle (Shepherd *et al.*, 1980). However, these changes in the lipoprotein lipids are not necessarily responsible

for the increased catabolic rate since modifications of the fatty acid composition of cell membrane phospholipid are associated with changes in the endocytotic rate of those cells (Schroeder, 1981).

However, despite these observations and the extensive use of cultured cells to elucidate the LDL receptor pathway in cultured cells (Goldstein and Brown, 1977), little use has been made of cultured cells in attempting to discover how polyunsaturated fatty acids produce their effects.

It was shown, in the previous chapter, that the fatty acid composition of fibroblast membrane phospholipids could easily be altered by growing the cells in the presence of the required fatty acid. To examine whether this had any effect on the LDL receptor, existing procedures for measuring the binding, internalisation and degradation of [^{125}I]LDL by cultured cells were used (Goldstein and Brown, 1974*b*; Goldstein *et al.*, 1976). After characteristing this assay with cells grown under normal conditions it was possible to examine any changes brought about by the growth of the cells with fatty acids. In particular, the binding of LDL to its receptor was examined since this was believed to be the rate-limiting step for the entire catabolic process (Goldstein and Brown, 1974*b*).

To examine more closely the relationships between the composition of cellular phospholipid and changes in LDL catabolism, the binding, internalisation and degradation of the lipoprotein were measured as one fatty acid was gradually replaced by another in the growth medium supplement.

4.2 Methods and materials

LDL was labelled with ^{125}I by the method of McFarlane (1958) as described by Bilheimer *et al.* (1975).

Glycine buffer consisted of 100 mM glycine / 100 mM NaCl / 63 mM NaOH (pH 10.0) and also contained sodium azide (100 µg/ml). A stock solution of ICl (3.24 mg/ml) was prepared. This was equivalent to 2.5 mg/ml of iodine. Since the equivalent of 5 µg of iodine were required for each mg of LDL protein this was diluted with 2 M NaCl before use to give the required amount of ICl in 50 µl. LDL was isolated from plasma, and 1 ml containing approximately 10 mg protein was dialysed against 1 litre of glycine buffer. In a typical preparation 20 µl of Na ^{125}I (2 mCi) were added to 500 µl of LDL containing approximately 5 mg protein. To this were added 50 µl ICl (containing 25 µg I after a 5-fold dilution with 2 M NaCl) and the contents of the tube were immediately shaken vigorously. The bulk of the free radioactive iodide was removed by running the reaction mixture through a 10 ml Sephadex G100 column (3.5 ml void volume). The fraction containing the labelled LDL was collected, dialysed against 4 changes of 1 litre Dulbecco's phosphate-buffered saline and sterilised by filtration through a 0.22 µm Millex filter. The solution was stored at 4°C until used, after aliquots had been taken to assay the protein concentration and to measure the amount of radioactivity associated with the lipoprotein. LDL labelled by this method usually gave approximately 200 counts/min/ng LDL protein.

This was diluted with cold LDL to give a solution giving approximately 30 counts/min/ng LDL protein which was used for experiments.

Binding, internalisation and degradation of LDL were measured by the methods of Goldstein and Brown (1974*b*), Goldstein *et al.* (1976) and Goldstein *et al.* (1983*b*). Fibroblasts for experiments were grown as described either in Section 2.1 or Section 3.4. After a 24 h preincubation in medium containing LPDS, radioactive LDL was added to the medium and the cells were incubated for the required time at 37°C. After this, 1.5 ml of the medium was removed and stored at -20°C until assayed for radioactive LDL degradation products (see below).

The cells were washed rapidly three times with 2 ml of ice-cold 150 mM NaCl/50 mM Tris/HCl (pH 7.4 at 4°C) containing bovine serum albumin (2 mg/ml). The cells were then incubated twice for 10 min at 4°C with 2 ml of the same washing solution followed by one rapid wash with 2 ml of ice-cold 150 mM NaCl/ 50 mM Tris/HCl (pH 7.4 at 4°C). The fibroblasts were then incubated at 4°C for 60 min in 2 ml of ice-cold 150 mM NaCl/ 10 mM HEPES containing heparin (10 mg/ml). Heparin forms a complex with LDL which leads to the lipoprotein becoming detached from fibroblast cell surface receptors. Therefore, with the extensive washing procedures which preceded this incubation, the amount of radioactivity released by heparin was a measure of the quantity of LDL bound at the cell surface. The fibroblasts were washed once more with 2 ml of ice-cold 150 mM NaCl/50 mM Tris/HCl

and then dissolved by incubation at room temperature for at least 15 min with 2 ml 0.1 N NaOH. The radioactivity in this solution represented the LDL which had been internalised by the cell but had yet to be completely degraded. An aliquot of the dissolved cells was also taken to assay the amount of fibroblast protein in each dish. When LDL is degraded in the lysosomes of cells the protein component is hydrolysed into its constituent amino acids (Goldstein and Brown, 1977). In the case of [^{125}I]LDL this results in the release of radioactive iodotyrosine which is secreted by the fibroblasts and accumulates in the culture medium. The degradation of LDL can therefore be assayed by measuring the amount of trichloroacetic acid-soluble, non-iodide radioactivity present in the medium after the labelled lipoproteins have been incubated with cells. The frozen medium was thawed and placed in an ice/water bath for 15 min. To 1.5 ml of medium were added 370 µl trichloroacetic acid (500 mg/ml). The tubes were shaken vigorously and allowed to stand in the ice/water bath for 30 min before centrifugation at 200 x *g* at 4°C for 20 min in a Mistral 6L centrifuge. This precipitated from the medium any radioactive LDL which had not been degraded. 1 ml of the supernatant was taken into a fresh tube, 14 µl KI (300 mg/ml) and 40 µl H_2O_2 (30% w/v) were added and the tubes shaken. After being allowed to stand for 5 min at room temperature any iodide present was oxidised to iodine and was removed by two extractions with 2.5 ml chloroform. The radioactivity measured in 750 µl of the supernatant was used to calculate the amount of LDL degraded by the fibroblasts

during the incubation period (Bierman *et al.*, 1974). The binding of LDL by fibroblasts at 4°C was measured as described by Goldstein *et al.* (1976). Following a 24 h preincubation the medium containing LPDS was replaced with 2 ml of the same medium, also containing LPDS but lacking $NaHCO_3$. After the cells had been allowed to cool for 30 min in a 4°C cold room, labelled LDL was added to the medium and the fibroblasts incubated for the required time at this temperature. The cells were washed as described above but, instead of incubating with heparin, the cells were dissolved in 0.1 N NaOH. Since no LDL was internalised or degraded at 4°C, the radioactivity detected in the sodium hydroxide was a measure of the LDL bound to the fibroblast surface.

Some LDL binds to the fibroblast surface independently of the LDL receptor. Allowance for this non-specific binding was made by measuring the amount of labelled LDL binding to the cell surface in the presnce of a 100-fold excess of unlabelled LDL. Similarly, the small proportion of labelled LDL which breaks down in the medium independently of the fibroblasts was allowed for by measuring the release of trichloroacetic acid-soluble, non-iodide radioactivity in the medium incubated at 37°C for the required time in the absence of cells.

Radioactivity was measured using an LKB Wallac 80,000 Gamma Sample Counter obtained from LKB-Produkter AB, Bromma, Sweden. Na ^{125}I, carrier free was purchased from Amersham International.

HEPES was purchased as a 1 M solution (pH 7.4) from Gibco Europe.

Trichloroacetic acid, ICl, KI and NaCl were from BDH Chemicals, hydrogen peroxide (30% w/v) was from Hopkins and Williams, Chadwell Heath, Essex, U.K. and chloroform from May and Baker. Sephadex G100 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Tris, bovine serum albumin (fraction V) and heparin (lithium salt, 160 USP units/mg) were purchased from Sigma Chemical Company.

4.3 Results

In the binding assay ^{125}I not associated with the cells was removed by extensive washings (Fig. 4.1(a)). The bulk of the LDL was removed in the course of this washing and the LDL which remained could only be removed by incubation with washing buffer containing heparin (Fig. 4.1(b)). The removal of LDL from its receptor by heparin was essentially complete after an incubation of 60 min (Fig. 4.2).

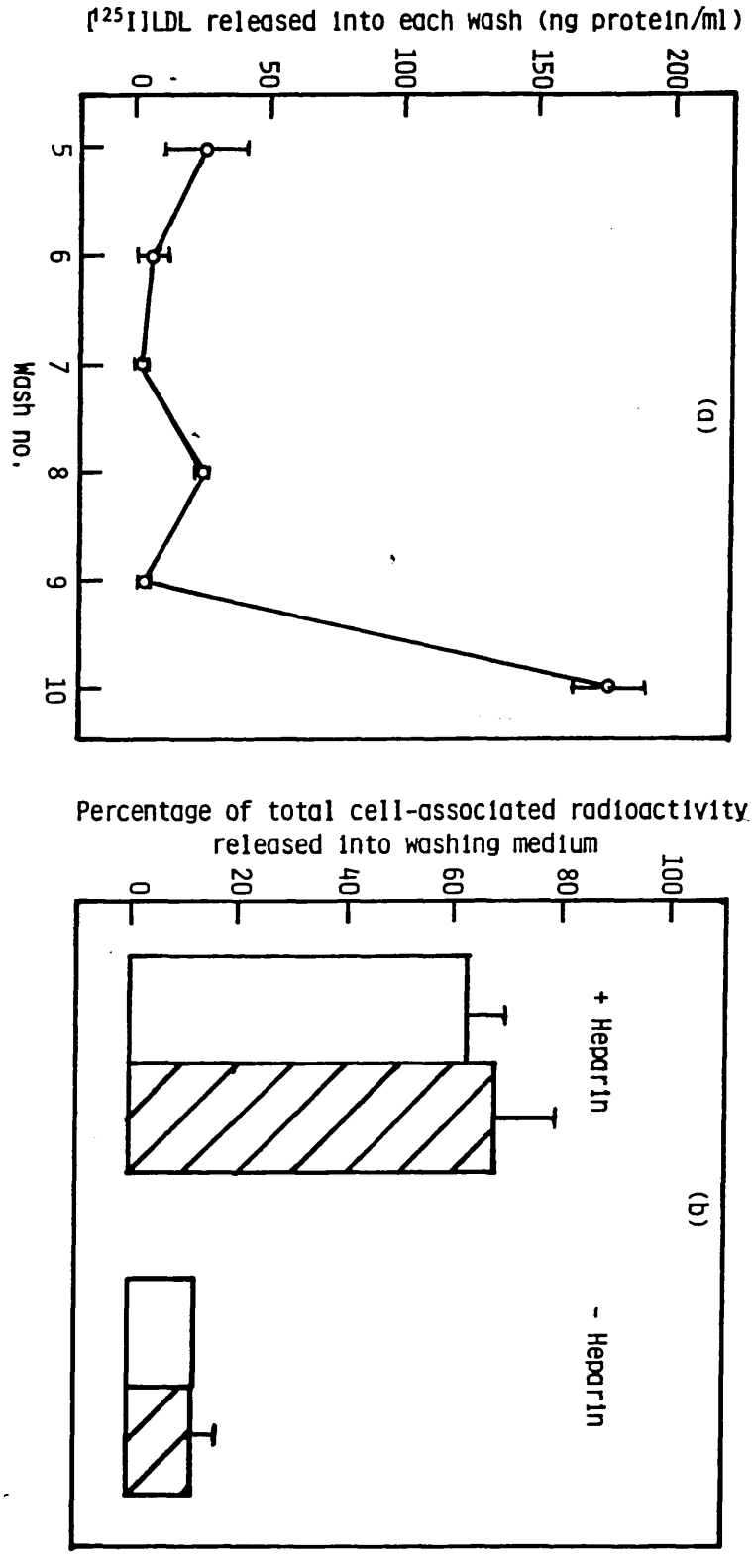
This washing procedure (Goldstein *et al.*, 1976) was followed in all further experiments except that the dishes of fibroblasts were not shaken on a rotary shaker during the incubation with heparin since this did not seem to be necessary (Fig. 4.1(b)).

Binding, internalisation and degradation of LDL by fibroblasts grown under normal conditions could now be examined. At both 4°C and 37°C, the binding process was complete within 2 h (Fig. 4.3(a)). More LDL was bound by cells at 37°C than at 4°C, although the amount of LDL bound by fibroblasts at 37°C

Figure 4.1 Procedure for washing away unbound [^{125}I]LDL in binding assay

(a) 4 Dishes (60 mm) of fibroblasts were incubated for 5 h at 37°C with [^{125}I]LDL (5 µl LDL protein/ml). The cells were washed rapidly 3 times with 2 ml 150 mM NaCl/50 mM Tris-HCl (pH 7.4) containing bovine serum albumin (2 mg/ml) and were then incubated for 2 periods of 10 min with 2 ml of the same buffer. The monolayers were washed twice with 2 ml of the same buffer without albumin and incubated for 1 h with 2 ml 150 mM NaCl/10 mM HEPES containing heparin (10 mg/ml). After a further wash with 2 ml 150 mM NaCl/50 mM Tris-HCl without albumin the cells were dissolved in 0.1 N NaOH. The amount of [^{125}I]LDL released into washes 5-10 was measured. Wash 8 was the heparin incubation, wash 10 was the addition of 0.1 N NaOH.

(b) 16 Dishes (60 mm) of fibroblasts were incubated for 2 h at 4°C with [^{125}I]LDL and washed as described above. 8 Dishes were incubated with 150 mM NaCl/10 mM HEPES containing heparin (10 mg/ml) during wash 8, while the remaining 8 dishes were incubated with 150 mM NaCl/10 mM HEPES, without heparin. 4 Dishes from each set were shaken at 60 oscillations/min on a rotary shaker (plain bar) with the remaining 4 dishes allowed to stand undisturbed (striped bar) during this incubation. The results are expressed as the percentage of total cell associated radioactivity (wash 8 + wash 10) released during wash 8. The amount of radioactivity released by cells incubated with heparin was significantly higher than the amount released by cells incubated without heparin ($p<0.001$, Student's t-test).


[125I]LDL released into each wash (ng protein/ml)
0
50
100
150
200
Wash no.
5
6
7
8
9
10
(a)
Percentage of total cell-associated radioactivity released into washing medium
0
20
40
60
80
100
+ Heparin
- Heparin
(b)

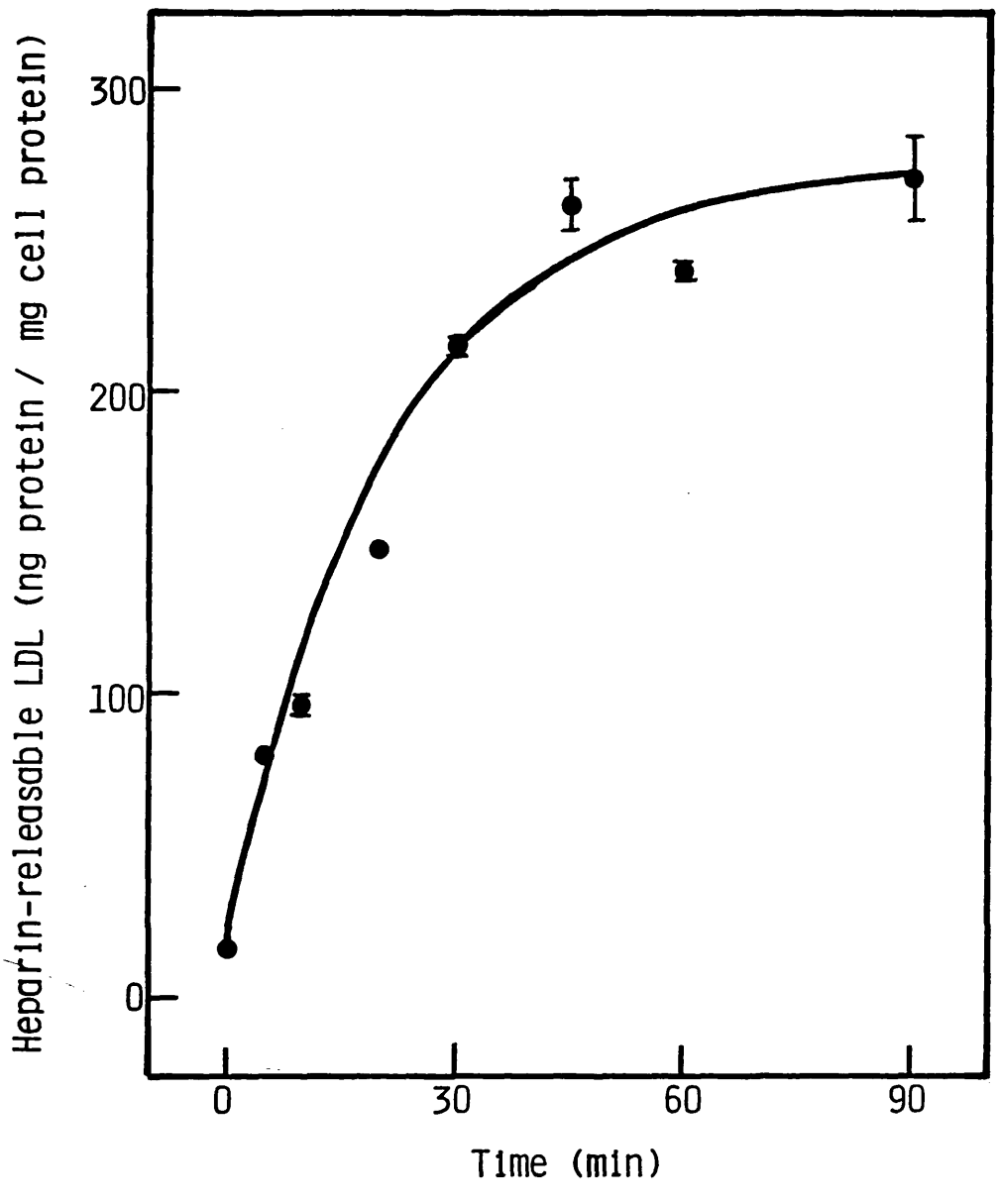
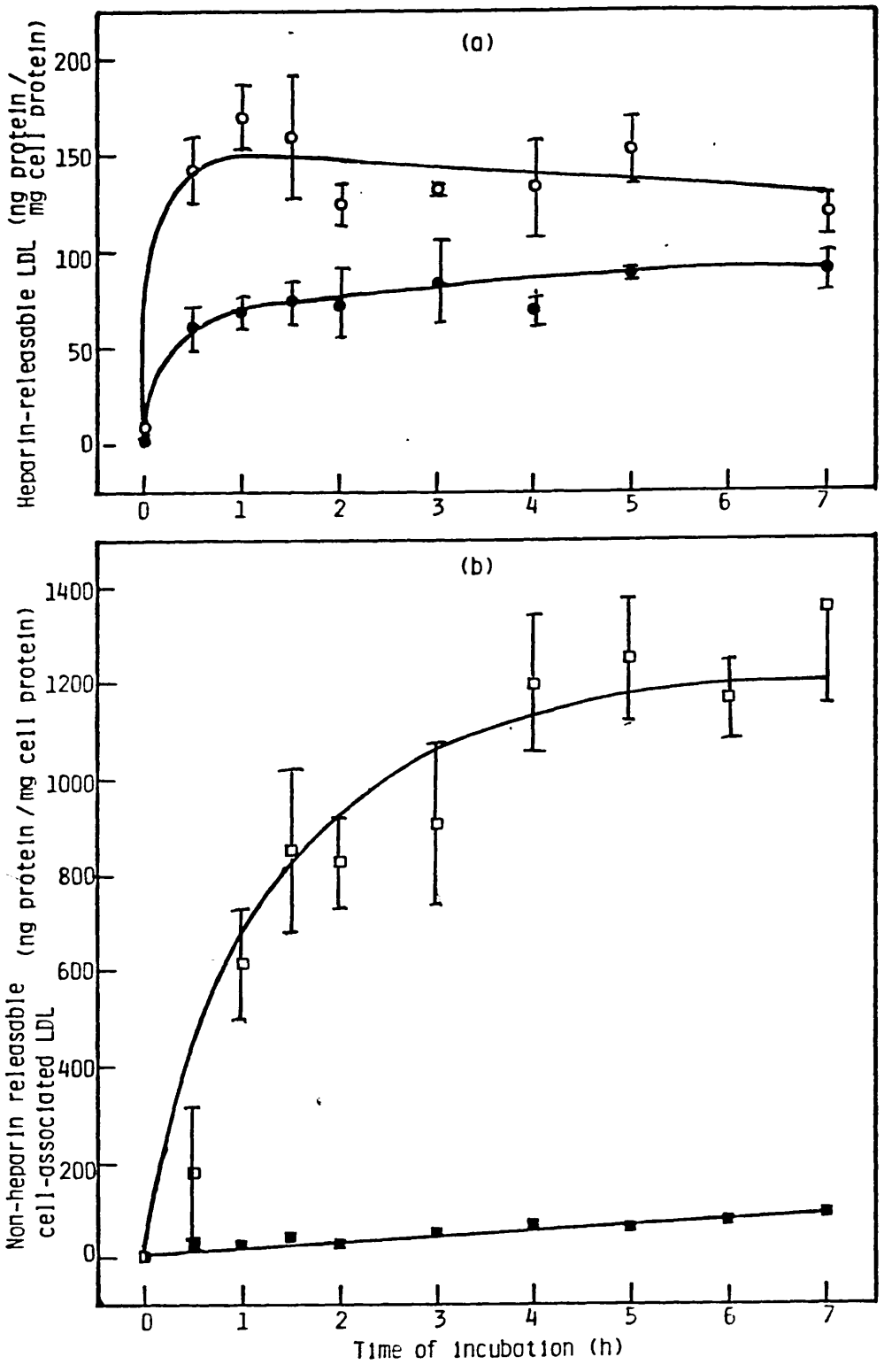


Figure 4.2 Effect of time on the amount of LDL released from the cell surface by heparin

Cells were incubated for 5 h at 37°C with [^{125}I]LDL (5 μg protein/ml). Lipoprotein which was not then associated with the cells was removed by washing the monolayer as described. The cells were then incubated for varying times at 4°C with 50 mM NaCl/10 mM HEPES containing heparin (10 mg/ml). The radioactivity released by this incubation was measured. The values are the average of duplicate dishes.

Figure 4.3 Effect of time of incubation on (a) binding and (b) internalisation of [^{125}I]LDL by fibroblasts at 4°C (closed symbols) and 37°C (open symbols)

Cells were grown for 6 days in medium containing foetal calf serum and for a further 24 h in medium containing lipoprotein-deficient serum. Some dishes then had their medium replaced with medium containing lipoprotein-deficient serum but lacking $NaHCO_3$ and were placed at 4°C for at least 30 min before the start of the experiment. At zero time [^{125}I]LDL (19.0 cpm/ng LDL protein) was added at a concentration of 30 μg LDL protein/ml to dishes at 37°C and at 5 μg/ml to dishes at 4°C. At various times, binding and internalisation of LDL by the fibroblasts was assayed by the methods described in the text. The points represent the means of duplicate dishes.


(a)
Heparin-releasable LDL (ng protein / mg cell protein)
200
150
100
50
0
0
1
2
3
4
5
6
7
(b)
Non-heparin releasable (ng protein / mg cell protein) cell-associated LDL
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
Time of incubation (h)

declined with time, presumably reflecting the down-regulation of cell-surface receptors occurring after fibroblasts have taken up cholesterol (Goldstein and Brown, 1977). At 4°C there was virtually no detectable internalisation or degradation (Figs. 4.3(b) and 4.4). However, at 37°C the amount of LDL internalised by the cells rose sharply over the first 2-3 h. The rate of increase then slowed and levelled off by 5-6 h (Fig. 4.3(b)). There was a 30 min lag before the products of the degradative process began to accumulate in the medium (Fig. 4.4). The rate of degradation then remained constant for at least a further 4 h before dropping slightly by 7 h, probably as a consequence of the observed decrease in the number of cell -surface receptors with time. The delay at the start of the incubation would give rise to an underestimation of the rate of degradation. This can obviously be minimised by continuing the incubation for as long as possible and therefore binding, internalisation and degradation of LDL by fibroblasts at 37°C were all measured after 5 h incubations. Binding at 4°C could be assayed after the fibroblasts had been incubated with LDL for 2 h. Since binding of LDL by the receptor has been believed to be the rate-limiting step for the entire catabolic process (Goldstein and Brown, 1974*b*), any observed changes brought about by fatty acids might be expected to result from changes in the properties of the LDL receptor. To gain an indication of the number of LDL binding sites and the affinity of those binding sites for the lipoprotein, the relationship between the concentration of LDL in the medium and the amount of lipoprotein bound was examined.

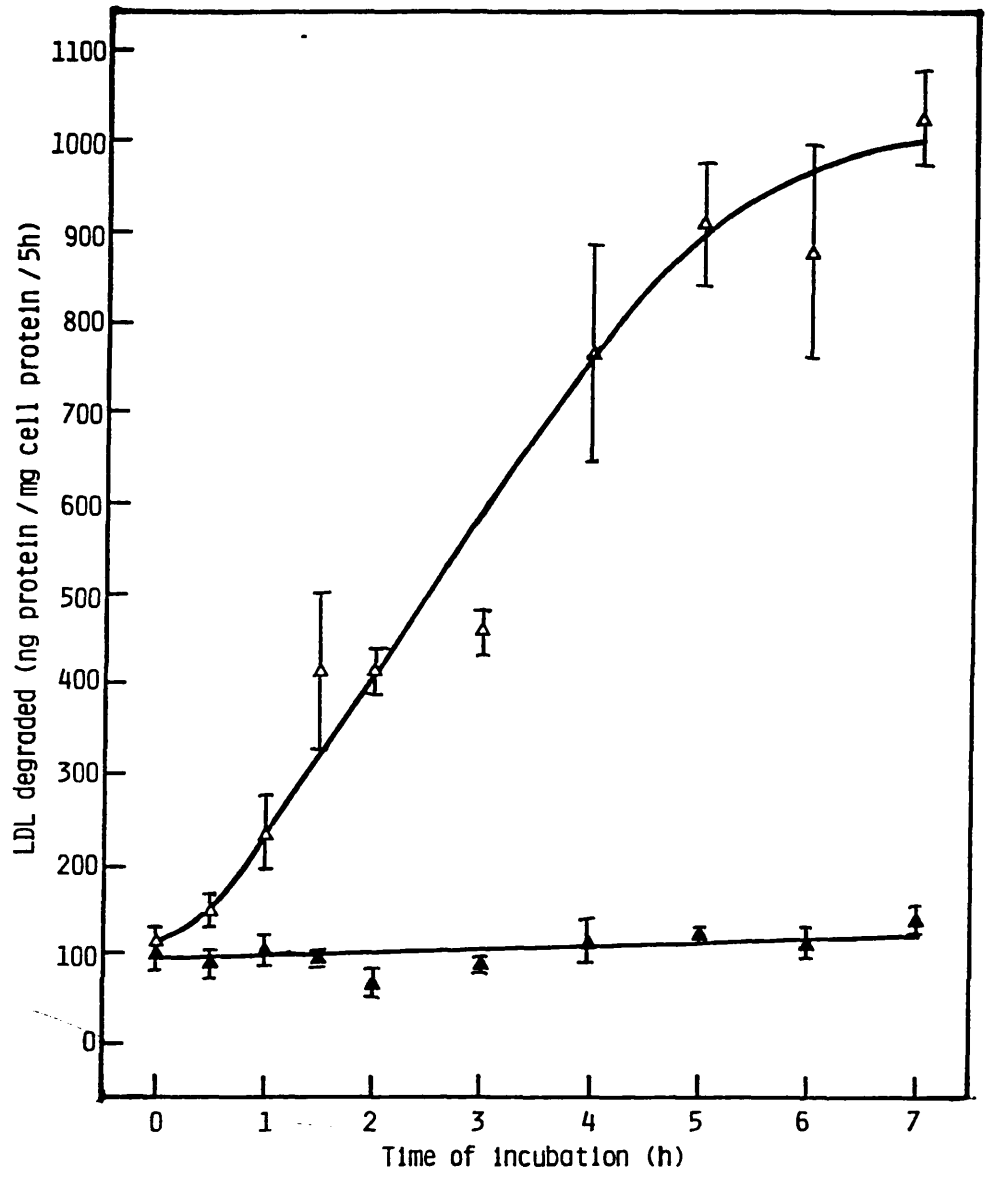


Figure 4.4 Effect of time of incubation on the degradation of [^{125}I]LDL by fibroblasts at 4°C (▲) and 37°C (△)

Cells were prepared as described in the legend to Fig. 4.3 and the amounts of non-iodide, trichloroacetic acid soluble radioactivity in the medium after various periods of incubation were assayed as described. The points represent the means of duplicate dishes.

A comparison of total cell-associated LDL at 4°C (Fig. 4.5(a)) with heparin-releasable LDL at 37°C (Fig. 4.5(b)) in cells grown under normal conditions gave curves similar to those obtained by Goldstein *et al.* (1976). At 4°C the cell-surface receptors were saturated at a lipoprotein concentration of 5 μg LDL protein/ml in the medium while at 37°C saturation did not occur until the concentration had reached approximately 30 μg LDL protein/ml. The concentration of LDL at which half maximal binding occurred was approximately ten times higher at 37°C than 4°C. However, the amount of LDL bound by fibroblasts, at saturating concentrations of the lipoprotein, was approximately halved as the temperature decreased from 37°C to 4°C. The higher maximal binding at 37°C indicates that there are more sites capable of binding LDL at 37°C, while the higher concentration of LDL at which half maximal binding occurs indicates that the affinity of those receptors for the lipoprotein is lower than those found at 4°C. The differences between binding at 4°C and 37°C, at saturating concentrations of LDL, were statistically significant (Table 4.1).

As the concentration of LDL in the medium changed, the internalisation and degradation of the lipoprotein varied in a similar way to 37°C binding, both these processes becoming saturated at about 30 μg LDL protein/ml (Figs. 4.6(a) and (b)). After 5 h at 37°C, in the presence of a saturating concentration of the lipoprotein, the amount of LDL which had been degraded over the incubation period was approximately the same as the amount which was inside the cell at that point in time and about 20-fold greater than the amount bound at the cell surface (Table 4.1).

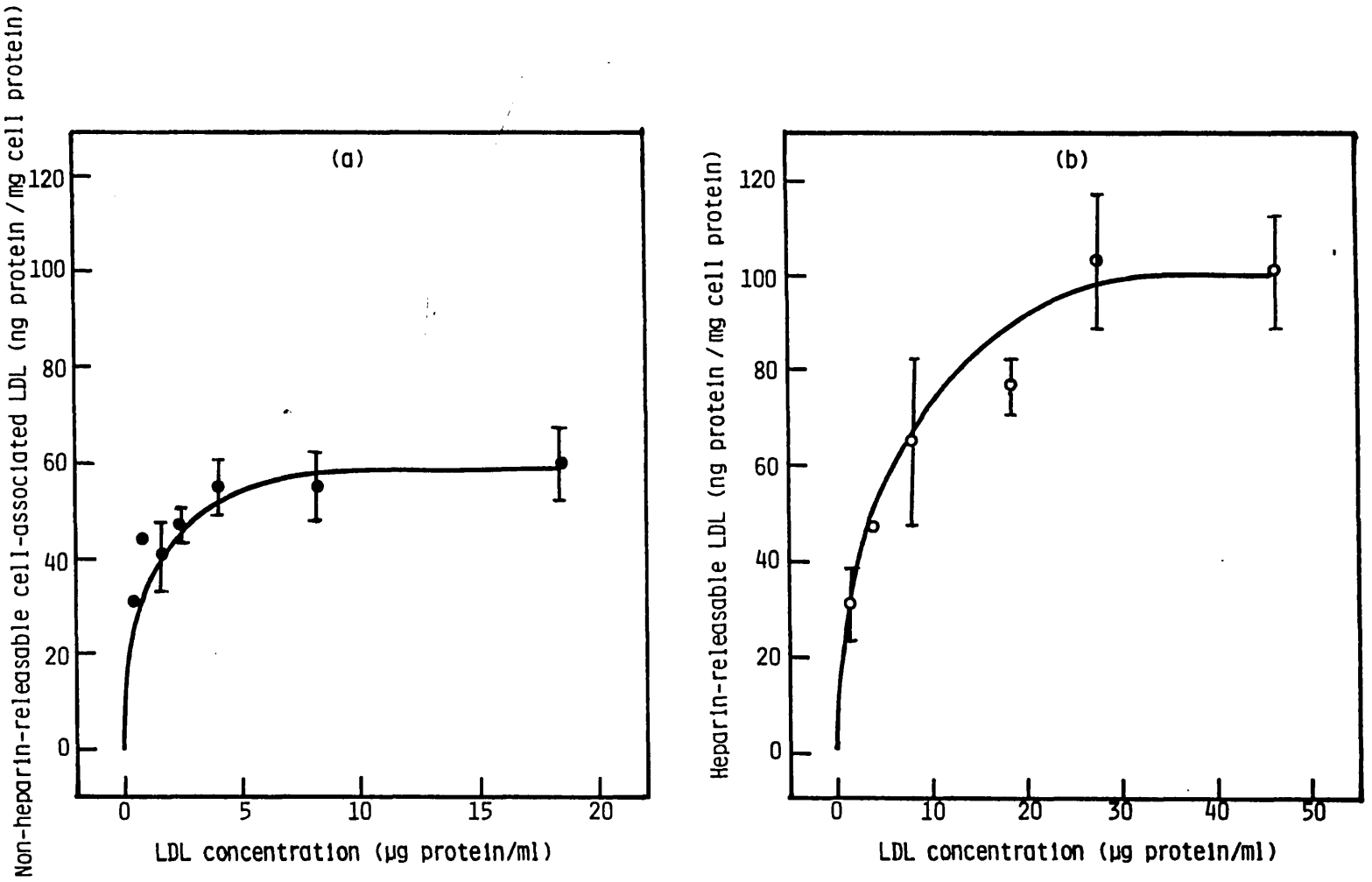


Figure 4.5 Effect of LDL concentration on the binding of [^{125}I]LDL by fibroblasts at 4°C (●) and 37°C (○)

Cells were prepared as described in the legend to Fig. 4.3. At 4°C, after a 2 h incubation in the presence of various amounts of [^{125}I]LDL (16.8 cpm/ng LDL protein) the total cell associated LDL was measured. At 37°C, after a 5 h incubation the heparin-releasable radioactivity was assayed. The points represent the means of duplicate dishes.

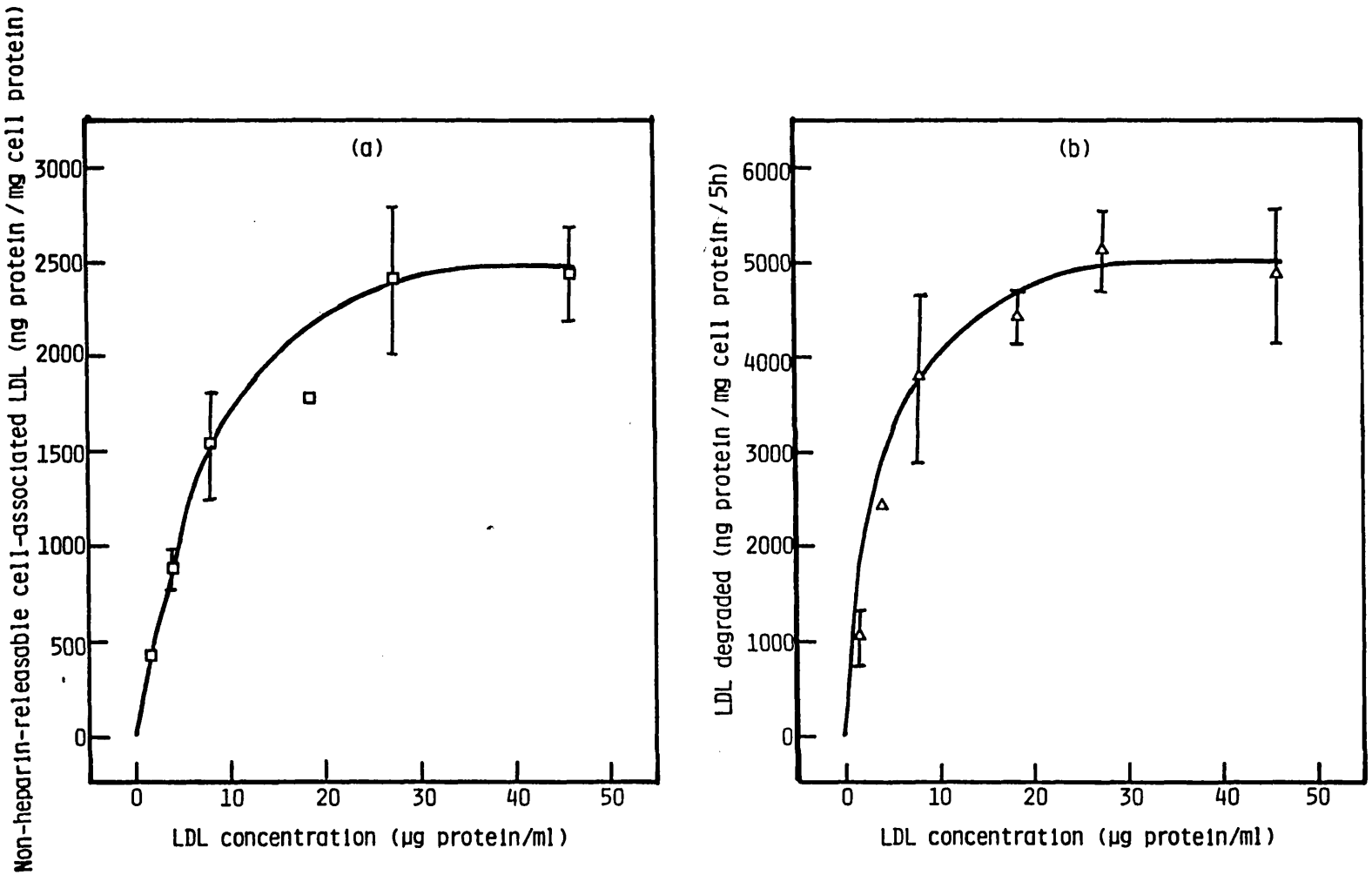


Figure 4.6 Effect of LDL concentration on the internalisation (□) and degradation (△) of [^{125}I]LDL by fibroblasts at 37°C

Cells were prepared as described in the legend to Fig. 4.3. After a 5 h incubation at 37°C in the presence of various concentrations of [^{125}I]LDL (16.8 cpm/ng LDL protein) the amounts of LDL internalised and degraded by the cells were assayed. The points represent the means of duplicate dishes.

Table 4.1

The effect of fatty acids on binding, internalisation and degradation of LDL

Fibroblasts were grown for 6 days in medium containing FCS without additional fatty acids or supplemented with 50 μM fatty acid. After a further 24-h preincubation with LPDS containing the same fatty acid supplements, dishes of fibroblasts were either incubated for 2 h at 4°C with 5 μg protein/ml of [^{125}I]LDL or for 5 h at 37°C with 30 μg protein/ml of [^{125}I]LDL/ml. After these incubations, total cell-associated [^{125}I]LDL at 4°C and binding, internalisation and degradation of [^{125}I]LDL at 37°C were assayed as described in Section 4.2. Values are the mean ± S.E.M. of the numbers of experiments (given in parentheses). The specific activity of the LDL varied between 17-68 cpm/ng of LDL protein. Internalisation of [^{125}I]LDL by cells grown with palmitate was significantly less than that by cells grown with either oleate or linoleate ($p<0.05$; paired t-test). Degradation of [^{125}I]LDL by cells grown with linoleate was significantly higher than degradation by cells grown with palmitate ($p<0.02$; paired t-test).

	Binding at 4°C (*ng LDL protein/ mg cell protein*)	*Binding at 37°C* (*ng LDL protein/ mg cell protein*)	*Internalisation at 37°C* (*ng LDL protein/ mg cell protein*)	*Degradation at 37°C* (*ng LDL protein/ mg cell protein/5h*)
No additions	44.6±5.3 (5)	59.4±12.5 (5)	948±199 (10)	1053±265 (10)
Palmitate	45.6±5.7 (5)	56.8±13.3 (5)	715±53 (7)	899±137 (7)
Oleate	51.8±5.5 (5)	53.6±5.1 (5)	1042±159 (7)	936±181 (7)
Linoleate	45.4±3.3 (5)	63.6±9.5 (5)	1138±300 (7)	1354±353 (7)

The ability of fibroblasts to catabolise LDL after growth in the presence of fatty acids was examined. The binding of the lipoprotein at both 4°C and 37°C was very similar to the binding by cells grown without supplementary fatty acid (Fig. 4.7). Although the 4°C binding by cells grown with oleate seemed higher than normal in this particular experiment the mean value from 5 experiments showed no significant difference (Table 4.1).

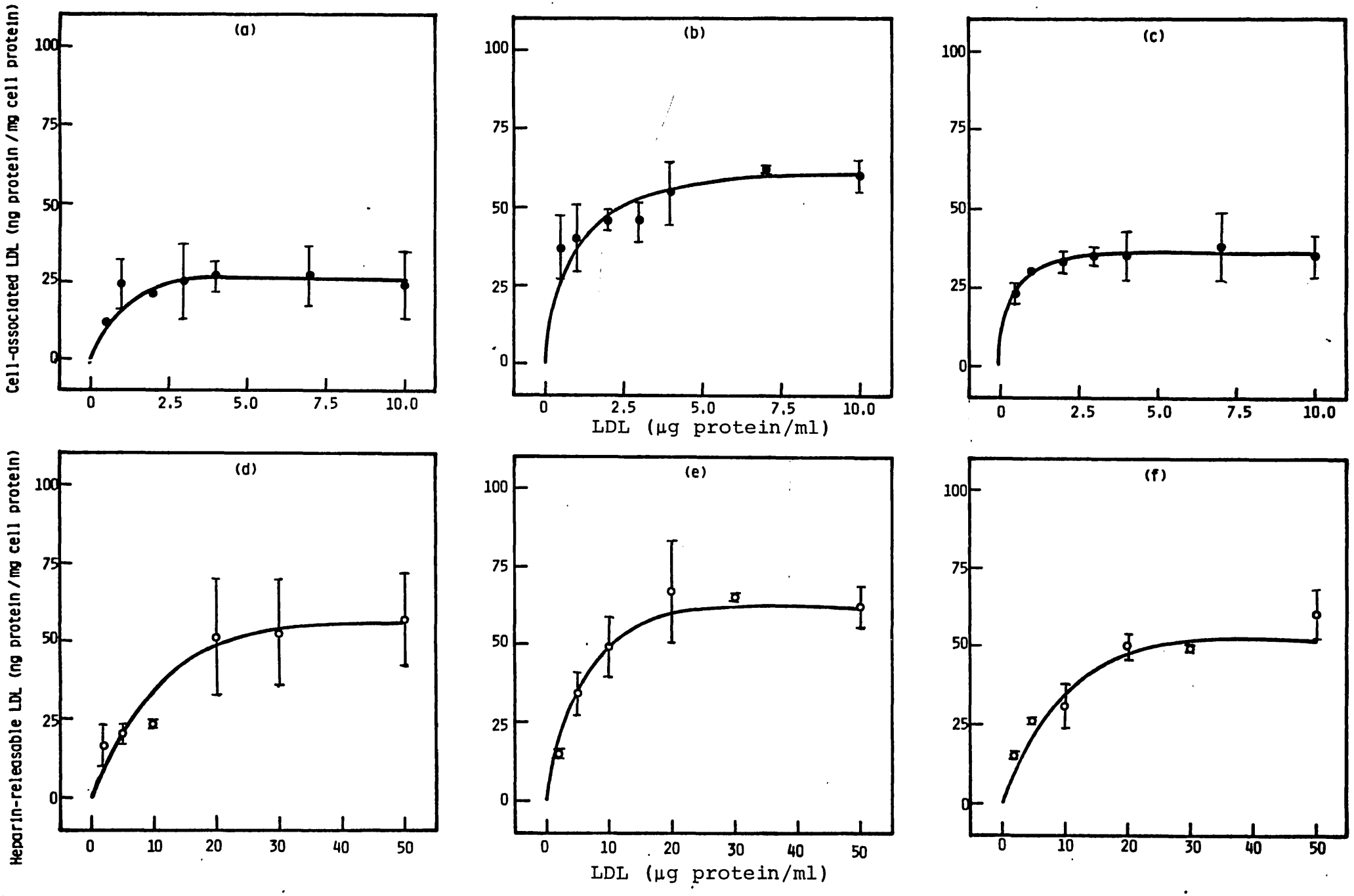
Estimates of the maximal binding and the concentration of LDL at which half maximal binding occurs at 37°C by cells grown with palmitate, oleate or linoleate showed that, at least under the conditions employed, the addition of these fatty acids to the growth medium failed to bring about any change in either the number or affinity of fibroblast LDL receptors (Fig. 4.8).

At 4°C the amount of LDL bound by cells in the presence of saturating concentrations of the lipoprotein did not differ significantly, irrespective of the fatty acid supplementing the medium (Table 4.1). Similarly, at 37°C, changes in the fatty acid supplement failed to produce a significant change in the maximum amount of LDL which was bound specifically by the fibroblasts. It is not possible to directly compare 4°C binding with 37°C binding, since the 37°C binding was measured after a 5 h incubation with LDL by which time down-regulation of receptor number was already evident. However, even after down-regulation the maximum specific binding at 37°C was still higher than the maximum specific binding at 4°C by the corresponding cells (Table 4.1).

In contrast to their lack of effect on binding, fatty acids added to the culture medium greatly affected both the internalisation and degradation of LDL by fibroblasts (Figs. 4.9(a) and (b)). These curves may be interpreted as showing that LDL was internalised and degraded by two processes, one that was essentially saturated by

Figure 4.7 Effect of LDL concentration on the binding of LDL at 4°C (a,b,c) and 37°C (d,e,f) by fibroblasts grown in medium supplemented with 50 μM palmitate (a,d), oleate (b,e) or linoleate (c,f)

Cells were grown for 6 days in medium containing foetal calf serum supplemented with the required fatty acid and for a further 24 h in medium containing lipoprotein-deficient serum supplemented with the same fatty acid. The medium of some dishes was replaced with medium containing lipoprotein-deficient serum and fatty acids but lacking $NaHCO_3$. These cells were cooled to 4°C and incubated for 2 h with various concentrations of [^{125}I]LDL (30.5 cpm/ng LDL protein) after which cell-associated LDL was assayed. Cells at 37°C were incubated for 5 h in the presence of various concentrations of LDL after which heparin-releasable LDL was assayed. The points are the means of duplicate dishes.


(a)
(b)
(c)
(d)
(e)
(f)
Cell-associated LDL (ng protein/mg cell protein)
Heparin-releasable LDL (ng protein/mg cell protein)
LDL (µg protein/ml)
LDL (µg protein/ml)
0
25
50
75
100
0
2.5
5.0
7.5
10.0
0
10
20
30
40
50

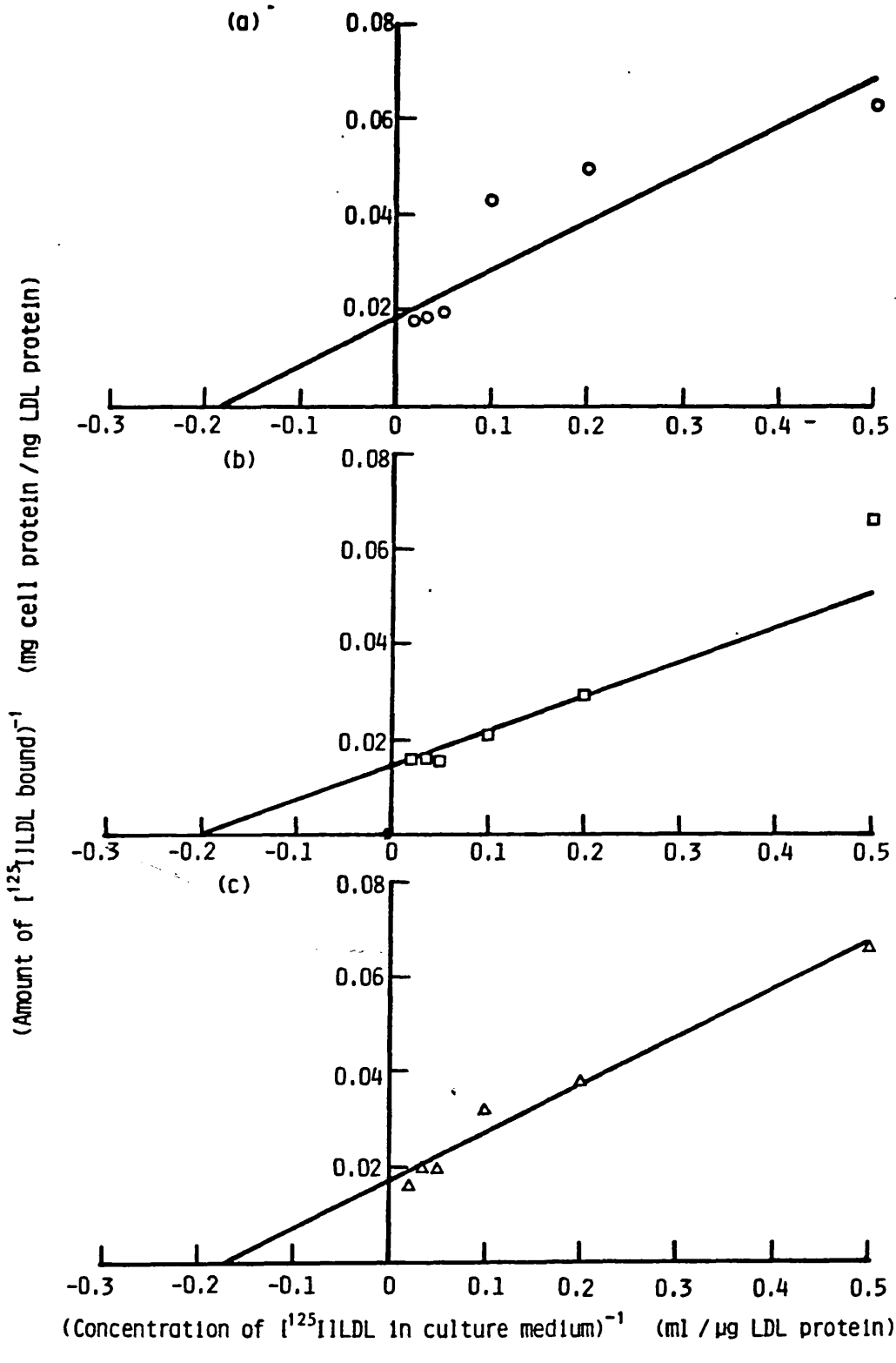


Figure 4.8 Calculating the maximal binding of LDL and the concentration of LDL at which half maximal binding occurs in fibroblasts grown with various fatty acids

The data from Fig. 4.7 were replotted in a double reciprocal form as described by Lineweaver and Burk (1934). From the resulting straight lines the maximal binding of LDL and the concentration of LDL at which half maximal binding occurs could be estimated for cells grown in palmitate (O), oleate (□) or linoleate (Δ).

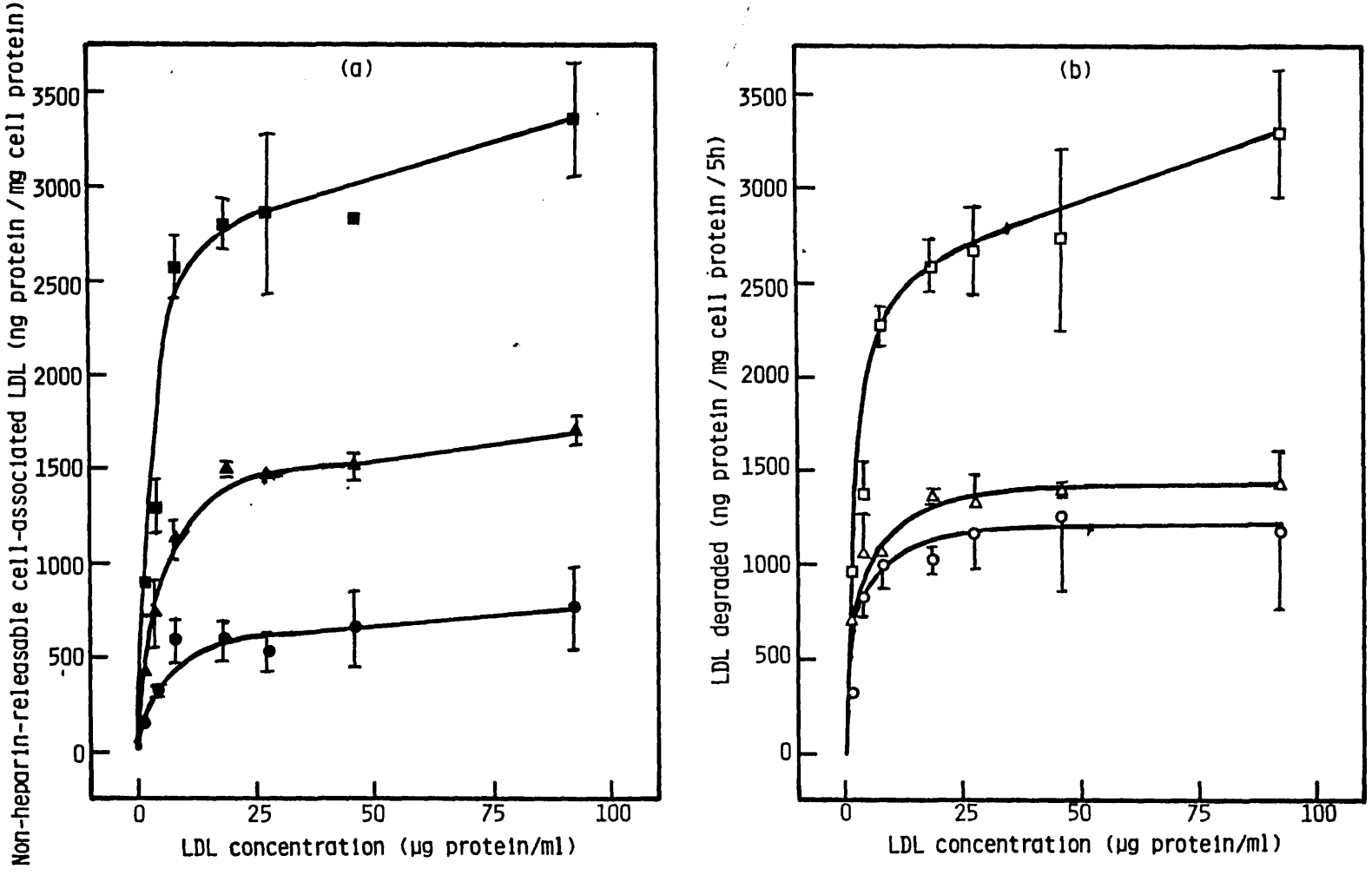


Figure 4.9 Effect of LDL concentration on the internalisation (a) and degradation (b) of LDL at 37°C by fibroblasts grown in medium supplemented with 50 µM palmitate (●,○), oleate (▲,△) or linoleate (■,□).

Cells were grown as described in the legend to Fig. 4.7 and incubated for 5 h at 37°C in the presence of various concentrations of [^{125}I]LDL (30.5 cpm/ng LDL protein), after which the quantities of LDL internalised and degraded by the fibroblasts were assayed. The points are the means of duplicate dishes.

30 μg LDL protein/ml and one which remained unsaturated at almost 100 μg/ml. The non-saturable internalisation and degradation, revealed at higher concentrations of LDL, were both directly proportional to the concentration of LDL and were much greater in fibroblasts grown in the presence of linoleate than in cells grown with either oleate or palmitate. Internalisation and degradation via the saturable process, mediated by cell-surface receptors, was also highest in cells grown in the presence of linoleate. The fatty acids present in the fibroblast growth medium did not perceptibly affect the apparent affinity for LDL of the processes responsible for internalisation or degradation. The maximal rate of saturable internalisation and degradation were taken as the difference between the observed rates at 30 μg LDL protein/ml and the non-saturable rate at the same concentration, estimated from the linear part of the concentration curve.

In the presence of a saturating concentration of LDL (30 μg protein/ml), the rate of internalisation of the lipoprotein by cells grown in palmitate was significantly lower than that by cells grown in either oleate or linoleate (Table 4.1). Similarly the maximum rate of degradation of LDL by cells grown in palmitate was significantly lower than that by cells grown in linoleate (Table 4.1).

In order to investigate in greater detail, the relationship between the catabolism of LDL and the degree of saturation of the phospholipid fatty acids, fibroblasts were grown in medium supplemented with mixtures of fatty acids so that while the

total fatty acid concentration remained constant, the proportions of different fatty acids were altered. As oleate gradually replaced palmitate in the growth medium, so the proportion of monounsaturated fatty acids in the fibroblast phospholipids also increased at the expense of saturated fatty acid (Fig. 4.10(a)). This change was entirely due to oleate replacing palmitate since the proportions of stearate (20%) and palmitoleate (4%) remained unchanged, as did the proportion of polyunsaturated fatty acids (8%). This caused an increase in the P/S ratio of the cellular phospholipid. As the proportion of oleate in the growth medium was increased, the binding, internalisation and degradation of LDL by fibroblasts all rapidly increased, reaching a peak when the fatty acid supplement consisted of 20 μM palmitate and 30 μM oleate (Fig. 4.10(b)). All three variables then decreased and, in 50 μM oleate, had returned to values similar to those in 50 μM palmitate. Changes in the amounts of binding, internalisation and degradation did not seem to follow changes either in the amount of one particular fatty acid or in the P/S ratio. However there was a strong correlation between degradation and binding (r=0.91, p<0.02) and also between degradation and internalisation (r=0.93, p<0.01), suggesting that changes in internalisation and degradation were in response to variations in the numbers of binding sites.

There was a completely different situation when linoleate replaced either oleate (Fig. 4.11) or palmitate (Fig. 4.12) in the growth medium. In each case, as the proportion of linoleate was increased, there was a large increase in the rate of degradation

Figure 4.10 Variation of phospholipid fatty acid composition (a) and LDL catabolism (b) of fibroblasts grown in medium supplemented with different proportions of palmitate and oleate

Cells were grown for 6 days in medium containing foetal calf serum supplemented with 50 μM fatty acids in the required proportions and for a further 24 h in medium containing lipoprotein-deficient serum supplemented with the same fatty acids. 3 Dishes were harvested and pooled for analysis of phospholipid fatty acids($n = 2$).
(a) shows changes in the amounts of saturated (●), monounsaturated (■) and polyunsaturated (○) fatty acids as well as the P/S ratio (□) as oleate replaced palmitate in the medium. A further 2 dishes were incubated for 5 h with [^{125}I]LDL (30 μg LDL protein/ml; 67.4 cpm/ng LDL protein) and the amounts of binding (△), internalisation (▽) and degradation (▲) of the lipoprotein were assayed.

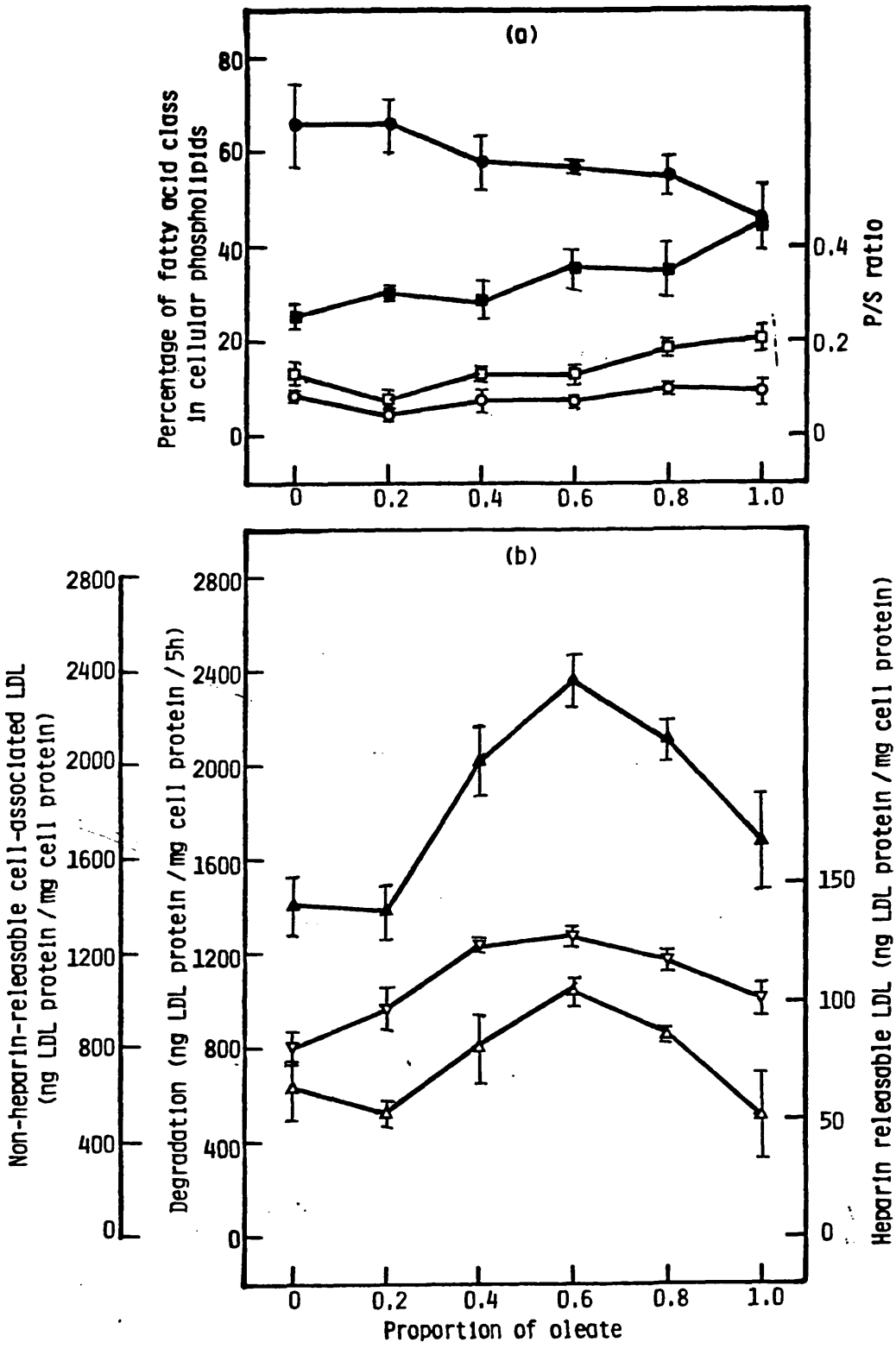

(a)
Percentage of fatty acid class in cellular phospholipids
80
60
40
20
0
0.4
0.2
0
P/S ratio
0
0.2
0.4
0.6
0.8
1.0
(b)
Non-heparin-releasable cell-associated LDL (ng LDL protein / mg cell protein)
Degradation (ng LDL protein / mg cell protein / 5h)
Heparin releasable LDL (ng LDL protein / mg cell protein)
2800
2400
2000
1600
1200
800
400
0
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
Proportion of oleate

Figure 4.11 Variation of phospholipid fatty acid composition (a) and LDL catabolism (b) of fibroblasts grown in medium supplemented with different proportions of oleate and linoleate

Cells were grown for 6 days in medium containing foetal calf serum supplemented with 50 μM fatty acids in the required proportions and for a further 24 h in medium containing lipoprotein-deficient serum supplemented with the same fatty acids. 3 Dishes were harvested and pooled for analysis of phospholipid fatty acids($n = 2$).
(a) shows changes in saturated (●), monounsaturated (■) and polyunsaturated (○) fatty acids as linoleate replaced oleate in the medium. A further 2 dishes were incubated for 5 h with [^{125}I]LDL (30 μg LDL protein/ml; 67.4 cpm/ng LDL protein) and the amounts of binding (△), internalisation (▽) and degradation (▲) of the lipoprotein were assayed.

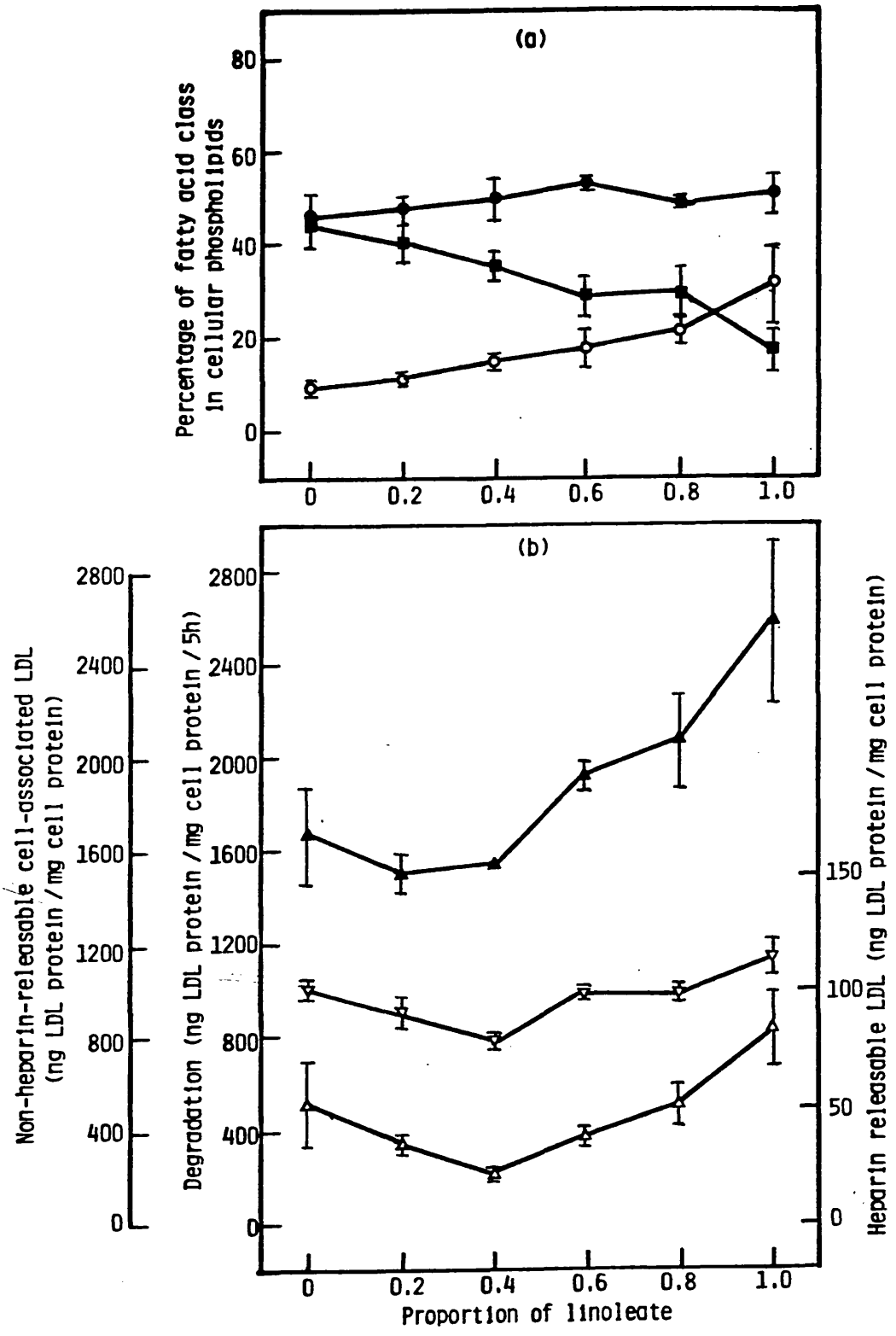
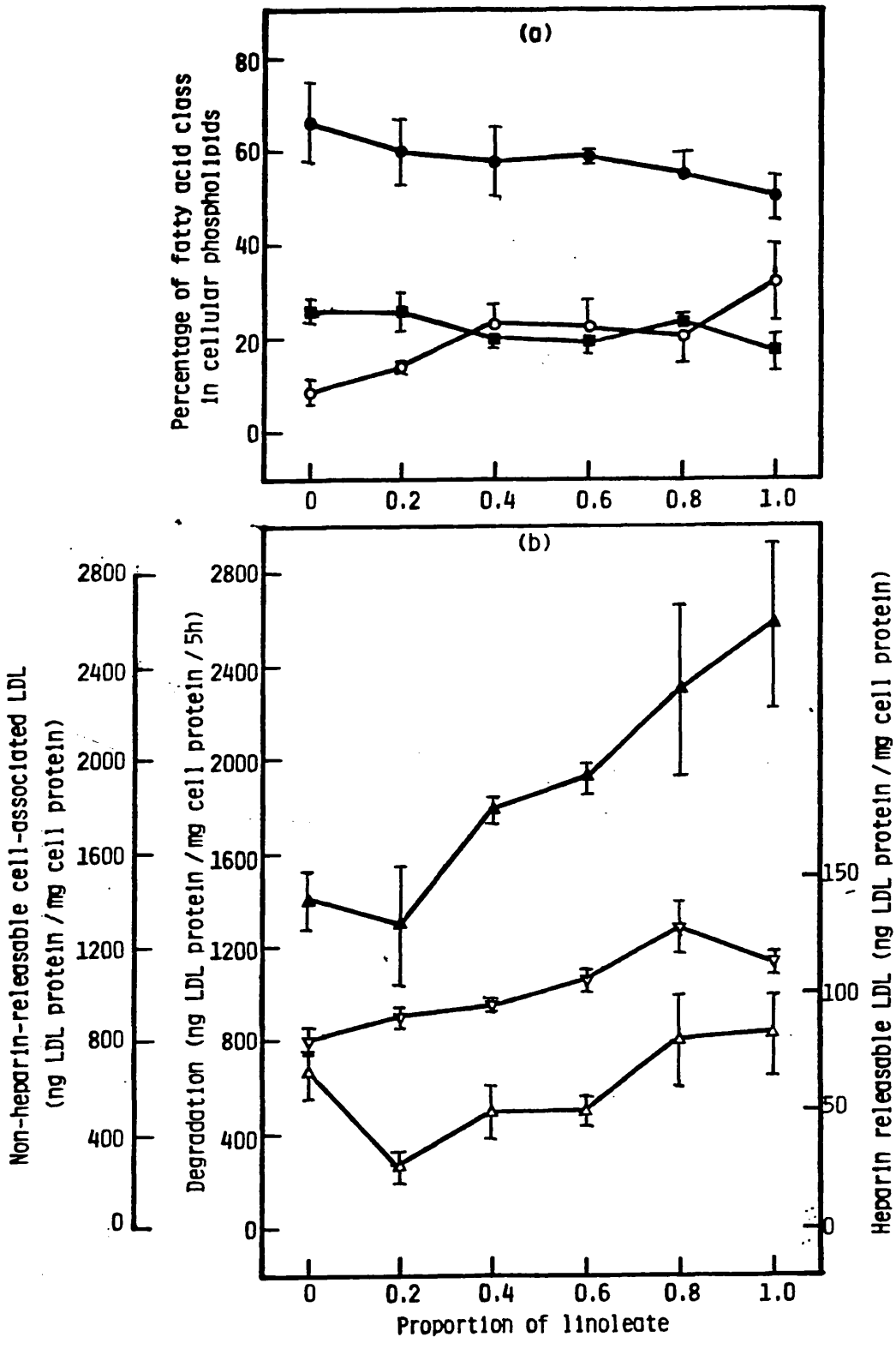

(a)
Percentage of fatty acid class in cellular phospholipids
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
(b)
Non-heparin-releasable cell-associated LDL (ng LDL protein / mg cell protein)
Degradation (ng LDL protein / mg cell protein / 5h)
Heparin releasable LDL (ng LDL protein / mg cell protein)
2800
2400
2000
1600
1200
800
400
0
150
100
50
0
Proportion of linoleate

Figure 4.12 Variation of phospholipid fatty acid composition (a) and LDL catabolism (b) of fibroblasts grown in medium supplemented with different proportions of palmitate and linoleate

Cells were grown for 6 days in medium containing foetal calf serum supplemented with 50 μM fatty acids in the required proportions and for a further 24 h in medium containing lipoprotein-deficient serum supplemented with the same fatty acids.

(a) shows changes in saturated (●), monounsaturated (■) and polyunsaturated (○) fatty acids as linoleate replaced palmitate in the medium(n=2). A further 2 dishes were incubated for 5 h with [^{125}I]LDL (30 μg LDL protein/ml; 67.4 cpm/ng LDL protein) and the amounts of binding (△), internalisation (▽) and degradation (▲) of the lipoprotein were assayed.


(a)
Percentage of fatty acid class in cellular phospholipids
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
(b)
Non-heparin-releasable cell-associated LDL (ng LDL protein / mg cell protein)
Degradation (ng LDL protein / mg cell protein / 5h)
Heparin releasable LDL (ng LDL protein / mg cell protein)
2800
2400
2000
1600
1200
800
400
0
150
100
50
0
Proportion of linoleate

but much smaller changes in the amounts of LDL bound or internalised by the cell. As linoleate replaced oleate in the growth medium, the proportions of these two fatty acids in cellular phospholipids underwent a reciprocal change, linoleate increasing at the expense of oleate. Since the proportions of the other major fatty acids remained approximately constant, the polyunsaturated fatty acids increased while the monounsaturated fatty acids decreased so that, although the proportion of saturated fatty acid remained constant, the P/S ratio increased (Figs. 4.11(a) and (b)). Similar changes occurred when linoleate replaced palmitate in the growth medium. Although linoleate mainly replaced palmitate in the cellular phospholipids there was also a slight decrease in the amount of oleate. Thus polyunsaturated fatty acids increased at the expense of both monounsaturated and saturated fatty acids (Fig. 4.12(a)) and, again, the net effect was a steadily increasing P/S ratio (Fig. 4.13(a)).

This raised the possibility that, under certain conditions, the rate of LDL degradation by fibroblasts may be related to the P/S ratio of the fatty acids in the cell membrane phospholipids. In Fig. 4.13(a) the rate of LDL degradation and the P/S ratio of cell membrane phospholipid each seemed to vary in a similar way with the proportion of linoleate in the medium, whether linoleate was replacing oleate or palmitate. The data shown in Fig. 4.13(a) were replotted in order to see how the rate of LDL degradation varied with the P/S ratio of the phospholipid fatty acids from the corresponding fibroblasts (Fig. 4.13(b)). The apparently

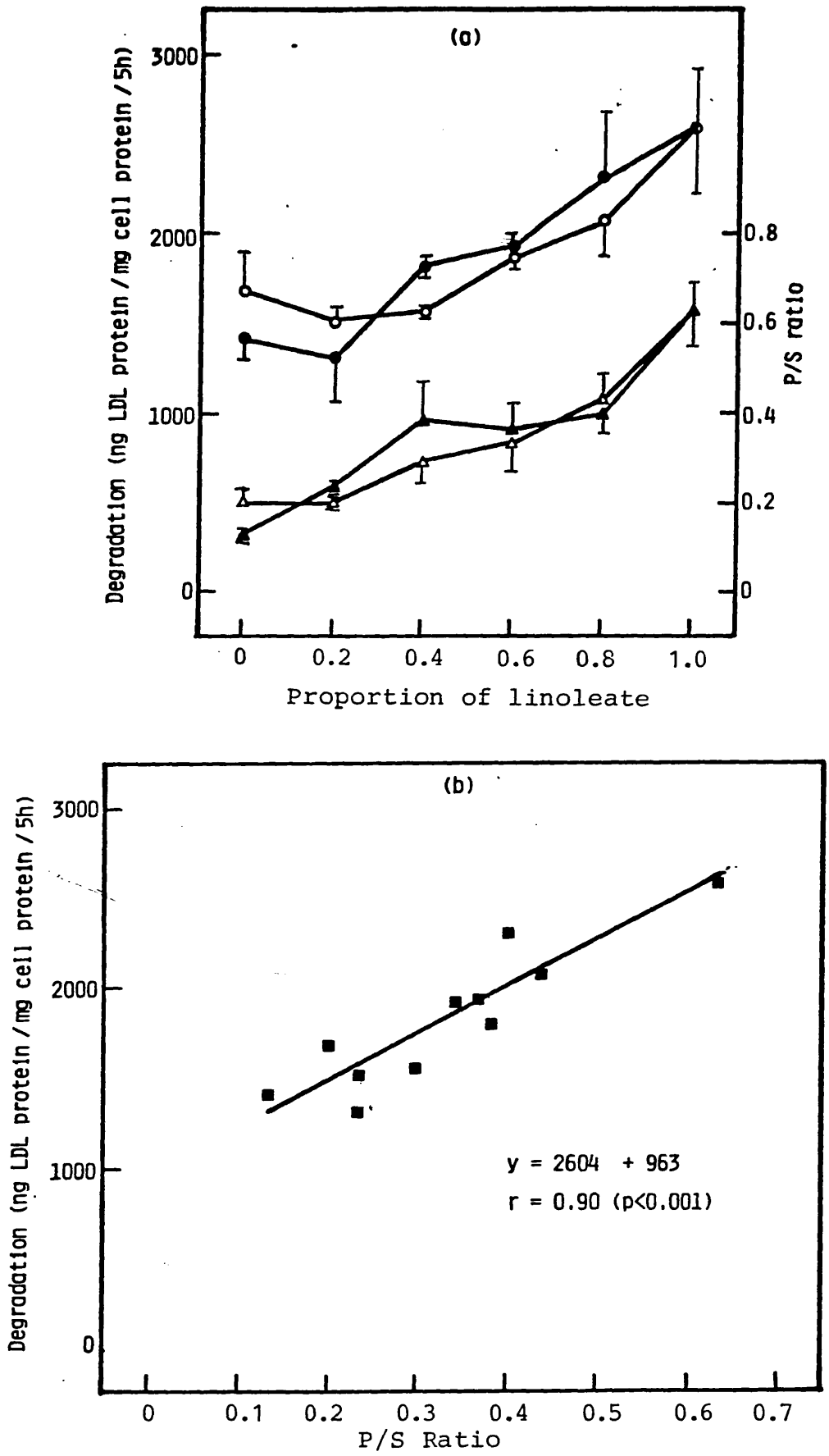


Figure 4.13 Comparison of the variations in the P/S ratio of membrane phospholipid fatty acids (Δ,▲) and LDL degradation (O,●) of fibroblasts grown in medium in which linoleate supplemented was gradually replaced by oleate (open symbols) or palmitate (closed symbols)

Cells were prepared as described in the legends to Figs. 4.10 and 4.11. The data shown in (a) were replotted in (b) and the linear regression line calculated. The correlation coefficient (0.90) was significantly different from 0 ($p<0.001$).

linear relationship between these parameters was analysed by statistical methods. The regression line was calculated as $y = 2604x + 963$, where y = rate of degradation and x = P/S ratio. The correlation coefficient 0.90 was highly significant ($p<0.001$) indicating a very close correlation between degradation and P/S ratio in these cells grown under these conditions.

4.4 Discussion

When normal fibroblasts are incubated in medium without lipoproteins, they express specific cell-surface receptors which enable them to bind, internalise and degrade LDL (Goldstein and Brown, 1977). All three of these processes can be measured by the methods described by Goldstein *et al.* (1976). Binding of LDL by fibroblasts at 4°C differed from binding at 37°C in two ways: the number of LDL particles which could be bound to the cell surface was greater at 37°C, while the affinity of the receptors for their ligand was greater at 4°C. These differences could reflect the variations in membrane conformation which are brought about by changes in the temperature of the membrane. Alternatively, since the binding at 37°C is measured after the cells have been incubated with LDL for 5 h while at 4°C the fibroblasts have their membrane structure effectively frozen before being exposed to the lipoprotein, these differences may indicate that the receptor can alter its structure depending on whether or not LDL is present in the medium.

The addition of fatty acids to the fibroblast growth medium, which had been shown to alter the fatty acid composition of membrane phospholipid, also caused changes in the rate at which the cells were able to internalise and degrade LDL. These differences were most pronounced between cells grown in the presence of palmitate (saturated fatty acid) and those grown with linoleate (polyunsaturated fatty acid). The catabolism of LDL by cells grown with oleate (monounsaturated fatty acid) proceeded at rates similar to those of cells grown without supplementary fatty acid and intermediate between those of cells grown with the other fatty acids.

Since binding is believed to be the rate-limiting step in the entire catabolic process (Goldstein and Brown, 1974*b*), any change in the degradation rate would be expected to result from changes in the ability of the cells to bind LDL. However, rather surprisingly, at both 4°C and 37°C the maximal binding, which gives an indication of the number of LDL receptors, and the concentration of LDL at which half maximal binding occurs, which gives an indication of the affinity of those receptors, do not seem to change and it is therefore unlikely that differences in the ability of the LDL receptor to bind LDL could explain the difference in the rates of degradation of LDL seen between cells grown with palmitate and those grown with linoleate.

LDL internalisation and degradation in these fibroblasts seemed to take place by two mechanisms; one saturable and believed to be the receptor-mediated pathway, the other non-saturable at 100 μg LDL protein/ml

and probably receptor-independent adsorptive endocytosis (Miller *et al.*, 1977). Both these processes take place at a greater rate in cells whose membrane phospholipids are enriched with linoleate and since the binding of LDL to its receptor does not seem to be affected, it is likely that some more general change in membrane properties is responsible. Nestel and Ma (1979) were unable to show any difference in the rates of LDL degradation between fibroblasts grown in 100 μM linoleate or in the same concentration of oleate, but they gave no details of the phospholipid fatty acid composition to enable a direct comparison to be made with the results presented here. In addition, the findings reported here show that, when compared with cells grown with linoleate, it is cells grown with palmitate and not those grown with oleate which exhibited the greatest decrease in the rate of LDL degradation.

In order to examine more closely the effect of fatty acids on LDL metabolism, fibroblasts were grown in medium supplemented with mixtures of two different fatty acids. As expected, when oleate replaced palmitate in the growth medium, it also replaced palmitate in the cell phospholipids. However, binding, internalisation and degradation all rose sharply, reaching a peak when palmitate and oleate were present in the supplement in approximately equal amounts before declining as the proportion of oleate continued to increase. Under these conditions internalisation and degradation seemed to follow changes in the number of receptors expressed at the cell surface although it is

not possible to offer an explanation of why the number of cell-surface receptors should increase in this way. However, fatty acids can affect glucose transport in cultured cells by altering the ability of insulin to bind to its receptor, which also enters cells via the coated pit (Grunfield *et al.*, 1981).

As linoleate replaced either palmitate or oleate in the growth medium an enhancement of the ability of cells to degrade LDL was closely correlated with an increase in the P/S ratio of phospholipid fatty acids. Since there was no corresponding change in the amount of LDL bound by the cells, the increased ability of the cells to degrade LDL seemed to result from changes in the membrane properties related to the increased proportion of linoleate in cellular phospholipids. Changes in the degree of saturation of phospholipid fatty acids can affect membrane fluidity (Burns *et al.*, 1979), the rate of endocytosis (Schroeder, 1981) and, in macrophages, the rates of phagocytosis and pinocytosis (Mahoney *et al.*, 1977).

Both receptor-mediated and receptor-independent LDL degradation increased in cells grown with linoleate. A possible interpretation of this observation is that a rise in the P/S ratio of the fatty acids in the membrane phospholipids may be accompanied by a non-specific increase in the rate of membrane turnover. This could result in not only an increased uptake of LDL by receptor-independent adsorptive endocytosis but also a general increase in the rate at which receptor-mediated endocytosis takes place. Alternatively, an increase in membrane fluidity may allow receptors to undergo the cycle of internalisation and return to the surface at a faster rate.

Chapter 5

ACYL-COENZYME A:CHOLESTEROL ACYLTRANSFERASE ACTIVITY IN FIBROBLASTS

5.1 Introduction

The development of an atherosclerotic lesion is associated with the accumulation of cholesteryl esters within the cells of the arterial wall (Ross, 1981). The cholesterol is thought to be derived from the catabolism of cholesterol-rich lipoproteins. Cholesterol that is surplus to the cells' requirements is prevented from building up within cells through the action of ACAT (E.C. 2.3.1.26) a membrane-bound enzyme found in the rough endoplasmic reticular fraction of the microsomal pellet (Goodman *et al.*, 1963; Balasubramaniam *et al.*, 1978). This enzyme catalyses the linkage of cholesterol with fatty acid to form cholesteryl esters which are stored as lipid droplets within the cell, and it is the presence of a large mass of these droplets which characterises the foam cells of an atherosclerotic lesion (Smith and Smith, 1976). ACAT activity measured in a range of freshly isolated animal tissues increases as a result of hypercholesterolaemia brought about by cholesterol feeding (Mathur *et al.*, 1981; Norum *et al.*, 1983).

Studies on the formation of cholesteryl esters in cultured cells have been limited to observing changes in the mass or in the net incorporation of radioactive fatty acids from the medium (Goldstein *et al.*, 1974; Brown *et al.*, 1975). This approach is clearly unsuitable for measuring the enzyme activity in cells grown in medium supplemented with large amounts of fatty acid. A detailed investigation of cholesteryl ester synthesis and its relationship to lipoprotein uptake requires a direct assay of ACAT activity and the method of Goodman *et al.* (1963) has been adapted for use with preparations of fibroblast membranes. The characteristics of the enzyme have been investigated and a comparison has been made under a variety of conditions, with substrate cholesterol derived from different sources, between ACAT activity in membrane preparations and the incorporation of oleic acid into cholesteryl esters by intact cells.

Since both ACAT and its substrate, cholesterol, are both membrane bound, there is a problem in comparing the amount of enzyme activity in two different membrane preparations. Changes in the measured activity could be brought about either by differences in the amount of active enzyme or by differences in the amount of cholesterol accessible to the enzyme. In liver microsomes, ACAT activity can be increased by incubating the membranes themselves with a cholesterol-rich lipoprotein (Hashimoto and Dayton, 1979) and this effect has been investigated with fibroblast membrane preparations. An equilibration of cholesterol between the cholesterol-rich donor lipoprotein and

fibroblast membranes could provide a means of modifying membranes from different sources so that they contained the same amount of cholesterol.

It was shown in the previous chapter that enrichment of fibroblast phospholipids with linoleic acid by growing the cells in the presence of that fatty acid coincided with an enhancement of the cells' ability to degrade LDL. The resulting increase in the availability of cholesterol as substrate for the enzyme ACAT might be expected to affect the rate of cholesteryl ester formation by the fibroblasts. There is also a possibility that ACAT, being a membrane bound enzyme, might be affected by the lipid composition of its membrane environment. In order to test these possibilities, fibroblasts were grown in the presence of various fatty acids, preincubated with LPDS and incubated for various times with LDL. ACAT activity was assayed in membranes prepared from these cells both before and after the isolated membranes had been equilibrated with cholesterol-rich donor lipoprotein.

5.2 Methods and materials

The fibroblasts used in these experiments were grown in dishes and harvested as described in Section 2.1 and Section 3.4.

A rapid and simple method for estimating the net rate of cholesteryl esterification in fibroblasts grown in medium without supplementary fatty acids was to incubate the cells for a short time

in the presence of [1-^{14}C]oleic acid complexed with bovine serum albumin and assay the amount of radioactivity incorporated into cholesteryl esters. Fraction V albumin was defatted by the method of Chen (1967) and complexed with oleate by the method of Spector *et al.* (1964). In a typical preparation, 25 g albumin was dissolved in 250 ml NaCl (9 g/l), 12.5 g activated, acid-washed charcoal was added and the pH adjusted to 3.0 by slowly adding 0.2 N HCl with constant stirring. The mixture was stirred for a further 75 min at 4°C before the charcoal was removed by filtration, first through Whatman filter paper No.5 and then through a 0.22 μm Millex filter. The pH was adjusted back to 7.0 by slowly adding 5 N NaOH with constant stirring and the solution was dialysed twice against 5 litres of NaCl (9 g/l). A 30 mM solution of the sodium salt of oleic acid was prepared by the method described in Section 3.2. The albumin solution was diluted with NaCl (9g/l) and to 460 ml containing 20 g (0.3 mmol) of protein, were added 40 ml of sodium oleate. This gave a solution in which four molecules of oleate were complexed with each molecule of albumin. This solution was stored at -20°C in 8 ml aliquots. For experiments, 50 μCi (0.86 μmol) [1-^{14}C]oleic acid dissolved in benzene were dried under N_2 and redissolved in 8 ml of the albumin/oleate solution to give a solution of 0.6 mM albumin/2.4 mM oleate (specific activity 2.5 μCi/μmol).

This solution was added to the medium in dishes of cells to give a final concentration of 100 μM oleate. The cells were then incubated for 1 h at 37°C before being washed and harvested.

The pellet of cells was resuspended in 300 µl H_2O, aliquots taken to measure the protein content and 200 µl taken to assay the amount of radioactively labelled cholesteryl ester. Cellular lipid was extracted by the method of Folch *et al.* (1957) and separated by thin-layer chromatography as described in Section 3.2. The radioactivity associated with the band containing cholesteryl esters was measured by liquid scintillation spectrometry. The scintillation fluid used was 2,5-diphenyloxazole (3 g/l) and 1,4-di[2-(5-phenyloxazolyl)]-benzene (100 mg/l) in toluene.

In order to assay ACAT activity in fibroblasts, pellets of cells prepared by the usual harvesting procedures were suspended in 10 mM potassium phosphate buffer (pH 7.4) containing 2 mM dithiothreitol and were left at 0°C for 10 min for the cells to lyse. The suspension was then homogenised (30 strokes) in a Potter-Elvehjem glass homogeniser with a teflon pestle. The homogenate was centrifuged at 30,000 rpm for 10 min at 4°C in a Beckman 42.2 Ti rotor. The pellet was resuspended in 200 µl of the same homogenising buffer.

For the equilibration of cholesterol between membranes and donor lipoprotein, 100 µl of homogenising buffer, containing 50 µg of membrane protein was mixed with 100 µl of phosphate-buffered saline containing 10 mg protein/ml of LDL from a homozygous FH subject (equivalent to approximately 20 mg cholesterol/ml). After 60 min at 37°C the membranes were reisolated by centrifugation at 30,000 rpm in a 42.2 Ti rotor as before and resuspended in 100 µl of homogenising buffer.

Liposomes were prepared by the method of Batzri and Korn (1973) as modified by Synouri-Vrettakou and Mitropoulos (1983). In a typical preparation 5 mg cholesterol and 10 mg phosphatidylcholine were dissolved in 2 ml ethanol, taken up in a 2 ml syringe and injected through a 19G needle into 50 ml PBS in a 100 ml conical flask under nitrogen. The suspension was concentrated and the ethanol removed using an Amincon ultrafiltration device containing a 100 Å disc filter under N_2 pressure (103 kPa) with rapid stirring.

ACAT activity in membrane suspensions was assayed by a method based on that of Goodman *et al.* (1963). Samples were assayed in duplicate, in a final volume of 50 µl containing 100 mM potassium phosphate buffer (pH 7.4), 2 mM dithiothreitol, 4 mM $MgCl_2$, bovine serum albumin (12 mg/ml), approximately 15 µg of membrane protein (30 µl of suspension) and 20 µM [1-^{14}C]-oleoyl-coenzyme A (oleoyl-CoA) (125 d.p.m./pmol). After a 15 min incubation at 37°C, the reaction was stopped by the addition of 1 ml of chloroform/methanol (2:1 v/v). Cholesteryl esters were extracted, separated by thin-layer chromatography and assayed for radioactivity as described above.

[1-^{14}C]Oleic acid and [1-^{14}C]oleoyl-CoA were purchased from Amersham International. 2,5-Diphenyloxazole and 1,4-di-[2-(5-phenyloxozolyl)]-benzene, both chromatographically purified for scintillation were obtained from Koch-Light Laboratories, Slough, Berks., U.K. Activated, acid-washed charcoal was from Sigma Chemical Company, and NaCl, $MgCl_2$, toluene and dithiothreitol were all from BDH Chemicals. The ultrafiltration device and

100 Å filters were obtained from Amincon, Woking, Surrey, U.K. and all other equipment or reagents were from the suppliers listed in Chapter 2.

5.3 Results

All of the ACAT activity in cultured fibroblasts was associated with cellular membranes. No activity could be detected in the supernatant fraction obtained after centrifugation of cell homogenates at 100,000 x *g*.

In order to study ACAT activity in the comparatively small amounts of material which can be obtained from fibroblasts grown in culture, it was necessary to modify the original assay method described by Goodman *et al.* (1963). By increasing the proportion of membranes and decreasing the total assay volume, it was possible to reliably assay ACAT activity in membranes prepared from only two 60 mm culture dishes. Since no extra cholesterol was added to the assay mixture, the cholesterol used as substrate by the enzyme was derived entirely from what was already present on the membranes. Under these conditions ACAT exhibited an apparent K_m for the second substrate of 8.5 μM oleoyl-CoA (Fig. 5.1(c)). The observed activity was directly proportional to the concentration of membrane protein up to approximately 20 μg protein/assay (Fig. 5.1(b)) and the rate of formation of cholesteryl oleate was constant for at least 15 min by membranes containing either a high or a low activity of ACAT (Fig. 5.1(a)). In all further assays of ACAT activity,

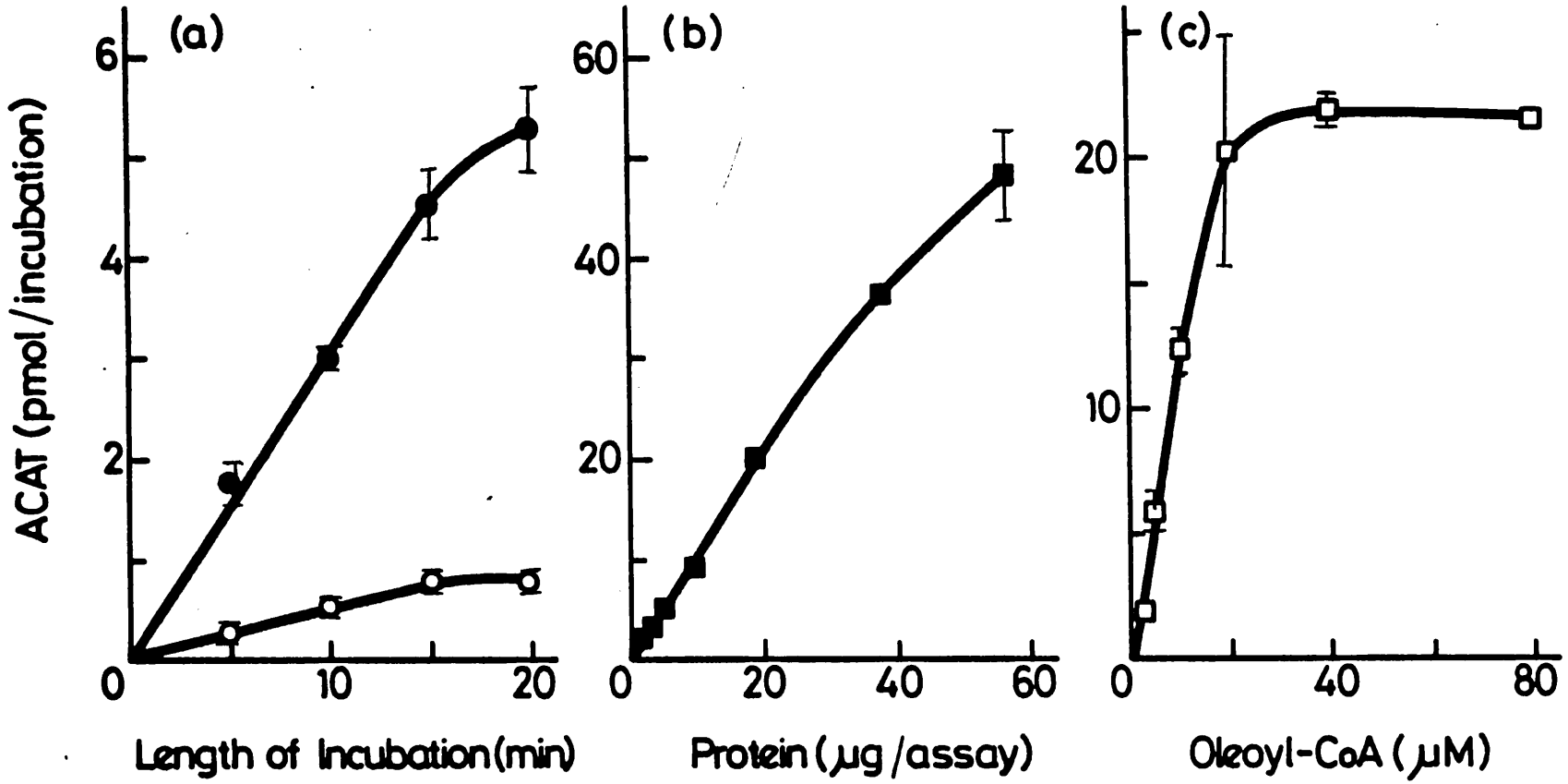


Figure 5.1 Characteristics of ACAT assayed in fibroblast membranes

Normal fibroblasts, preincubated for 24 h with LPDS were harvested either immediately (○), 7 h after the addition of 100 μg of protein/ml of LDL (●) or 24 h after the addition of 50 μg/ml cholesterol (■,□). Membranes were prepared from cells pooled from ten 60 mm dishes and ACAT was assayed as described in Section 5.2.

(a) Assays contained 13 μg of membrane protein with 20 μM oleoyl-CoA and were incubated at 37°C for the times indicated.

(b) Assayed containing different amounts of membrane protein with 20 μM oleoyl-CoA were incubated at 37°C for 15 min.

(c) Assays containing 20 μg of membrane protein and different concentrations of oleoyl-CoA were incubated at 37°C for 15 min.

Each point represents the average value of duplicate assays.

15 μg of membrane protein were incubated with 20 μM oleoyl-CoA for 15 min. ACAT activity was low, and remained low in membranes prepared from fibroblasts that had been incubated for 24 h or more in medium containing LPDS. The addition of LDL to these cells caused a rapid increase in enzyme activity, followed by an equally rapid fall to almost the original value (Fig. 5.2(a)). The magnitude of the increase depended upon the concentration of LDL added, but with each concentration the greatest activity was observed 7-8 h after the addition of the lipoprotein. There was an apparently saturable relationship between this maximal activity and the concentration of LDL in the incubation medium (Fig. 5.3(a)). ACAT activity in fibroblast membranes was also increased if non-esterified cholesterol was added to the culture medium. Activity reached a maximum 24 h after the addition of cholesterol (Fig. 5.2(b)) and remained high for at least a further 24 h. Cholesterol, unlike LDL, was able to increase ACAT activity in fibroblasts from a homozygous FH subject. There was a similar concentration-dependent increase in activity in both types of cell which was apparently saturated at a concentration of 100 μg of cholesterol/ml (Fig. 5.3(c)). The ability to stimulate cholesterol esterification was not limited to cholesterol supplied from outside the cell since incubation of fibroblasts with mevalonic acid, an intermediate in the pathway of cholesterol synthesis, also caused a concentration-dependent increase in ACAT activity, of similar magnitude to that produced by LDL (Fig. 5.3(b)). In order to be able to relate the changes

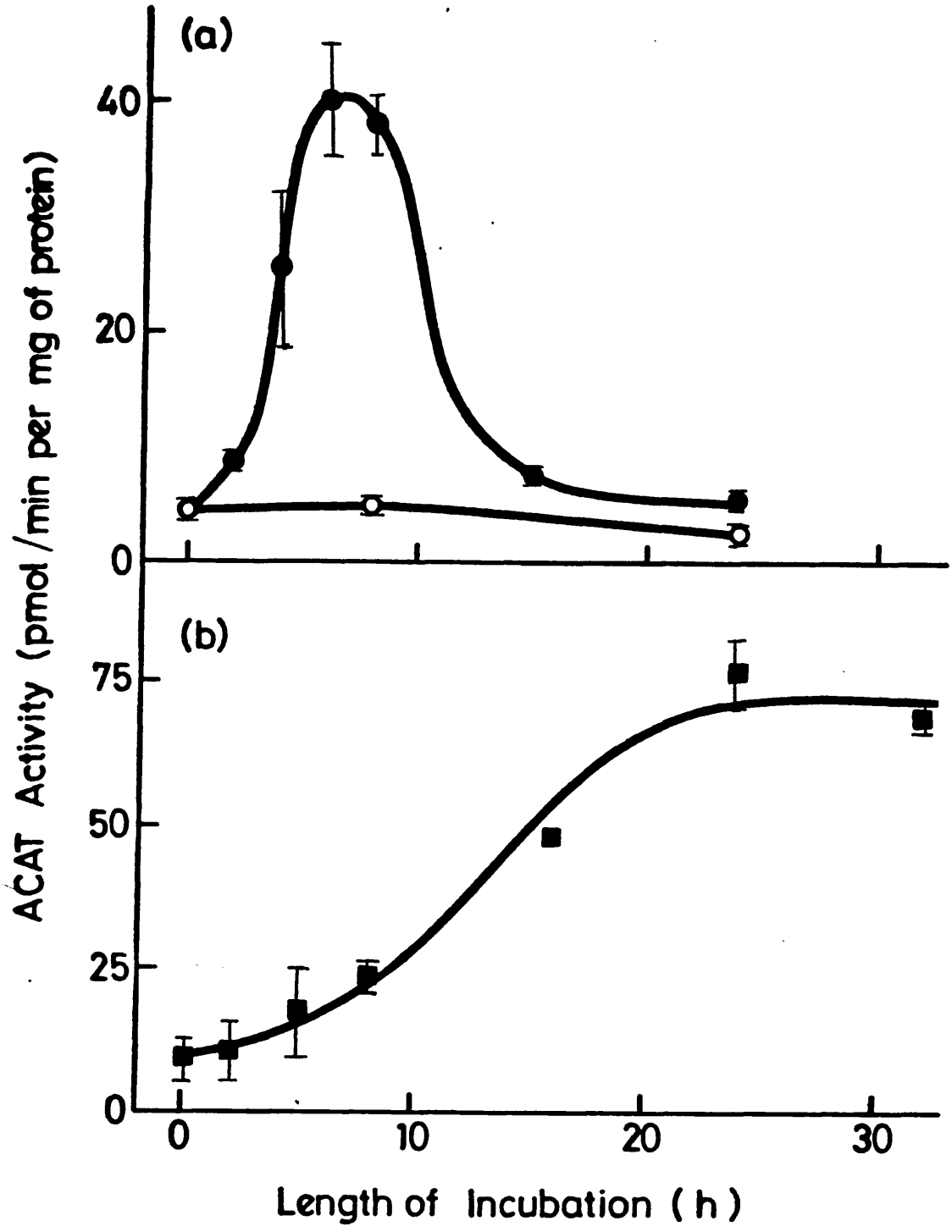


Figure 5.2 Effects of LDL (a) and cholesterol (b) on ACAT activity

Normal fibroblasts were preincubated for 24 h with LPDS and then for various periods with LPDS alone (○) or LPDS containing either 100 μg of protein/ml of LDL (●) or 50 μg/ml of cholesterol (■). At each time, ACAT activity was assayed in membranes prepared from cells from two duplicate 60 mm dishes and the values shown are the averages of duplicate assays.

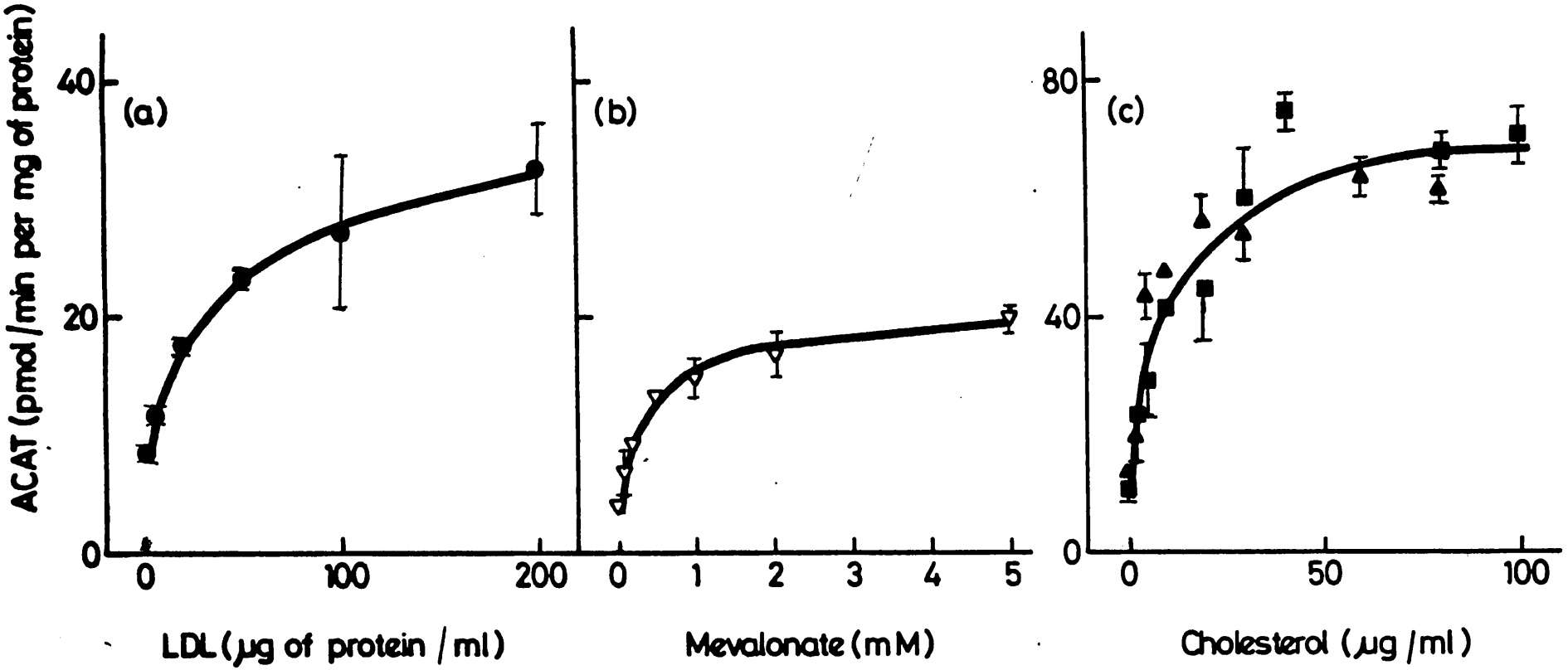


Figure 5.3 Relationships between ACAT activity and the concentration of LDL (a), mevalonic acid (b) and cholesterol (c) in the incubation medium

Fibroblasts were preincubated with LPDS for 24 h before the addition of the required concentrations of LDL (●), mevalonic acid (▽) or cholesterol (■,▲).

(a) Membranes were prepared from normal cells 7 h after the addition of LDL.

(b) Membranes were prepared from normal cells 24 h after the addition of mevalonic acid.

(c) Membranes were prepared from normal cells (■) or cells from a homozygous familial hypercholesterolaemic subject (▲) 24 h after the addition of cholesterol.

ACAT activity in the membrane preparations was assayed as described in Section 5.2 and values shown are the averages of duplicate assays.

in ACAT activity to changes in net cholesterol esterification in intact cells, the ability of fibroblasts to incorporate [^{14}C]oleate into cholesteryl esters was investigated. The process had an apparent K_m of approximately 5 μM for oleate in the growth medium (Fig. 5.4(a)) and the rate at which oleate was esterified with cholesterol remained constant for at least 3 h (Fig. 5.4(b)). In all other experiments, the amount of [^{14}C]oleate incorporated into cholesteryl esters was assayed after a 1 h incubation in the presence of 100 μM oleate. Since there was essentially no lag before the [^{14}C]oleate appeared in cholesteryl esters, the incorporation gave an indication of the net rate of cholesteryl ester formation at that time.

Incorporation of oleate changed in a similar way to ACAT activity in response to LDL or cholesterol (Fig. 5.5). Incubation of fibroblasts with LDL produced a transient increase in the rate of incorporation which reached a maximum after 8 h, whereas cholesterol produced a more gradual, but prolonged effect. If LDL was added at the same time as a low concentration of cholesterol the rate of incorporation of oleate into cholesteryl esters at any time was equivalent to the sum of the rates observed with each alone (Fig. 5.5). As with ACAT, the effect of LDL upon the rate of oleate incorporation into cholesteryl esters was concentration-dependent and saturable (Fig. 5.6). Fibroblasts from a heterozygous FH subject incorporated oleate into cholesteryl esters at approximately one-half of the rate observed with normal cells, while fibroblasts from a homozygous FH subject incorporated

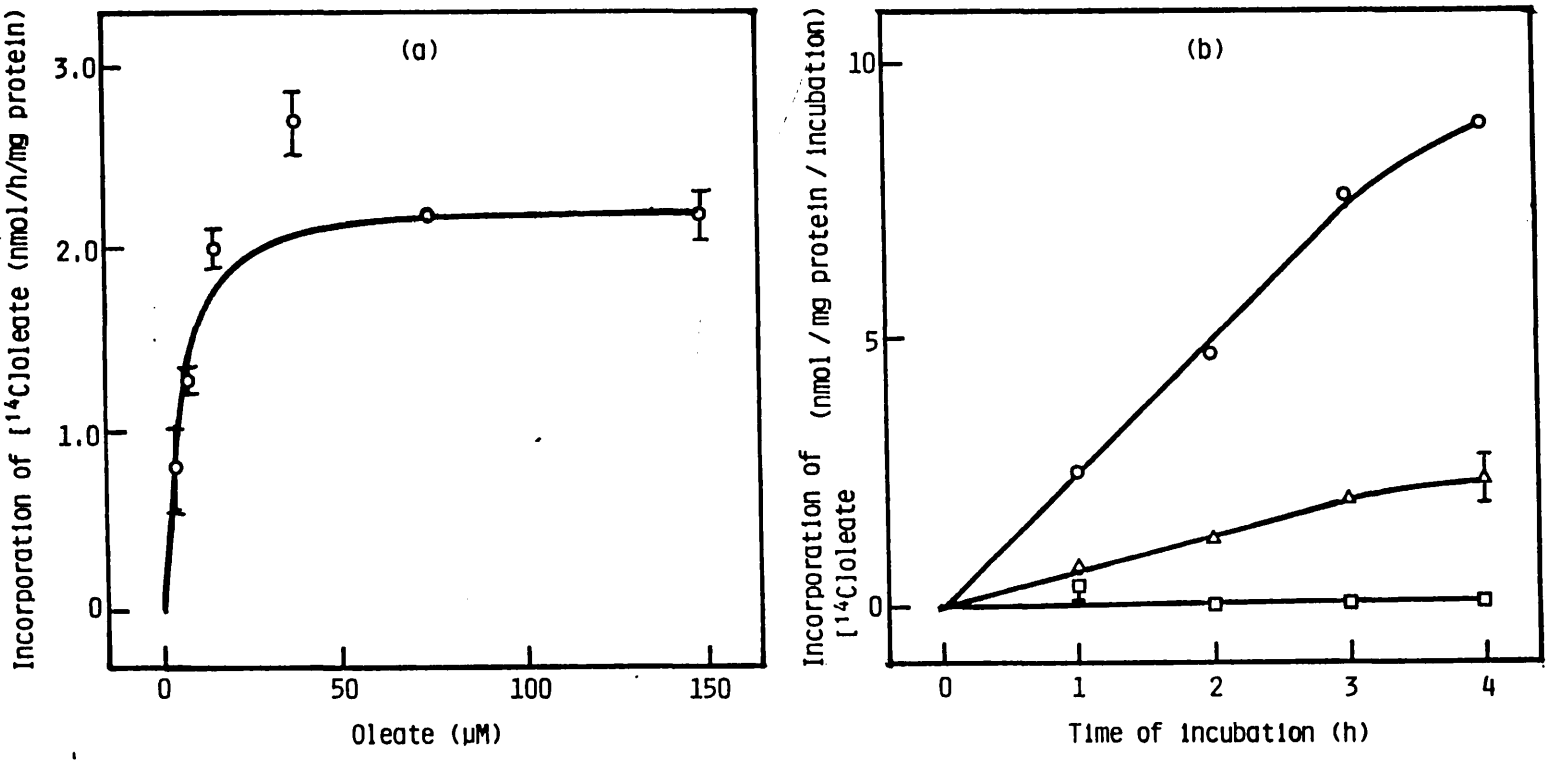


Figure 5.4 Characteristics of [^{14}C]oleate incorporation into cholesteryl esters by cultured human fibroblasts

Cells were preincubated for 24 h in LPDS and the incorporation of [^{14}C]oleate (9.4 dpm/pmol) into cholesteryl esters was assayed, as described in Section 5.2, either immediately (□) or after a further incubated with LDL for 7 h (○) or 24 h (△). (a) Fibroblasts in dishes were incubated for 3 h in the presence of various concentrations of oleate. (b) Fibroblasts in dishes were incubated for various times in the presence of 150 μM oleate. Each point is the average value from duplicate 60 mm dishes.

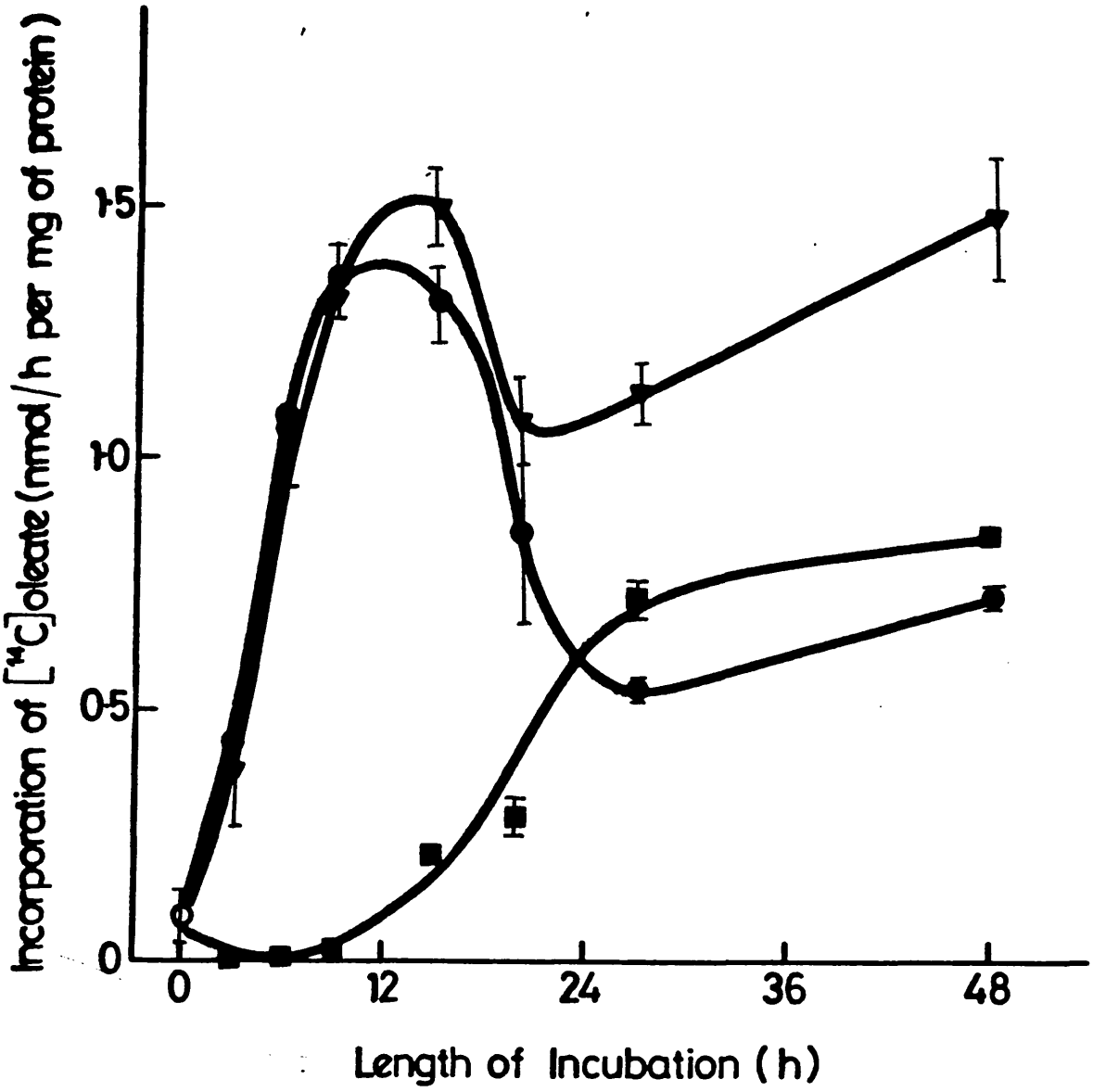


Figure 5.5 Effect of LDL (●), cholesterol (■) and LDL plus cholesterol (▼) on the rate of incorporation of [^{14}C]oleate into cholesteryl esters by cultured fibroblasts

Normal fibroblasts were preincubated for 24 h with LPDS and then for various periods with 100 μg of protein/ml of LDL, 20 μg/ml of cholesterol or a combination of the two. At the times indicated, [1-^{14}C]oleate complexed with albumin (3.5 d.p.m./pmol) was added to the incubation medium to give a final concentration of 150 μM oleate, and the incubations continued at 37°C for 1 h. The cells were then harvested and the radioactivity present in cholesteryl esters was determined as described in Section 5.2. Values shown are the averages of assays from duplicate 60 mm dishes.

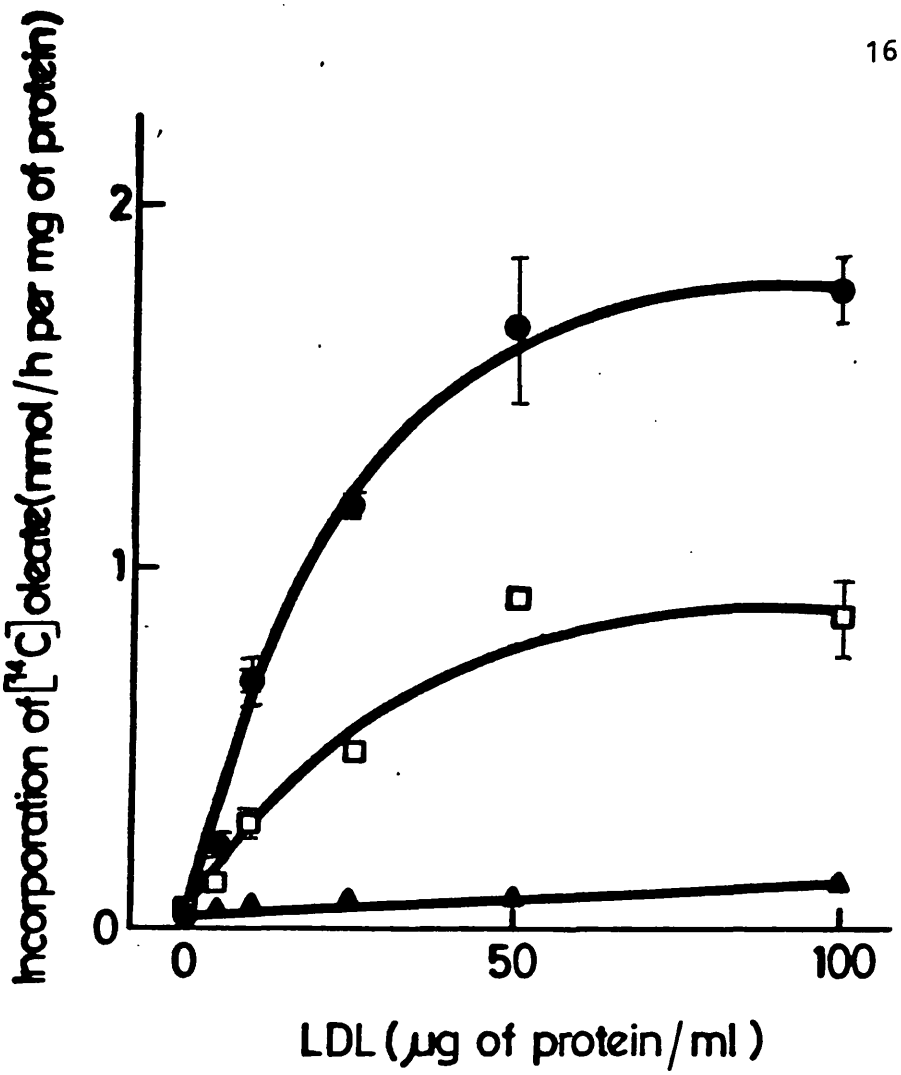


Figure 5.6 Relationship between the concentration of LDL and incorporation of [^{14}C]oleate into cholesteryl esters by normal fibroblasts (●) and fibroblasts from heterozygous (□) and homozygous (▲) familial hypercholesterolaemic subjects

Cells were preincubated for 24 h with LPDS before the addition of LDL. After 7 h at 37°C the cells were incubated for a further hour at 37°C with [1-^{14}C]oleate and the incorporation into cholesteryl esters was determined as described in Section 5.2. Values are averages of duplicate incubations.

very little oleate into cholesteryl esters, even at the highest concentration of LDL (Fig. 5.6).

The two columns on the left of Table 5.1 show a comparison between values of ACAT activity in fibroblast membranes and the rate of oleate incorporation by intact cells, obtained using concentrations of LDL or cholesterol that saturated each process. Generally the values for net oleate incorporation were similar to the corresponding values for ACAT activity, indicating that the enzyme had not been particularly purified during the preparation of the membranes. When fibroblasts had been incubated with LPDS alone the rate of oleate incorporation into cholesteryl esters measured in intact cells was relatively less than the ACAT activity measured in isolated membranes, presumably as a result of cholesterol esterase activity in intact cells. This suggests that, in intact cells, the esterase only had a significant effect upon the net incorporation when the rate of cholesterol esterification was low and that its influence on ACAT activity, assayed in membrane preparations, was diminished even further. ACAT activity in membranes from fibroblasts incubated for 24 h with a saturating concentration of cholesterol was approximately double the maximum activity observed 7 h after the addition of LDL (Table 5.1).

Under the conditions of the ACAT assay, with no added substrate cholesterol, an increase in observed activity could have resulted from an increase in the membrane bound substrate pool, from an increase in the total catalytic activity present on the membranes, or from both. To distinguish between these

Table 5.1

The effects of LDL and cholesterol on [^{14}C]oleate incorporation by intact cells and ACAT activity in isolated membranes

Norman fibroblasts were preincubated for 24 h with LPDS and then for the periods shown in the same medium containing 100 μg protein/ml LDL or 50 μg/ml of cholesterol. At the appropriate times cells were incubated for 1 h with [1-^{14}C]-oleate to determine the rate of [^{14}C]oleate incorporation into cholesteryl esters or were assayed for membrane ACAT activity before and after equilibration of the membranes with donor lipoprotein (see Section 5.2). Values shown are the means ± S.E.M. for the numbers of separate incubations (shown in parentheses). *Values significantly higher than those of controls incubated with LPDS alone ($p<0.05$; Student's t-test). All values obtained after incubation for 24 h with cholesterol were significantly higher ($p<0.05$; Student's t-test) than the corresponding values obtained after incubation for 7 h with LDL.

Incubations	*[1-^{14}C]Oleic acid incorporated into cholesteryl esters (pmol/min per mg protein)*	*ACAT activity (pmol/min per mg protein)*	
		Unequilibrated membranes	*Equilibrated membranes*
LPDS alone	2.5±0.9 (15)	7.5±1.2 (10)	15.1±3.9 (7)
3 h with LDL		12.6±3.2 (4)	13.8±2.1 (4)
7 h with LDL	32.9±8.9 (7)*	28.1±2.8 (9)*	17.7±2.6 (7)
16 h with LDL		7.6±0.6 (2)	16.5±2.7 (6)
24 h with cholesterol	52.7±7.5 (3)*	66.6±7.0 (8)*	30.5±2.9 (4)*

possibilities it would be necessary to modify the cholesterol content of the membranes after they had been isolated so that activities could be compared under conditions in which each contained the same amount of cholesterol in the substrate pool. Hashimoto and Dayton (1979) have shown that incubation with cholesterol-containing lipoproteins increased the cholesterol content and ACAT activity of liver microsomes. To investigate whether there was a similar effect upon the fibroblast enzyme, isolated membranes were incubated with LDL from a homozygous FH subject (donor lipoprotein), which was the most cholesterol-rich lipoprotein that was available (1.08 mol unesterified cholesterol/mol phospholipid). After this incubation the membranes were reisolated and assayed for ACAT. ACAT activity in reisolated membranes depended on the concentration of donor lipoprotein present during the incubation rather than the activity in the membranes before the incubation (Fig. 5.7(a)). Whether the initial activity was large or small, a low concentration of donor reduced ACAT activity to a similar low value. As the donor concentration was raised, the enzyme activity in the reisolated membranes increased until the concentration reached approximately 4 mg of protein/ml after which it remained constant. Incubation with a high concentration of donor lipoprotein increased the activity of the less active membrane preparations but led to a reduction in the activity of the originally more active preparations.

These experiments suggest that the cholesterol in the membranes, and particularly that in the substrate pool for ACAT,

Figure 5.7 Effect on ACAT activity of incubation membrane preparations with cholesterol-rich donor particles

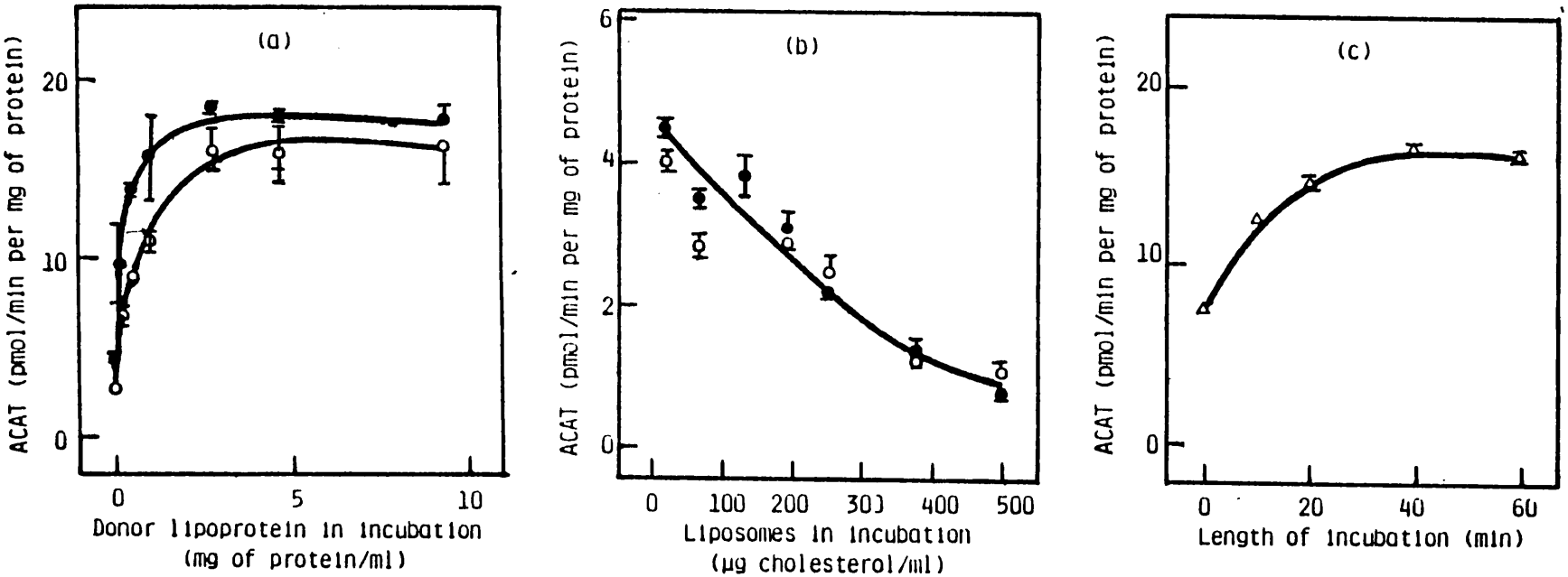
Membranes were prepared from normal cells that had been preincubated for 24 h with LPDS (O,Δ) or for a further 7 h with 100 μg of protein/ml of LDL (●). For each condition cells were pooled from ten 60 mm dishes.

(a) Samples containing 50 μg of membrane protein were incubated for 60 min at 37°C with the indicated concentration of donor lipoprotein as described in Section 5.2. Membranes were reisolated and assayed for ACAT activity.

(b) Samples containing 50 μg of membrane protein were incubated for 60 min at 37°C with the indicated concentration of liposomes (0.97mol unesterified cholesterol/mol phospholipid). Membranes were reisolated and assayed for ACAT activity.

(c) Samples containing 50 μg of membrane protein were incubated for periods shown with 5 mg of protein/ml of donor lipoprotein. Membranes were reisolated and assayed for ACAT.

The points represent the averages of duplicate assays. Before incubation, ACAT activity in membranes from cells incubated with LPDS and from cells incubated with LDL was 6.6 and 26.4 pmol/min per mg of protein, respectively.


(a)
ACAT (pmol/min per mg of protein)
20
10
0
0
5
10
Donor lipoprotein in incubation
(mg of protein/ml)
(b)
ACAT (pmol/min per mg of protein)
6
4
2
0
0
100
200
300
400
500
Liposomes in incubation
(μg cholesterol/ml)
(c)
ACAT (pmol/min per mg of protein)
20
10
0
0
20
40
60
Length of incubation (min)

could exchange and equilibrate with the donor lipoprotein cholesterol, although under the conditions employed the substrate pool could not be saturated. This, in turn, suggested that the activity of membranes incubated with another cholesterol-rich donor might be even higher. To this end membranes were incubated with liposomes (0.97 mol unesterified cholesterol/mol phospholipid). However, the results were disappointing with the activity measured in membranes after equilibration being less than the lowest initial activity (Fig. 5.7(b)), and in all future experiments membranes were equilibrated with FH LDL.

Any net transfer between donor lipoprotein and fibroblast membranes was complete well within the standard 60 min incubation (Fig. 5.7(c)). In subsequent experiments, incubations to achieve cholesterol equilibration always contained approximately 250 μg of membrane protein/ml with 5 mg of protein/ml of donor lipoprotein.

In order to discover whether the increase in ACAT activity observed when fibroblasts were incubated with LDL or cholesterol could be entirely explained by an increase in the supply of cholesterol to the substrate pool, the membrane preparations were equilibrated with donor lipoprotein, reisolated and assayed. The rise and fall in ACAT activity produced by LDL, observed in unequilibrated membranes, were not apparent after equilibration (Table 5.1). Each preparation then had the same activity irrespective of the conditions of culture. In contrast, equilibrated membranes from membranes from cells incubated

for 24 h with cholesterol exhibited a much greater ACAT activity than equilibrated membranes from cells incubated with LPDS alone (Table 5.1).

One aim of this work was to discover whether the increase in LDL degradation by fibroblasts that accompanied the enrichment of cellular phospholipids with linoleic acid was associated with an increase in cholesteryl ester synthesis. To this end, ACAT activity was compared in membranes isolated from cells grown in medium supplemented with 50 μM palmitate, oleate or linoleate and from cells grown in medium without additional fatty acid. After incubation of fibroblasts with LPDS, ACAT activity in unequilibrated membranes from cells grown with linoleate or oleate showed a significant, two-fold increase over that of cells grown with palmitate or without a fatty acid supplement (Table 5.2). LDL produced the normal transient increase in ACAT activity which, in each case, was greatest 7 h after the addition. This maximal ACAT activity in cells grown with linoleate or oleate was also almost twice as great as the corresponding enzyme activity in cells grown with palmitate (Table 5.2). Moreover, it was not simply the absolute values of ACAT activity which were increased in cells grown with the unsaturated fatty acids. The magnitude of the increase brought about by the 7 h incubation was also 1.5- to 2-fold greater in cells grown with linoleate or oleate than those grown with palmitate.

This increase in ACAT activity was still apparent even after the membranes had been fully equilibrated with the donor

Table 5.2

The effect of LDL on ACAT activity in membranes from normal fibroblasts grown in unsupplemented medium or medium supplemented with 50 μM fatty acid

Fibroblasts were grown for 6 days in medium supplemented with fatty acids as described and then for 24 h in medium containing LPDS with the same fatty acids. Cells were harvested immediately or 7 h after the addition of 100 μg of protein/ml of LDL. Membranes were prepared and assayed for ACAT activity before and after the equilibration of the membranes with donor lipoprotein. Values are the mean ± S.E.M. of five separate experiments.

	ACAT Activity (pmol/min per mg of protein)			
	No Additions	*50 μM Palmitate*	*50 μM Oleate*	*50 μM Linoleate*
Unequilibrated membranes				
LPDS*	8.3±1.1	7.0±1.7	15.8±2.4	15.8±3.1
LDL*	25.5±4.1	20.1±4.0	41.5±8.1	35.8±6.5
Increase†	17.1±3.7	13.1±4.5	25.6±8.2	18.7±6.0
Equilibrated membranes				
LPDS§	15.7±3.2	12.1±1.2	21.6±0.6	18.2±2.3
LDL*	13.5±1.7	16.7±3.2	34.0±3.2	29.9±1.4

*Values for cells grown with palmitate or without additional fatty acid are significantly lower ($p<0.05$; Student's t-test) than those for cells grown with oleate or linoleate.
†Value for cells grown with palmitate are significantly lower ($p<0.05$; paired t-test) than values for cells grown with oleate or linoleate.
§Value for cells grown with palmitate is significantly lower ($p<0.05$; Student's t-test) than values for cells grown with oleate or linoleate.

lipoprotein. The ACAT activities in the equilibrated membranes from fibroblasts grown with linoleate or oleate were again approximately twice the activities of fibroblasts grown with palmitate whether the cells had been incubated with LPDS only or for a further 7 h with LDL.

Interestingly, while the ACAT activities of equilibrated membranes from cells grown with palmitate, like those from cells grown without supplementary fatty acids, were the same whether the cell was incubated with LPDS only or for 7 h with LDL, this was clearly not the case when the fibroblasts were grown with oleate or linoleate. Indeed, after growth with either of these fatty acids, the ACAT activity of fully equilibrated membranes from cells incubated for 7 h with LDL was 50% greater than the activity of equilibrated membranes from cells grown only in LPDS.

5.4 Discussion

Measurement of the accumulation of radioactivity in cellular cholesteryl esters during incubation with [^{14}C]oleic acid (Goldstein *et al.*, 1974*a*) has proved a quick and easy method of estimating cholesteryl esterification in cultured cells. However, the values obtained are the net difference between cholesteryl ester formation, catalysed by ACAT, and its subsequent hydrolysis by cholesteryl esterase. By a simple modification of standard methods it has been possible

to assay ACAT activity in the small amounts of membrane material available from fibroblasts. A comparison between values of cellular incorporation and the direct membrane assay showed that, in many circumstances, measuring the net oleate incorporation during short, pulse incubations would provide an adequate indication of changes in the rate of cholesterol esterification. Esterase activity only significantly affected the results if the rate of esterification was low. However, the direct ACAT assay was required for a more detailed study of cholesterol esterification and was essential for investigating activity in cells grown in medium supplemented with free fatty acids. The variation of ACAT activity in the membrane assay with the concentration of [^{14}C]oleoyl-CoA added, and the linearity of the relationship between ester formation and time, even with membranes from cells grown with high concentrations of fatty acid, indicated that any dilution of [^{14}C]oleoyl-CoA added as substrate by unlabelled, membrane associated fatty acyl-CoA was negligible.

When LDL is added to fibroblasts preincubated without lipoproteins, the rapid increase in both oleate incorporation into cholesteryl esters and membrane ACAT activity reflected the initially high rate of uptake and degradation of the lipoprotein. As the number of LDL receptors, and thus the supply of cholesterol, was reduced, the rate of cholesteryl ester formation reached a peak and then fell. Cells from homozygous FH subjects, which cannot express functional LDL receptors, did not show any increase in the rate of cholesteryl ester formation on incubation with LDL. Conversely, cells that had been grown with linoleate, which

degrade LDL at a greater rate than those grown in normal medium or with palmitate, exhibited a correspondingly greater ACAT activity in their isolated membranes. However, it was not only receptor-mediated uptake of LDL which could increase ACAT activity. The addition of mevalonic acid, which stimulates endogenous cholesterol synthesis, had a similar effect, while the uptake of non-esterified cholesterol from the medium, by a process which was not subject to any metabolic control, could increase ACAT activity to a value which was approximately twice as high as the maximum observed with LDL.

The rate at which cholesterol is deposited within the cells is clearly an important factor in regulating the rate of cholesteryl ester formation. This is further supported by the observation that when LDL was added to cells at the same time as cholesterol, each stimulated the incorporation of oleate into cholesteryl esters independently of the other so that the rate at any time was equivalent to the sum of the rates observed with each alone.

Given an increase in the supply of cholesterol, there are a number of ways in which an increase in activity could be achieved. The cholesterol used as substrate by ACAT, like the enzyme itself, is membrane associated (Balasubramaniam *et al.*, 1978*b*). Since it is only a small proportion of the total membrane cholesterol (Balasubramaniam *et al.*, 1978*a*), once the membranes have been isolated there is only a limited amount of substrate available to the enzyme. For this reason, the initial rate of reaction was measured in all membrane preparations and, under these

conditions, the activity observed depended on the amount of enzyme present in the preparation, its degree of activation and the amount of cholesterol in the substrate pool. Incubation of the membranes with donor lipoprotein resulted in an apparent equilibration of cholesterol between the lipoprotein and the membranes, including the substrate pool, providing an opportunity to gain some insight into the mechanisms by which ACAT activity was modulated. There was a saturable relationship between the activity observed after equilibration and the concentration of the donor lipoprotein. Although incubation with a saturating concentration of the donor did not elicit the maximum possible ACAT activity, it provided conditions in which membrane preparations, that differed before incubation only in the size of the substrate pool, would give the same activity when subsequently assayed. This was the case with membranes from cells that had been grown in normal medium, preincubated with LPDS and then incubated with LDL for various times. After equilibration the membranes had the same activity irrespective of the activity in the membranes before equilibration. Thus, the differences in ACAT activity in unequilibrated membranes could be explained entirely by differences in the size of the substrate pool. There was no evidence for any alteration in the amount of enzyme present nor for any change in membrane structure leading to a change in enzyme activity or a different distribution of membrane cholesterol during the equilibration with donor. Doolittle and Chang (1982*a*) have reported experiments with ACAT reconstituted into artificial membranes

that also indicate that the effects of LDL are not associated with changes in the amount of enzyme present. It is not possible to exclude the possibility that cholesterol can itself modulate enzyme activity as well as being substrate.

When fibroblasts were grown in medium supplemented with linoleate, even the effect of LDL could not be entirely explained by an increase in the size of the substrate pool. Membranes prepared from cells grown with linoleate had a greater ACAT activity than those prepared from cells grown with palmitate, both in cells incubated with just LPDS and in cells incubated for a further 7 h with LDL. Moreover, the increase in activity brought about the 7 h incubation of the fibroblasts with LDL was greater in cells grown with linoleate than those grown with palmitate.

This confirms in cultured cells the effect of dietary fat on ACAT activity in intact animal tissues (Mitropoulos *et al.*, 1980; Spector *et al.*, 1980; Field and Salome, 1982; Johnson *et al.*, 1983). This increase in ACAT activity appears to be a general effect of polyunsaturated fatty acid and not simply a specific effect of linoleate (Johnson *et al.*, 1983).

These differences in activity were still apparent after the membranes had been equilibrated with donor lipoprotein. Indeed, equilibrated membranes from fibroblasts grown with linoleate, unlike those from cells grown with palmitate or in normal, unsupplemented medium, exhibited a further increase in ACAT activity if the cells had been incubated with LDL. Similarly, membranes prepared from cells that had been incubated

for 24 h in medium with non-esterified cholesterol had a greater ACAT activity after equilibration than those from cells left in LPDS.

While it would appear that in both cases there was an increased supply of cholesterol, mere expansion of the substrate pool would seem to be insufficient to entirely account for these results and a further change, either to the enzyme itself or possibly to the organisation of the membrane, must be postulated. When faced with an exceptional increase in the supply of cholesterol, whether from prolonged uptake of non-esterified cholesterol or from the accelerated degradation of LDL in cells grown with linoleate, the fibroblasts were able to increase their potential catalytic activity so that surplus cholesterol was esterified and did not accumulate and interfere with normal membrane function. This increase in catalytic activity could involve induction of ACAT synthesis or an activation of existing enzyme through a change in membrane fluidity (Spector *et al.*, 1980; Field and Salome, 1982; Suckling *et al.*, 1982), or through a covalent modification such as phosphorylation (Suckling *et al.*, 1983*a*,*b*).

It was somewhat puzzling that, following incubation with LPDS, which should reduce the supply of cholesterol, membranes from linoleate-enriched cells should still have a higher ACAT activity than those from palmitate-enriched cells. This may possibly be because during the preceding growth period in FCS the increased rate of LDL degradation may have brought about an increased maximum potential ACAT activity which failed to decay during the 24 h in LPDS.

Whatever the actual mechanisms involved, the enhanced rate of LDL degradation shown by fibroblasts grown with linoleate leads to a corresponding increase in the rate of cholesterol esterification by the cells aided by an increase in the potential ACAT activity of their membranes.

In contrast, the potential ACAT activity in fibroblasts cultured under normal conditions and preincubated with LPDS is sufficient to esterify the cholesterol released after the addition of LDL without the need for any further synthesis or activation of the enzyme.

It will be noted from Tables 4.1 and 5.2 that the effect of oleate on LDL degradation and ACAT activity does not fit in entirely with this hypothesis. Cells grown with oleate or linoleate had similar amounts of ACAT activity under whatever conditions they were compared, yet cells grown with oleate degraded considerably less LDL than those grown with linoleate. No explanation can be offered for this observation beyond the possibility that oleate has a specific effect on ACAT activity. However, it should perhaps be noted that the higher than expected ACAT activity of cells grown with oleate is not due to the presence of extra substrate oleate which would reduce the specific activity of [^{14}C]oleoyl coenzymeA giving a lower measured enzyme activity.

Chapter 6

DISCUSSION

During the onset of atherosclerosis, which is one of the major causes of death in the United Kingdom, lipid-filled foam cells appear in the coronary arteries. As the amount of lipid, chiefly cholesteryl esters, increases, these cells swell into the lumen of the artery, restricting the flow of blood to heart muscles (Ross, 1981). The probability of any individual suffering from this disease may be influenced by a number of factors. However, atherosclerosis rarely develops unless the plasma cholesterol concentration exceeds 160 mg/ml (Goldstein and Brown, 1977) and the observation that people with high plasma concentrations of LDL were more likely than usual to suffer heart disease (Stamler, 1973) has made LDL metabolism the subject of intense interest, both *in vivo* and *in vitro*.

The hypocholesterolaemic effect of dietary polyunsaturated fatty acids (Kinsell *et al.*, 1952; Keys *et al.*, 1965) appears to be effected, at least in part, by an increase in the fractional catabolic rate of LDL *in vivo* (Shepherd *et al.*, 1980). This observation has not been followed up *in vitro* despite the large amount of research carried out into other aspects of the LDL receptor pathway in cultured cells. Therefore, the aim of the work presented in this thesis was to investigate how the

catabolism of LDL in cultured human fibroblasts is affected by the addition of fatty acids to the growth medium. Of particular interest was the relationship between the fatty acid composition of cellular phospholipid and the ability of the fibroblasts to catabolise LDL.

Modification of the fatty acid composition of cellular phospholipid can lead to changes in membrane fluidity (Quinn, 1976) which accompany changes in the properties of membrane-associated proteins (Horowitz *et al.*, 1974; Solomonsen *et al.*, 1976; Shinitzky and Rivnay, 1977). For this reason, the effects of palmitate, linoleate and oleate were examined. Palmitate, a saturated fatty acid, would be expected to bring about a decrease in membrane fluidity; linoleate, a poly-unsaturated fatty acid, would be expected to increase membrane fluidity; while oleate, a monounsaturated fatty acid, should have little overall effect on the fluidity (Quinn, 1976). Changes in the fatty acid composition of membrane phospholipids were achieved, with varying degrees of success, by growing cells in the presence of the required fatty acid, under the appropriate conditions. The proportion of linoleate, initially low, could be increased four-fold by growing the fibroblasts in medium supplemented with this fatty acid. On the other hand, the proportion of palmitate, high to begin with, could be increased only marginally when cells were grown with this saturated fatty acid, while the proportion of oleate could be increased by approximately one-third in cells grown in the presence of oleate. It was the proportion of palmitate and, to a lesser extent, oleate

which decreased to accommodate increases in the proportions of other fatty acids. The ability of fibroblasts to incorporate fatty acids from the growth medium into their phospholipids ensured that cells with a wide range of phospholipid fatty acid compositions could be obtained with little difficulty. This allowed a close examination of the relationship between the fatty acid composition of cellular phospholipid and various aspects of LDL catabolism.

The LDL receptor is one of a class of proteins associated with the cell surface membrane whose function is to bind specific ligands and take them into the cell by a process known as receptor-mediated endocytosis. Work on the LDL receptor has led to an understanding, not only of how this lipoprotein is metabolised, but also of the more general mechanisms responsible for receptor-mediated endocytosis (Goldstein *et al.*, 1979; Brown *et al.*, 1983*a*). Recently, work by Brown, Goldstein and their coworkers has started to uncover some aspects of LDL receptor structure, its amino acid sequence and the control of its synthesis (Beisiegel *et al.*, 1981; Schneider *et al.*, 1983; Cummings *et al.*, 1983; Russell *et al.*, 1983, 1984; Tolleshaug *et al.*, 1982, 1983). Like many other cell-surface proteins, the LDL receptor is a transmembrane protein, which is to say that it has domains at both the cytoplasmic and external faces of the membrane connected by a sequence of hydrophobic amino acid residues which span the lipid bilayer. Given this intimate connection with the membrane, changes in the composition of the cellular phospholipid might be expected to influence the behaviour of the receptor.

Interestingly, fatty acids added to the medium appeared to influence LDL degradation by two separate mechanisms. When oleate gradually replaced palmitate in the medium, the rate of degradation increased until the two fatty acids were approximately equimolar. As the proportion of oleate increased further the rate of degradation decreased. These changes, although apparently unconnected with changes in the proportion of any fatty acid or group of fatty acids in the cell phospholipid, correlated very closely with the number of binding sites expressed at the cell surface. Although no explanation can be offered as to why the number of binding sites increased under these conditions, it appears that, in this case, the increased rate of degradation was a direct consequence of the increased binding capacity of the cells. This was expected since cell-surface binding is believed to be the rate-limiting step in the degradative process (Goldstein and Brown, 1974*b*).

In complete contrast was the effect of linoleate on LDL catabolism. Degradation of the lipoprotein by both the saturable and non-saturable pathways was increased. Fibroblasts grown with linoleate could degrade LDL by the saturable pathway at a rate 50% greater than that of fibroblasts grown with palmitate but, surprisingly, there was no accompanying change in either the amount of LDL bound by the cell or the apparent affinity of the receptors for the lipoprotein. As linoleate replaced oleate or palmitate in the medium, the increasing rate of LDL degradation was accompanied by and correlated very closely with an increase in the P/S ratio of the fatty acids in membrane phospholipids.

This suggested that the enhanced rate of LDL degradation could have been caused by a change in membrane properties related to changes in the composition of the phospholipid fatty acids. Possibly, a rise in the P/S ratio of phospholipid fatty acids could be accompanied by a non-specific increase in the rate of membrane turnover leading to an enhnacement of LDL degradation by both the non-saturable, adsorptive endocytotic mechanism and the saturable receptor-mediated pathway.

The cholesterol released by the degradation of LDL is used by the cell in membrane synthesis. Cholesterol in excess of the cell's requirements is re-esterified by the enzyme ACAT. Since ACAT is a membrane-associated enzyme its activity may be influenced by changes in its lipid environment. In addition, when fibroblasts are grown in medium supplemented with linoleate, the enhanced rate of LDL degradation with the consequential increase in the supply of cholesterol as substrate for ACAT could also have been expected to affect the activity of the enzyme. To test these possibilities an existing assay for the enzyme (Goodman *et al.*, 1963) was modified for use with fibroblast membrane preparations. ACAT activity varied in response to changes in cholesterol supply in much the same way as net cholesteryl ester synthesis in intact cells when measured by the incorporation of [^{14}C]oleate into cholesteryl esters. Whether measured in membrane preparations or in intact cells, the enzyme's activity could be influenced by cholesterol from a variety of sources and, when LDL and unesterified cholesterol were both added to the culture medium, each achieved its effect on cholesteryl ester formation independently of the other.

The changes in the initial rates of ACAT activity assayed in membrane preparations could be due to changes in the amount of substrate cholesterol present, the amount of enzyme present, or the degree of activation of the enzyme. By equilibrating the membranes with donor lipoprotein and re-isolating them, it was possible, within each growth condition, to assay the enzyme in the presence of a constant amount of substrate. This showed that cells grown with LPDS and those incubated for a further 7 hours with LDL had the same potential ACAT activity and that differences observed in the enzyme activity of the unequilibrated membranes were due to differences in the availability of cholesterol as substrate. More prolonged exposure to high concentrations of cholesterol leads to a greater potential ACAT activity, although it is not possible to comment on whether this arises through synthesis of new enzyme or activation of existing enzyme. Cells with phospholipids enriched with linoleate not only degraded LDL at a higher rate than cells grown with palmitate but also had greater ACAT activity, both after 24-hour preincubation with LPDS and after a further 7-hour incubation with LDL. The size of the increase brought about by LDL was also greater. The changes in ACAT activity of the membranes following equilibration with donor lipoprotein showed that the increased synthesis of cholesteryl esters by cells grown with linoleate could only be partly explained by an increased supply of substrate cholesterol. While sufficient ACAT enzyme is present in fibroblasts to cope with normal fluctuations in the supply of substrate cholesterol,

cells grown with linoleate are degrading LDL at a greater rate than normal by both the receptor-dependent and receptor-independent pathways so that these cells are exposed to a prolonged increase in the supply of substrate cholesterol. Since cells grown with unesterified cholesterol seem capable of increasing their maximum potential ACAT activity, it would seem reasonable to suppose that cells grown in the presence of polyunsaturated fatty acids would have the same capability.

Although the mechanism by which linoleate affects LDL receptor function and ACAT activity *in vitro* is unclear, the results presented here clearly show that alterations to the fatty acid composition of fibroblast membrane phospholipid are associated with considerable changes in the ability of these cells to process LDL and these findings suggest a possible explanation for the hypocholesterolaemic effect of polyunsaturated fatty acids *in vivo*.

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