THE EFFECT OF POLYUNSATURATED FATTY ACIDS ON CHOLESTEROL METABOLISM

SEBELY PAL

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By

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ABBREVIATIONS

ACAT	Acyl CoA:cholesterol acyl transferase
AGLDL	Acetylated LDL
BSA	Bovine serum albumin
CE	Cholesteryl esters
CEH	Cytoplasmic cholesteryl ester hydrolase
CHD	Coronary heart disease
DHA	Docosahexanenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
HDL	High density lipoprotein
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A
hr	hour
LA	Linoleic acid
LDL	Low density lipoprotein
LPDS	Lipoprotein deficient serum
mg	milligram
PC	Phosphotidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
SM	Spingomyelin
t1/2	half times
VLDL	Very high density lipoprotein

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ABSTRACT

Atherosclerosis is characterized by deposition of cholesterol and esterified cholesterol in cells of the vascular intima. These lipid laden cells are called foam cells due to their microscopic appearance and arise primarily from smooth muscle cells and monocyte macrophages. It is believed that the transformation of these cells into foam cells is a key step in the initiation of atherosclerosis. Therefore, to better understand the process of atherogenesis it is important to investigate the mechanisms that may regulate cholesterol esterification and hence the formation of foam cells.

Polyunsaturated fatty acids (PUFAs) in the diet can substantially reduce the incidence of coronary heart disease. However, dietary ω-3 polyunsaturated fatty acids found primarily in marine oils have been shown to be better in reducing the risk of atherosclerosis than other polyunsaturated fatty acids. Unfortunately, it is unclear how ω-3 PUFAs are involved in delaying the atherogenic process. It is possible that these fatty acids may be antiatherogenic by inhibiting cholesterol esterification or increasing cholesterol removal from cells thereby inhibiting the initial step of foam cell formation.

The major objective of this study was to investigate the impact of different polyunsaturated fatty acid enrichment of nonhepatic tissues on cholesterol esterification and

cholesterol efflux. Human fibroblasts and macrophages were enriched with either linoleic acid (18:3, ω-6) or eicosapentaenoic acid (20:5, ω-3) by supplementing the culture medium. The incorporation of radiolabelled oleoyl CoA into cholesterol esters was reduced by 44% when human fibroblasts were enriched with ω-3 polyunsaturated fatty acids compared with cells enriched with ω-6 polyunsaturated fatty acids. Also, macrophages enriched with ω-3 PUFAs had significantly lower (52%) total cholesterol content than those enriched with ω-6 PUFAs. Cholesterol efflux was measured in cells enriched with ω-3 PUFAs and ω-6 PUFAs. In the presence of HDL, the rate of efflux of radiolabelled cholesterol from cells enriched with ω-3 PUFAs was substantially faster (2x) than efflux from cells enriched with ω-6 PUFA enriched cells. The data suggests that ω-3 PUFAs may be antiatherogenic by inhibiting cholesterol esterification and by accelerating cholesterol efflux from nonhepatic cells.

1. INTRODUCTION

Cholesterol was first identified almost two centuries ago and was characterized as "the waxy, white component of gallstones" (Stallones, 1980). In 1816, Chevreul, a French scientist, gave cholesterol its original name "cholesterine", from the Greek words chole (bile) and steros (solid) (Bloch, 1987). Later, this compound was found to have a reactive hydroxyl group and the name was changed to "cholesterol" (Bloch, 1987). In the early 1900s, extensive efforts focused on the structure of the sterol molecule. At the time these structural studies were in progress, remarkable findings about the role of cholesterol in atherosclerosis were also being made. In 1906, Aschoff first drew attention to the high content of cholesterol in atherosclerotic aortas and in 1910, Windaus showed that the cholesterol deposited in atherosclerotic arteries was chiefly present as cholesteryl esters (Goodman, 1989).

Cholesterol has long been recognized as an essential component in many biological systems. It appears to play key structural and, possibly functional, roles in cellular membranes (Gibbons et al., 1982). Cholesterol is also a major precursor for many steroid hormones and bile acids. Because of the obvious biological importance of cholesterol, intensive efforts were made to elucidate the synthetic pathway of cholesterol in mammals, culminating with the Nobel prize

awarded to Konrad Bloch for defining the biosynthetic pathway from acetate to cholesterol (Bloch, 1987).

It is the role of cholesterol in the development of coronary artery disease, however that has spurred the greatest interest in cholesterol metabolism. Coronary artery disease has been called the "black plague" of the 20th century (Havel, 1988). Atherosclerosis is the leading cause of premature natural death in North America today and it is widely accepted that an elevated plasma cholesterol level is one of the major risk factors for atherosclerosis (Small, 1988).

1.1 Lipoproteins and Coronary Artery Disease

Cholesterol, like most lipids, is transported in body fluids by macromolecular complexes called lipoproteins (Stryer, 1988). Considerable interest in lipoprotein metabolism has been generated in the past few decades because of the apparent causal relationships between the levels of certain serum lipoproteins and the incidence of cardiovascular disease (Vance, 1990). For example, low density lipoproteins (LDL) carry a major portion of cholesterol in the form of cholesteryl ester in human plasma and there is a strong positive relationship between the level of LDL in plasma and the risk of coronary artery disease (McNamara, 1987; Nordoy and Goodnight, 1990; Vance, 1990). LDL is the major lipoprotein responsible for the delivery of cholesterol to peripheral tissues including those of the vasculature. The

fact that cholesterol in the arterial wall is derived from LDL has led to this lipoprotein being referred to as "bad cholesterol" or "bad lipoprotein" (Brown and Goldstein, 1986; Steinberg, 1983).

The high density lipoproteins (HDL) are also cholesterol rich particles. Evidence suggests that HDL plays an important role in reverse cholesterol transport (the movement of cholesterol from non-hepatic tissues to the liver for catabolism and excretion) (Glomset, 1968). Therefore, high levels of HDL appear to lower the relative risk of coronary disease and are thought to be antiatherogenic (Castelli et al., 1977; Frick et al., 1989; Nordoy and Goodnight, 1990, Riechl and Miller, 1989).

It appears that the deposition of cholesterol within the arterial wall plays a central role in the pathogenesis of atherosclerotic plaque (Goodman, 1989; Steinberg, 1983; Vance, 1990). The ability of LDL to deliver cholesterol to arteries and of HDL to remove cholesterol from arteries could account for the close relationship between these plasma lipoprotein levels and coronary disease (Nordoy and Goodnight, 1990; Riechl and Miller, 1981).

1.2 Theories of Atherogenesis

A striking feature of atherosclerosis is the massive accumulation of esterified cholesterol that forms "the heart of" the atheromatous plague. The name, "atheroma" itself

stems from the Greek word meaning gruel, which was first used by pathologists in the 18th century to describe a thickened area in the wall of a major artery which exuded a yellow grumous lipid upon sectioning. This lipid is now known to be nearly pure cholesteryl ester (Goldstein and Brown, 1977).

The literature pertaining to the development of atherosclerotic lesions, exemplifies the emergence of two distinct schools of thought about the biochemical and physiological basis underlying the early stages in atherogenesis. These two hypotheses have generally been termed the "endothelial injury hypothesis" and the "lipid infiltration hypothesis" (Steinberg, 1983).

The endothelial injury hypothesis suggests that damage to the monolayer of endothelial cells lining the artery may initiate plaque formation (Ross and Glomset, 1976). Loss of the structural integrity of the endothelium may cause the release of platelet derived growth factors (PDGF) which could stimulate smooth muscle cell proliferation and secretion of other growth factors. Thus, repeated episodes of endothelial damage and smooth muscle cell proliferation can lead to development of lesions (Ross and Glomset, 1976; Ross, 1981).

According to the classical lipid infiltration theory, lipoproteins, especially LDL, infiltrate the arterial intima prior to endothelial damage (Small, 1977; Steinberg, 1983). Endothelial cells, smooth muscle cells and monocyte macrophages in the intima appear to accumulate lipids,

particularly cholesterol, from these LDL particles and possibly from chylomicron remnants, very low density lipoproteins (VLDL) and β-VLDL (Steinberg, 1983). Thus, the lipid infiltration hypothesis implies that circulating cholesterol (principally in the form of LDL) is central to the atherogenic process, and at lower plasma levels, atherogenesis does not proceed (Steinberg, 1983).

It is now understood that atherosclerosis is a "multifactorial disease" involving a complex array of circulating blood proteins, lipoproteins and cells and their interactions with the cells and matrix proteins of the arterial wall (Witztum, 1990). The result is the "unified" hypothesis proposed by Steinberg, which states that a variety of factors, including endothelal injury and lipid infiltration, combine to produce arterial lesions (Steinberg, 1983). In fact, it is now recognized that endothelial injury may not be the initial event, but rather a subsequent event that makes an important contribution to the development and progression of fatty streaks within the arterial wall (Steinberg, 1983; Witztum, 1990). Evidence suggests that in response to elevated plasma LDL, the earliest event appears to be the focal accumulation of LDL within the intima of an apparently normal artery (Schwenke and Carew, 1989). This accumulation of LDL appears to be the first identifiable event in the lesion prone sites of the aorta. Monocyte macrophages then adhere to the luminal surface of the overlying

endothelium at the sites of LDL accumulation. Subsequent to endothelium binding, monocyte macrophages migrate into the subintimal space under an intact endothelium. Here they take on the classic appearance of foam cells. This term arises from the foam-like appearance of these cells in electron micrographs resulting from enrichment of the macrophages with cholesteryl esters (Steinberg, 1983). As more monocytes enter the intima, more foam cells are generated. Increases in the size and number of these macrophages eventually results in the rupture of the endothelium. At this stage, macrophages are exposed to the circulating blood elements, allowing platelet adherence and aggregation to occur. Release of factors such as PDGF serve both to recruit smooth muscle cells into the intimal layer and to stimulate their proliferation (Steinberg, 1983; Witztum, 1990). The accumulated cholesterol, cells and debris constitutes an atherosclerotic plaque which in time can narrow the lumen of the artery and lead to occlusion (Brown and Goldstein, 1986). Whatever the relative contributions of lipid infiltration and endothelial damage to the development of arterial lesions, the accumulation of cholesteryl ester in the arterial intima is a crucial event in atherogenesis.

1.3 Low Density Lipoproteins and Coronary Artery Disease

In a severely diseased artery, it is not unusual to see as much as a 10-fold increase in the content of unesterified cholesterol and more than a 50-fold increase in

the cholesteryl ester content (St. Clair, 1976). The close relationship between elevated cholesteryl ester in the arteries and atherosclerosis, has generated considerable interest in the mechanism of delivery of LDL cholesterol and the accumulation of cholesteryl esters in non-hepatic tissues. The mechanism of LDL uptake from the circulation by the LDL receptor pathway was established by Brown and Goldstein. They were awarded the Nobel prize in 1985 for their investigations of receptor-mediated endocytosis of LDL (Brown and Goldstein, 1986). The discovery of the LDL receptor has had a profound impact on cell biology, on clinical investigations and therapeutics, and on the study of atherosclerosis in general (Brown and Goldstein, 1986; Steinberg, 1988).

LDL is a large spherical particle whose fluid core contains neutral lipid, mostly cholesteryl ester (Brown and Goldstein, 1986). This core of cholesteryl ester is surrounded by a monolayer of phospholipid and unesterified cholesterol molecules. Embedded in the phospholipid is a large protein molecule, apoprotein B-100. The studies of Brown and Goldstein suggested that LDL was taken up by cells after apoprotein B-100 was recognized and bound by a specific receptor on the cell surface (Brown and Goldstein, 1986). These receptors are clustered in specialized regions of the cell membrane known as clathrin coated pits. After binding of LDL, the pits form invaginations and pinch off to form membrane bound structures in the cytoplasm, called coated

vesicles or endosomes. Acidification of these vesicles is believed to promote separation of ligand and the receptor. The receptor recycles back to the cell surface and the endosome containing the LDL fuses with lysosomes (Brown and Goldstein, 1986). Lysosomal acid lipases hydrolyze the LDL components, liberating unesterified cholesterol which enters the cellular cholesterol pool(s). Accumulation of cholesterol inhibits a key enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), in the pathway of endogenous cholesterol biosynthesis to prevent the synthesis of additional cholesterol. The influx of cholesterol also reduces the number of LDL receptors by suppressing transcription of the receptor gene (Brown and Goldstein, 1986). Finally, LDL derived cholesterol promotes the storage of cholesterol in the cell by activating acyl CoA:cholesterol acyl transferase (ACAT). This enzyme in the membrane of the endoplasmic reticulum, esterifies a fatty acid to the β-OH of cholesterol and the resulting cholesteryl esters are deposited as cytoplasmic droplets (Brown and Goldstein, 1986). This highly regulated LDL-receptor pathway described by Brown and Goldstein does not, however, fully account for the accumulation and deposition of large quantities of cholesterol in cells that is associated with atheroma and therefore the relevance of this pathway to atherogenesis has been questioned (Brown and Goldstein, 1986). Several lines of evidence suggest that the uptake of native LDL by macrophages in the

arterial wall does not occur to any significant extent (Steinberg, 1983; Steinberg, 1988; Witztum, 1990). Exposure of macrophages to LDL in culture leads to a decrease in the number of LDL receptors and thus, as in other cells, uptake of LDL is downregulated.

It has been shown in cell cultures that macrophages cannot be transformed into foam cells simply by exposure to high levels of native LDL (Witztum, 1990). Furthermore, humans with homozygous familial hypercholesterolemia (HFH) and the receptor deficient WHHL rabbit (an animal model for HFH) develop severe and rapid atherosclerosis with marked foam cell formation despite a total absence of functional LDL receptors (Witztum, 1990). If the LDL receptor is not involved in the mechanism by which macrophages take up excessive amounts of LDL then the obvious question is what are the mechanisms for foam cell formation and the atherogenicity associated with high plasma LDL?

Brown and Goldstein have reported that acetylated LDL is taken up by monocyte-macrophages by a pathway that is not downregulated (Goldstein et al., 1979). Acetyl LDL is taken up rapidly by macrophages in culture, transforming these cells into lipid-laden cells that resemble foam cells <u>in vivo</u>. The acetyl LDL is taken up by a saturable, specific process, implying the presence of an acetyl-LDL receptor that has now been designated "the scavenger receptor" (Steinberg, 1986; Witztum, 1990). The scavenger receptor pathway does not

appear to be regulated by the cholesterol content of the cells. Other chemically modified forms of LDL have also been shown to compete with acetyl LDL for the scavenger receptor. These include methylated LDL (Gajdusek et al., 1980), acetoacetylated LDL (Fogelman et al., 1980) and malonaldehyde-conjugated LDL (Fogelman et al., 1980). These chemically modified forms of LDL all have the lysine epsilon amino groups of apo-B-100 blocked.

Endothelial cells, arterial smooth muscle cells and macrophages can modify LDL by an oxidative mechanism to a form that becomes recognized by the high affinity acetyl LDL receptor (Leake and Esfahani, 1989). Oxidized LDL is also taken up by these cells and results in cholesteryl ester accumulation (Steinberg, 1988). Evidence is mounting that LDL can be oxidized within atherosclerotic lesions in vivo (Steinbrecher et al., 1984; Witztum, 1990). It is thought that oxidation of phospholipids within the plasma membrane of cells propagates the oxidative chain reaction in LDL.

Thus, high plasma LDL levels may be sufficient as a single initiating factor for the development of atherosclerosis (Steinberg, 1988, Witztum, 1990). Increased LDL levels could cause increased LDL infiltration into the intima and enhanced oxidative modification by smooth muscle ells, endothelial cells and monocyte macrophages (Steinberg, 1988; Witztum, 1990). Uptake of this oxidized LDL via the scavenger receptors would lead to the accumulation of

cholesteryl esters within the smooth muscle cells and macrophages. The deposition of cholesteryl esters within these cells in the arterial wall may transform the cells into foam cells, that eventually lead to the formation of atherosclerotic lesions (Steinberg, 1988). Therefore, it is the initial stage of atherosclerosis, involving the deposition of cholesteryl esters and the formation of foam cells, that must be inhibited to prevent coronary artery disease (Witztum, 1990). Moreover, since it is now apparent that arterial lesions are detected even in the first year or two of life, the efforts to reduce atherogenesis must be initiated in young children and be maintained throughout one's life (Witztum, 1990).

1.4 Dietary Fat and Atherosclerosis

Although many factors can influence the relative risk of coronary artery disease, the effects of dietary fat on atherogenesis have received much of the attention. A relationship between dietary fat and coronary disease was suggested as early as 1916 (Goodman, 1989). In recent years, a host of large lipid clinic studies have clearly demonstrated that individuals consuming diets rich in saturated fatty acids exhibit a much higher incidence of coronary artery disease than individuals eating diets rich in polyunsaturated fatty acids (PUFAs) (Goodnight et al., 1982; Grundy and Ahrens, 1970; McNamara, 1987; Nestel, 1987). These findings have been

confirmed by controlled feeding trials in several experimental animals (Balasubramaniam et al., 1985; Bieberdorf and Wilson, 1965; Garg et al., 1988; Krause and Hartman, 1984). It has been established that the biochemical basis for the reduction in atherogenesis by dietary polyunsaturated fatty acid lies in the marked decrease in plasma cholesterol, particularly LDL cholesterol, observed when saturated fatty acids in the diet are replaced by polyunsaturated fatty acids (Baudet et al., 1984; Shepherd et al., 1980).

It now appears that different types of polyunsaturated fatty acids may differ in their effectiveness in preventing arterial lesions. As early as 1952, Nelson observed that feeding coronary disease patients large amounts of fatty fish significantly improved the 20-year survival rates from 8% to 35% (Nelson, 1972). Although Nelson's work went virtually unnoticed, in 1970 Bang, Dyerberg and coworkers reported that the incidence of coronary artery disease among the Eskimo population of Northern Greenland was less than 10% of that in Eskimos living in Denmark (Bang and Dyerberg, 1972). They suggest that the absence of atherosclerosis in the Greenland Eskimos might be attributable to the high content of ω-3 polyunsaturated fatty acids in the Eskimo's marine based diet. These fatty acids have the first double bond located three carbons from the terminal methyl group. In contrast, the ω-6 polyunsaturated fatty acid in vegetables and vegetable oils, the major sources of polyunsaturated fatty

acids in most Western countries, have the first double bond inserted at carbon 6 from the terminal methyl group (Harris, 1985; Ziboh and Miller, 1990). The ability of diets rich in ω-3 polyunsaturated fatty acids to protect against atherosclerosis has since been confirmed in several controlled animal studies (Davis et al., 1987; Wiener et al., 1986), although some reports have suggested that dietary ω-3 polyunsaturated fatty acids do not reduce arterial lesions (Thiery and Siedel, 1987).

Dietary ω-3 fatty acids have a variety of effects on plasma lipid and lipoproteins (Nestel, 1990). Extensive research has focused on triglyceride metabolism since ω-3 PUFAs have been shown to consistently lower plasma triglyceride levels in virtually all human and animal experiments (Harris, 1989; Land, 1986; Nestel, 1990). Fish oil feeding has been shown to diminish lipogenesis, increase ketogenesis and fatty acid oxidation and result in an overall reduction in triglyceride secretion by the liver and in lower plasma VLDL (Bronsgeest-Schonte et al., 1981; Wong et al., 1985; Lang and Davis,1990).

Numerous studies have reported both increases and decreases in plasma LDL cholesterol upon fish oil supplementation in humans and experimental animals (Goodnight et al., 1982; Harris, 1989; Nestel, 1987; Nestel, 1990). In general though, it would appear that plasma total cholesterol and LDL cholesterol levels in animals fed ω-3 PUFAs are

similar to the levels in animals fed ω-6 PUFAs (Harris, 1989; Nestel, 1990). The effect of ω-3 PUFAs on HDL levels have also been inconsistent, but it would appear that HDL levels in plasma are not altered by fish oil supplemented diets when compared with vegetable oil diets (Kromhout et al., 1985; Nestel, 1987; Nordoy and Goodnight, 1990).

The usefulness of fish oils in treating hypercholesterolemia is doubtful, except when excess cholesterol is in VLDL as in type V hyperlipoproteinemia (Nestel, 1987). Yet, most existing knowledge confirms that dietary fish oils offer considerably greater protection against the development of atherosclerosis. Therefore, the beneficial effects of ω-3 PUFAs are probably not mediated through alterations in lipoprotein levels.

Evidence suggests that ω-3 PUFAs affect cholesterol metabolism, specifically cholesterol esterification in hepatic and non-hepatic tissues. CaCo-2 cells, (derived from human colon adenocarcinoma) in which the membrane fatty acid composition has been enriched with eicosapentanenoic acid had significantly lower rates of cholesterol esterification than did cells modified by palmitic acid (Murthy et al., 1988). Similar findings have also been reported in hepatocytes and liver microsomes that have been enriched with eicosapentanenoic acid (Rustan et al., 1988). Preliminary experiments have also suggested that dietary ω-3 PUFAs lower the esterification of cholesterol in liver microsomes isolated

from rabbits fed fish oil supplemented diets. Liver microsomes isolated from fish oil fed rabbits contained significantly less cholesteryl ester by mass than liver microsomes from rabbits fed vegetable oil supplemented diets (Goodyear and Davis, personal communication).

Inhibition of cholesterol esterification by ω-3 PUFAs in the cells of the intima, such as the smooth muscle cells and monocyte-macrophages, would result in reduced cholesteryl ester deposition, delayed foam cell development and inhibition of the initial stage of atherosclerotic lesions. However, investigations of the effects of ω-3 PUFAs on cholesterol esterification in mammals have been limited to intestine, liver and cells derived from these two tissues. Intestinal cells and hepatocytes are involved in the packaging and secretion of lipoproteins and therefore may not adequately represent cholesterol metabolism in non-hepatic tissue. It is, therefore, important that the possibility of such effects of ω-3 PUFAs on non-hepatic cells be examined.

1.5 Objectives

My objective was to study the effect of ω-3 and ω-6 PUFA enrichment of non-hepatic cells (human fibroblasts (D551) and mouse macrophages (J774)), on cholesterol metabolism. Experiments involved specific fatty acid enrichment of the cellular lipids in human fibroblasts and mouse macrophages and subsequent mass and radiochemical measurement of cholesteryl

esterification after fatty acid enrichment. To study the effect of fatty acid enrichment on cholesterol metabolism, efflux of cholesterol from ω-3 and ω-6 PUFA enriched cells was also measured. This involved enriching fibroblasts with these fatty acids and radiolabelling these cells with acetate to label the cholesterol pool enabling us to measure cholesterol efflux in the presence of an acceptor (HDL).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Lipids

All lipids were obtained from Sigma Chemical Company, St. Louis, MO, USA. These were used without further purification. Lipid purity was checked using thin layer chromatography (t.l.c.) and gas liquid chromatography (g.l.c.).

2.1.2 Radioisotopes

[oleoyl-1-^{14}C] Oleoyl-coenzyme A, (58.3 mCi/mmol), and [^{3}H]-acetic acid sodium salt, (1 mCi/mmol) were obtained from DuPont-NEN, Lachine, Quebec.

2.1.3 Chemicals

Unless specified, all chemicals used were reagent grade. Ammonium acetate, ammonium sulphate, cupric sulphate, chloroform (HPLC grade), hexane, methanol (HPLC grade), sodium phosphate (dibasic), potassium bromide, potassium sodium tartrate (crystal) and sodium chloride were purchased from Fisher Scientific Company. Tris, EDTA (disodium salt), ophthaldialdehyde, bovine serum albumin, cholesterol and DL-dithiothreitol were products of Sigma Chemical Company, St. Louis, MO, USA.

2.2 Methods

2.2.1 Fibroblasts

Normal skin fibroblasts (Detroit 551, American Type Culture Collection, Rockville, MD, USA) were grown in Eagle's Minimum Essential Medium (MEM) containing 10% Fetal Calf Serum (FCS), (3%) bicarbonate, and pen(100 IU/ml)-strep(100 µg/ml)-funigizone (25 µg/ml) (Flow Laboratories, Inc., McLean, VA, USA). Cells were seeded at 5 x 10^5 cells in 25 cm^2 flasks or in 175 cm^2 flasks and were grown at 37° C in 4% CO_2 and air.

2.2.1.1 Preparation of Fatty Acid Enrichment Medium for Human Fibroblasts

Fatty acid enrichment medium for human fibroblasts was prepared using a modification of the procedure reported by Spector (Spector et al., 1981). The sodium salts of various fatty acids in phosphate buffered saline (PBS, Flow Laboratories, Inc.) were added to Fetal Calf Serum that had been dialyzed against PBS, pH 7.4 for 24 hours. This solution was then added to MEM to give final concentrations of 10% FCS and 60 µg/ml of fatty acid salt.

2.2.1.2 Fatty Acid Enrichment in Human Fibroblasts

Cells were grown to 2 x 10^6 cells in 25 cm^2 flasks in MEM containing bicarbonate, 10% FCS and pen-strep-funigizone. This medium was then removed and replaced by the fatty acid enrichment medium, prepared as described above.

The cells were then incubated at 37° C for 120 hours in this medium. To activate the enzyme ACAT, cells were loaded with cholesterol in the form of LDL (Brown and Goldstein, 1983). Dialyzed human LDL (100 μg of LDL protein/ml) was added to the enrichment medium 18 hours before cells were harvested (at 102 hours). Human fibroblasts were washed two times with PBS and collected in 1 ml of PBS with a rubber policeman. Cells were pelleted at 12,000 x g for 1 minute and the supernatant was discarded. The cells were resuspended in a known volume of either PBS or ACAT buffer (50 mM Tris, 10 mM EDTA, pH 7.4) (Gavigan and Knight, 1983). Cell protein was estimated by the procedure of Lowry et al. (Lowry et al., 1951).

2.2.1.3 Assessment of Fatty Acid Enrichment

To determine the degree of fatty acid enrichment in human fibroblasts, cells were harvested and pelleted. They were then resuspended in 250 μl of PBS. Cellular lipids were extracted by using the procedure reported by Bligh and Dyer, first by the addition of 750 μl of chloroform:methanol (1:2, v/v). The protein was then pelleted at 12,000 x g for 5 minutes and discarded. Chloroform:water (1:1) (500 μl) was added to the supernatant. The top layer was discarded after centrifugation at 12,000 x g for 5 minutes and the lower fraction was evaporated under N_2 gas. The lipids were dissolved in 50 μl of chloroform:methanol (1:1) and were applied to silica gel plates (Rediplate, Fisher Scientific,

Dartmouth, N.S.) as a single spot. The plates were developed in a neutral lipid solvent system (hexane:diethyl ether:acetic acid, 85:15:2, v/v/v). The lipid spots were identified on the chromatograms under uv light after staining with 2',7'-dichlorofluorescein. The polar lipid (origin) was scraped from the plate and was extracted from the silica with 200 µl of chloroform:methanol (1:1, v/v).

The methyl ester derivatives of the fatty acids from the polar lipid fraction were analyzed by gas liquid chromatography (g.l.c.) (Keough and Davis, 1979). Separation of methyl esters was carried out on an H/P 5890 gas chromatograph fitted with a Supelcowax 10 fused capillary column (30m, 0.53 mmID and 1.0 µm film thickness) using helium as the carrier gas (flow rate was 15 ml/min) and operated isothermally at a temperature of 190° C. The fatty acids were quantified, using heptadecanoic acid (50 µg) as the internal standard.

2.2.1.4 Determination of Cellular Esterification in Whole Cell Lysates of Human Fibroblasts

Following a five-day enrichment with either linoleic acid or eicosapentaenoic acid, human fibroblasts was collected individually by scraping the flasks with a rubber policeman. Cells were pelleted at 12,000 x g and resuspended in 60 µl of ACAT buffer (50 mM Tris, 10 mM EDTA, pH 7.4). A 10 µl aliquot of cell suspension was then taken for protein determination

(Lowry et al., 1951).

The measurement of cholesterol esterification activity in whole cell lysates required the use of a radiolabelled fatty acid CoA. A study has shown that the best substrate for ACAT activity measurements is oleoyl CoA followed by palmitoyl, stearyl and linoleoyl CoAs in order of decreasing specificity (Suckling and Strange, 1985). Oleoyl CoA was used in these studies. The human fibroblasts were lysed by suspension in hypotonic ACAT buffer. The extent of cell lysis was determined by measuring cytoplasmic lactate dehydrogenase activity and trypan blue exclusion.

To measure cholesterol esterification in enriched human fibroblasts, ACAT buffer containing 0.5 mg/ml Bovine Serum Albumin (BSA) and 0.2 μCi/ml of [^{14}C]-oleoyl CoA (58.3 mCi/mmol) was added to cell lysates (40 μg) to give a final volume of 250 μl (Billheimer, 1985; Lichenstein and Breehner, 1980). This mixture was incubated at 37°C for 5 minutes and the reaction was stopped by the addition of 750 μl of chloroform:methanol (1:2, v/v).

The protein fraction was pelleted at 12,000 x g and discarded. The supernatant was retained and the lipids were extracted by the addition of 500 μl of chloroform:water (1:1). The upper layer was discarded and the lower portion was evaporated using N_2 gas. The lipid residue was dissolved in 100 μl of chloroform and was chromatographed on silica gel plates in hexane:diethyl ether:acetic acid (85:15:2, v/v/v).

The lipids were visualized in iodine vapor. The cholesteryl ester spot was scraped and counted for the presence of cholesteryl-[^{14}C]-oleate in a liquid scintillation counter (with automated quench correction). The rate of cholesterol esterification was expressed as picomoles of cholesteryl [^{14}C]-oleate formed per milligram of total extract protein per hour.

2.2.2 Macrophages

Mouse macrophages (J774, American Type Culture Collection, Rockville, MD, U.S.A.) were grown in RPMI 1640 (Flow Laboratories, Inc., Mclean, VA., U.S.A.) containing 10% FCS, bicarbonate, and pen-strep-fugizone in 25 cm^2 flasks and were grown at 37° C in 4% Co_2 and air.

2.2.2.1 Preparation of Fatty Acid Enrichment Medium for Macrophages (J774)

In the latter part of this study, we became aware of a simpler method for enriching macrophages. Fatty acid enriched medium for macrophages was prepared by adding the sodium salt of various fatty acids in an ethanol solution to RPMI (containing 10% FCS and pen-strep-fungizone) to give a final concentration of 60 µg/ml of fatty acid salt. Ethanol concentrations in the medium did not exceed 0.5% and 0.5% ethanol was added to medium in control flasks to ensure that cell growth was not affected.

2.2.2.2 Fatty Acid Enrichment of J774 Macrophages

Macrophages were grown until they were near confluence in RPMI containing bicarbonate, 10% FCS and pen-strep-fungizone. The medium was replaced by fatty acid enrichment medium, prepared as described above. The cells were incubated at 37°C for 36 hours in this medium. To stimulate cholesterol esterification in macrophages, cells were incubated for 18 hours with 50 µg/ml acetylated LDL (Goldstein et al., 1979). Macrophages were washed two times with PBS and then collected in 1 ml of PBS with the aid of a rubber policeman. Cells were pelleted at 12,000 x g for 1 minute and the supernatant was discarded. The cells were resuspended in a known volume of PBS. Cell protein was estimated by the procedure of Lowry (Lowry et al., 1951). Fatty acid enrichment in macrophages was assessed by the same method used to analyze the fatty acid composition in human fibroblasts.

2.2.3 Human Fibroblast Microsomes

2.2.3.1 Preparation of Microsomes from Human Fibroblasts Enriched with Eicosapentaenoic Acid and Linoleic Acid

Microsomes from human fibroblasts were isolated as described before (Davis and Poznansky, 1986). Cells were grown in 175 cm^2 flasks and enriched with various fatty acids (as described previously). Cells were washed two times with

PBS and collected by scraping with a rubber policeman. Cells were pelleted at 3500 x g for 5 minutes, the supernatant was discarded and the pellet was resuspended in 10 ml of 0.88 M sucrose/50 mM Tris/0.5% BSA (Fraction V, Sigma), pH 7.4.

The cell suspension was homogenized by hand (8-10 strokes) in a Dounce Homogenizer. Nuclei and unbroken cells were removed by centrifugation at 500 x g for 10 minutes and the supernatant was centrifuged at 20,000 x g for 20 minutes to remove mitochondria. The postmitochondrial supernatant was diluted with 2.5 volume of buffer A (50 mM phosphate/10 mM dithiotreitol, pH 7.4) and centrifuged at 105,000 x g for 2 hours. The microsomal pellets were resuspended in buffer A at 105,000 x g for 90 minutes and suspended in ACAT buffer.

NADPH-cytochrome c reductase activity was determined in microsomes isolated from human fibroblasts incubated with various fatty acids (Lake, 1987).

2.2.3.2 Determination of Fatty Acid Enrichment in Microsomes, Isolated from Human Fibroblasts Grown in the Presence of Eicosapentaenoic Acid and Linoleic Acid

Microsomes from fatty acid enriched cells were isolated by the procedure described in the previous section. To determine the extent of fatty acid enrichment, microsomes (100 μg) were extracted in 750 μl of methanol:chloroform (2:1) and pelleted at 12,000 x g (pellet was discarded).

Chloroform:water (1:1) was added to the supernatant and again centrifuged at 12,000 x g. The lower fraction was retained and evaporated under a stream of nitrogen gas. The lipids were dissolved in 100 µl of chloroform:methanol (1:1). 50 µl was applied to a silica gel plate and developed in a neutral lipid solvent system (see section 2.2.1.3) to estimate the fatty acid composition of the total polar lipid fraction. To determine the degree of fatty acid enrichment in the different phospholipid classes, 50 µl of total lipid extract was applied to a silica gel plate as a single spot and developed in a polar lipid solvent system (chloroform:methanol:water, 65:25:4, v/v/v). The phosphotidylcholine spots and phosphatidylethanolamine spots were identified on the chromatograms under uv light after staining with 2',7' dichlorofluorescein. The lipid spots were scraped from the plate and extracted from the silica gel with 200 µl of chloroform:methanol (1:1, v/v). The fatty acid methyl esters were analyzed by gas liquid chromatography as described previously.

2.2.3.3 Determination of Microsomal ACAT Activity

ACAT activity was determined in microsomes isolated from human fibroblasts incubated with various fatty acids. ACAT activity was measured by adding 150 µl of ACAT buffer containing 0.3 µCi of [^{14}C]-oleoyl CoA (58.3 mCi/mmol) and 1.3 mg BSA to 100 µl of microsomes (30 µg of protein) (Lichenstein

and Breehner, 1980). This mixture was incubated at 37°C for 20 minutes and the reaction was terminated by the addition of methanol:chloroform (2:1). Lipids were extracted and chromatographed as described previously. Cholesterol ester spots were scraped and counted in a liquid scintillation counter.

2.2.4 Cholesterol Efflux

2.2.4.1 Radiolabelling of Endogenous Cellular Cholesterol

The endogenous cholesterol pool in human fibroblasts was radiolabelled by the addition of 1 μCi/ml of [^{3}H]-acetic acid, sodium salt (1 μCi/μmol), to the medium used to enrich cells with fatty acid. The enrichment medium containing the radiolabelled compound was incubated with the cells for 5 days, at which point the medium was replaced by MEM (containing bicarbonate, pen-strep-funigizone and 5% lipoprotein deficient serum (LPDS)) (Phillips et al., 1980).

2.2.4.2 Cholesterol Efflux from Human Fibroblasts Enriched with Various Fatty Acids

Cholesterol efflux was measured in cells that were enriched with linoleic acid and eicosapentaenoic acid. Endogenous cellular cholesterol was radiolabelled as outlined above. Efflux experiments were initiated by the addition of an acceptor particle, high density lipoprotein (250 μg HDL protein/ml) in fresh culture medium (Lund-Katz et al., 1982).

At time intervals up to 24 hours, the medium was collected and the cells were washed twice with PBS. The cells were scraped from multiwell plates with a rubber policeman subsequent to the addition of PBS and were pelleted by spinning at 12,000 x g for 5 minutes at 24° C. The pellet was suspended in 260 µl of PBS, and 10 µl was taken for protein determination (Lowry et al., 1951). The lipids were extracted by the addition of 750 µl methanol:chloroform (2:1), protein pelleted at 12,000 x g for 5 minutes at 24° C, and the supernatant retained. Chloroform:water (1:1) (500 µl) was added to the supernatant and the lower portion was saved and evaporated under a stream of N_2 gas. The lipids were chromatographed on silica gel plates in hexane:diethyether:acetic acid (85:15:2, v/v/v). The unesterified and esterified cholesterol bands were identified in iodine vapor and were scraped and the radioactivity determined by liquid scintillation counting. The lipids in the medium were also extracted, chromatographed and cholesterol and cholesteryl esters were determined in the same manner.

2.2.5 Lipoproteins

2.2.5.1 Lipoprotein Preparation

Human plasma was obtained from the Canadian Red Cross, St. John's, NF. Human LDL (d 1.019 - 1.063 g/ml) and HDL (d 1.125 - 1.215 g/ml) were isolated from the plasma by ultracentrifugation according to the method of Havel (Havel,

1988). Lipoproteins were dialyzed against (150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) for 24 hours before use. The method of Lowry was used to determine the amount of lipoprotein-protein per ml (Lowry et al., 1951).

2.2.5.2 Preparation of Acetylated LDL

Human LDL was prepared as described above. LDL was acetylated by adding 1 ml of 0.15 M NaOH containing 16 mg of LDL protein to 1 ml of saturated solution of sodium acetate with continuous stirring in an ice water bath (Basu et al., 1976). Acetic anhydride (2 ml) was added in multiple small aliquots (.2 ml) over a period of one hour at 4° C. After the last addition, the solution was left at 4° C for an additional thirty minutes. Acetylated LDL solution was then dialyzed at 4° C for 24 hours against (0.15 NaCl, .3 mM EDTA, pH 7.4). Lipoprotein protein was estimated by the Lowry method (Lowry et al., 1951).

2.2.6 Analyses

2.2.6.1 Determination of Cellular Cholesterol Content

To quantify the content of cellular and microsomal cholesterol, human fibroblast cells and microsomes were isolated and extracted as described previously. To determine the cellular cholesterol content of J774 macrophages, cells were isolated (as described previously) and extracted (using the same procedure used to extract human fibroblasts).

Extracted lipids were chromatographed on silica gel plates in hexane:dimethylethylether:acetic acid (85:15:2, v/v/v). Cholesterol and cholesteryl ester spots were identified in iodine vapor and collected by scraping. The recovery of free and unesterified cholesterol was determined by cholesterol analysis using the O-phthaldehyde method (Rudel and Morris, 1973). 900 µl of O-phthaldehyde reagent, with a concentration of 0.5 mg/ml in glacial acetic acid, was added to each sample. The solution was mixed thoroughly and incubated for 10 minutes at room temperature. Concentrated sulfuric acid (450 µl) was added and the solutions immediately mixed. The absorbance was read at 550 nm, 10 minutes after the addition of the concentrated sulfuric acid (Rudel and Morris, 1973).

2.2.6.2 Analysis of Fatty Acyl CoA Pool in Human Fibroblasts Enriched with Various Fatty Acids

The fatty acyl CoA pool size was measured to investigate whether fatty acid enrichment of human fibroblast influenced cholesterol esterification by the modification of metabolic pools. The fatty acyl CoA pool size was estimated using a modified version of the procedure established by Bartlett (Watnough et al., 1989). Human fibroblasts were enriched with various fatty acids and were then harvested by scraping, pelleted at 12,000 x g for 5 minutes at 24° C and resuspended in 1 ml of PBS. The suspension was quenched with 200 µl of acetic acid and 20 nmol of [^{14}C]-oleoyl CoA was then

added as an internal standard to estimate percent recovery. A portion (50 µl) was removed for determination of acid soluble radioactivity. Each sample was transferred to an extraction tube containing 100 µl of saturated $(NH_4)_2SO_4$ and placed in a boiling water bath for two minutes and then allowed to cool to room temperature. Each sample was extracted twice with 10 volumes of diethylether to remove free fatty acids and then extracted for 1 hour with 8 ml of methanol:chloroform (2:1, v/v) with continuous agitation. After centrifugation (40,000 x g for 10 minutes at 4°C) the supernatant was retained. The pellet was resuspended in 3 ml of methanol:chloroform (2:1, v/v) and recentrifuged. The solvent was removed from the recombined supernatants with a stream of N_2 gas at 24° C until the volume was 2 ml. The pH was adjusted to 6-7 by adding 2.0 ml of 1 M-ammonium acetate, and the sample was freeze dried overnight. The residue was extracted with 2.4 ml of methanol, centrifuged at 90,000 x g and the supernatant retained. The pellet was resuspended in 0.8 ml of methanol, recentrifuged and the combined supernatants diluted to a final volume of 4.0 ml with 0.8 ml of deionized water. This was applied to a DEAE-Sephacel (acetate form) column poured in a 10 ml disposable pipette. The column was washed with 5 ml of methanol:water (4:1, v/v). The fraction containing acyl CoA was eluted with 6 ml of methanol:water (4:1, v/v) containing 0.5 M-ammonium acetate and 10 mM acetic acid. This fraction was then diluted with 2-

3 ml of deionized water and freeze dried overnight. The residue was dissolved in 1 ml of chloroform:methanol (1:2), centrifuged at 12,000 x g for 10 minutes at 24° C and the pellet discarded. The supernatant was retained and a 200 µl aliquot was assayed for radioactivity in a liquid scintillation counter to determine acyl CoA recovery. The remainder of the supernatant was evaporated under a stream of nitrogen at 24° C, 5 µg of heptadecanoic acid was added and the lipids were then transmethylated overnight and analyzed by g.l.c. (under same conditions described previously). The acyl CoA pool size was quantified using heptadecanoic acid as an internal standard.

3. RESULTS

3.1 Fatty Acid Enrichment of Human Fibroblasts

Human fibroblasts were incubated with either linoleic acid or eicosapentanenoic acid enrichment medium for five days. Cells incubated with medium supplemented with 60 μg/ml of linoleic acid (18:2, ω-6) had a higher relative content of linoleic acid and little ω-3 unsaturated fatty acid (Table 1). When cells were incubated with eicosapentanenoic acid enrichment medium, the cellular phospholipids contained much less linoleic acid (Table 1). However, cellular phospholipids were not enriched with eicosapentanenoic acid but rather were enriched with the immediate elongation product of EPA, docosapentaenoic acid (22:5, ω-3) (Table 1). The relative content of docosahexaenoic acid (22:6, ω-3) was similar at approximately 4.1% in the cells incubated with either linoleic acid or eicosapenaenoic. There were no significant differences in the relative contents of other fatty acids in cellular phospholipids (data not shown).

Cellular protein was similar in flasks of cells incubated with either linoleic or eicosapentaenoic acid supplemented medium but varied from batch to batch from approximately 30 μg-100 μg of protein per flask. The average cell number per flask for both groups was estimated to be approximately 2×10^6 cells/flask.

Microsomal phospholipids from human fibroblasts

TABLE 1. Fatty acid content of phospholipid fraction from human fibroblasts incubated with linoleic acid or eicosapentaenoic acid supplemented medium (n=4)

FATTY ACID	LINOLEIC ACID ENRICHED (% OF TOTAL FATTY ACIDS)	EICOSAPENTAENOIC ACID ENRICHED (% OF TOTAL FATTY ACIDS)
18:2 (ω-6)	10.0 ± 0.8	2.9 ± 0.4
20:5 (ω-3)	1.6 ± 0.9	1.0 ± 0.2
22:5 (ω-3)	1.3 ± 0.3	9.8 ± 2.6
22:6 (ω-3)	4.1 ± 0.8	4.2 ± 1.2

incubated with medium supplemented with either linoleic acid or eicosapentaenoic acid were analyzed for their fatty acid content (Table 2). Microsomal membrane phospholipids from human fibroblasts incubated with linoleic supplemented medium were enriched with linoleate (15.5% of total microsomal phospholipid fatty acid). However, when eicosapentaenoic acid was added to the medium, the cells elongated this fatty acid to docosapentaenoic acid that appeared in the microsomal phospholipids (9.4% of total microsomal phospholipid fatty acid). The relative content of docosahexaenoic acid was 2.3% and 1.9% in microsomes from cells incubated with linoleic acid and eicosapentaenoic acid, respectively. In subsequent experiments, when referring to cells that have been incubated in eicosapentaenoic acid supplemented medium, they will be referred to as cells enriched with docosapentaenoic acid.

To further investigate the incorporation of ω-3 and ω-6 PUFAs into cellular lipids, the phospholipid fraction of microsomes (isolated from cells incubated with either linoleic acid or eicosapentaenoic acid supplemented medium) was further separated by thin layer chromatography into different phospholipid classes. Table 3 shows the relative percents of ω-3 and ω-6 PUFAs in either phosphotidylethanolamine (PE) or phosphotidylcholine (PC) fraction of microsomal phospholipids. No difference was observed in the content of ω-6 PUFAs in either the PC or the PE fraction of microsomal phospholipids when cells were incubated with linoleic acid or

TABLE 2. **Fatty acid content of phospholipid fraction from human fibroblast microsomes isolated from fibroblasts incubated with linoleic acid or eicosapentaenoic acid supplemented medium (n=4)**

FATTY ACID	CELLS INCUBATED WITH LINOLEIC ACID (% OF TOTAL FATTY ACIDS)	CELLS INCUBATED WITH ACID (% OF TOTAL FATTY ACIDS)
18:2 (ω-6)	15.5 ± 1.2	5.0 ± 0.4
20:5 (ω-3)	-	-
22:5 (ω-3)	2.1 ± 0.2	9.4 ± 0.6
22:6 (ω-3)	2.3 ± 0.4	1.9 ± 0.3

TABLE 3. Fatty acid content in phosphotidylethanolamine (PE) and phosphotidylcholine (PC) fraction of microsomal phospholipids. Microsomes were isolated from cells incubated with either linoleic acid (LA) or eicosapentaenoic acid (EPA) supplemented medium (n=2).

FATTY ACID	MICROSOMES ISOLATED FROM CELLS INCUBATED WITH LA (% OF TOTAL FATTY ACID)				MICROSOMES ISOLATED FROM CELLS INCUBATED WITH EPA (% OF TOTAL FATTY ACID)			
	PC FRACTION		PE FRACTION		PC FRACTION		PE FRACTION	
ω-6 PUFAs	13.5	12.7	10.7	13.0	4.4	3.0	3.6	5.3
ω-3 PUFAs	6.2	6.00	11.3	10.6	8.8	11.0	21.5	31.4

eicosapentaenoic supplemented medium. However, a greater percentage of ω-3 PUFAs were incorporated into the PE fraction of microsomal phospholipids when cells were incubated with either linoleic or eicosapentaenoic acid supplemented medium.

Microsomal marker enzyme activity, NADPH-cytochrome c reductase was measured in microsomes isolated from human fibroblasts incubated with different polyunsaturated fatty acids. The activity of NADPH-cytochrome c reductase was similar in microsomes enriched with either ω-3 or ω-6 PUFAs (the activity of NADPH-cytochrome c reductase was 74.4 nmoles/mg protein and 72.9 nmoles/mg protein, respectively [Experiment 1] and 40.7 nmoles/mg protein and 52.7 nmoles/mg protein, respectively [Experiment 2]).

3.1.1 Effect on Cholesterol Levels

Cellular cholesterol levels, both unesterified and esterified were measured in human fibroblasts to assess the effects of fatty acid modification on cellular cholesterol. The cholesteryl ester levels in cells incubated with linoleic acid supplemented medium and eicosapentaenoic acid supplemented medium were significantly different. Figure 1 clearly shows that incubation of cells with eicosapentaenoic acid decreased the cellular cholesteryl ester content in these cells by 56% compared with cells enriched with linoleic acid. However, there appeared to be no significant difference in the level of free cholesterol in cells incubated with either fatty

acid (Figure 1). Statistical analysis was done by two-sided student t-test in this experiment and all subsequent experiments thereafter.

Figure 1: Cholesteryl ester (open bar) and unesterified cholesterol (closed bar) content of human skin fibroblasts enriched with linoleic acid or docosapentaenoic acid. Error bars are S.D. *$p<0.005$ ($n = 4$), $p>0.01$ (not significant) student t-test (two sided).

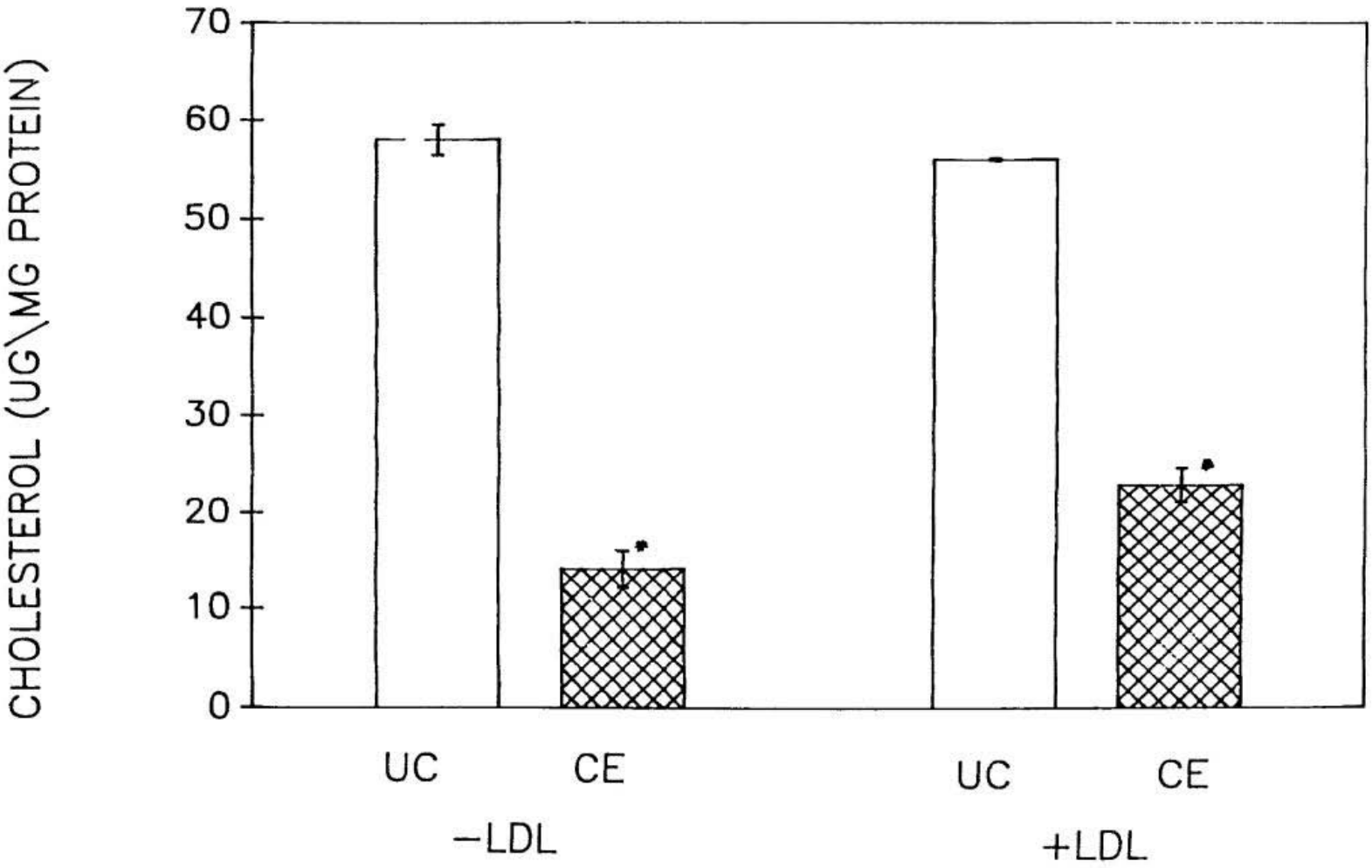
CHOLESTEROL (umol/mg protein)
1.000
0.800
0.600
0.400
0.200
0.000
*
*
Linoleic
Acid Rich
Docosapentaenoic
Acid Rich

3.2 Measurements of Cholesterol Esterification in Lysates of Human Fibroblast

3.2.1 Stimulation of Cholesteryl Esterification by Native LDL

We could not detect cholesteryl esterification in fibroblasts that were incubated in MEM + 5% LPDS (data not shown). To stimulate cholesterol esterification in human fibroblasts, the cells were incubated with 100 µg/ml of LDL protein for 18 hours (Brown, 1975). Cells incubated with MEM +5% LPDS and LDL contained 40% more cholesterol ester compared with cells that were incubated with only MEM and 5% LPDS (Figure 2).

Figure 2 Cholesterol content of cells incubated with (+LDL) and without LDL (-LDL). Open bars represent unesterified cholesterol (UC) and crossed bars represent cholesteryl ester (CE) content. Error bars are S.D. *$p<0.001$ ($n = 4$).


CHOLESTEROL (UG\MG PROTEIN)
70
60
50
40
30
20
10
0
UC
CE
UC
CE
−LDL
+LDL

3.2.2 Linearity of Cholesterol Esterification with Increasing Protein Concentrations and Time in Unenriched Human Fibroblasts

To investigate the effect of ω-3 and ω-6 PUFAs on cholesteryl esterification, incorporation of [^{14}C]-oleoyl CoA into cholesteryl esters was measured. Figure 3 shows that esterification activity in whole cell lysates appeared to be linear with increasing time up to 10 minutes. ACAT activity in whole cells also increased in a linear fashion with increasing amounts of protein, up to 60 μg of cell protein (Figure 4). In all subsequent measurements of cholesterol esterification, approximately 40 μg of cell protein was used per assay and assay mixtures were incubated for 5 minutes.

Bovine Serum Albumin was added to the reaction mixture to maintain a molar ratio of oleoyl CoA:albumin of 4:1. Serum albumin is required in the assay medium to prevent the high concentration of oleoyl CoA from inhibiting ACAT presumably because of the detergent-like effects of oleoyl CoA that might disrupt the cell membrane (Bilheimer, 1985).

Estimation of cell lysis by the hypotonic ACAT buffer was done by measuring cytoplasmic lactate dehydrogenase (LDH) activity. A small but significant difference was found in LDH activity between ω-3 and ω-6 PUFA enriched cell lysate suspensions. (LDH activity was 58.9 ± 10.1 nmoles/min/ml and 42.9 ± 6.05 nmoles/min/ml, respectively. ($n = 4$), $p<0.05$.) The number of viable cells in ω-3 and ω-6 PUFA enriched

fibroblasts were similar when stained with trypan blue (56.86 ± 1.50% and 58.6 ± 4.59% were stained with trypan blue, respectively.)

Figure 3: Linearity of [^{14}C]-oleoyl CoA incorporation into cholesteryl esters with time, in whole cell lysates (human fibroblasts).

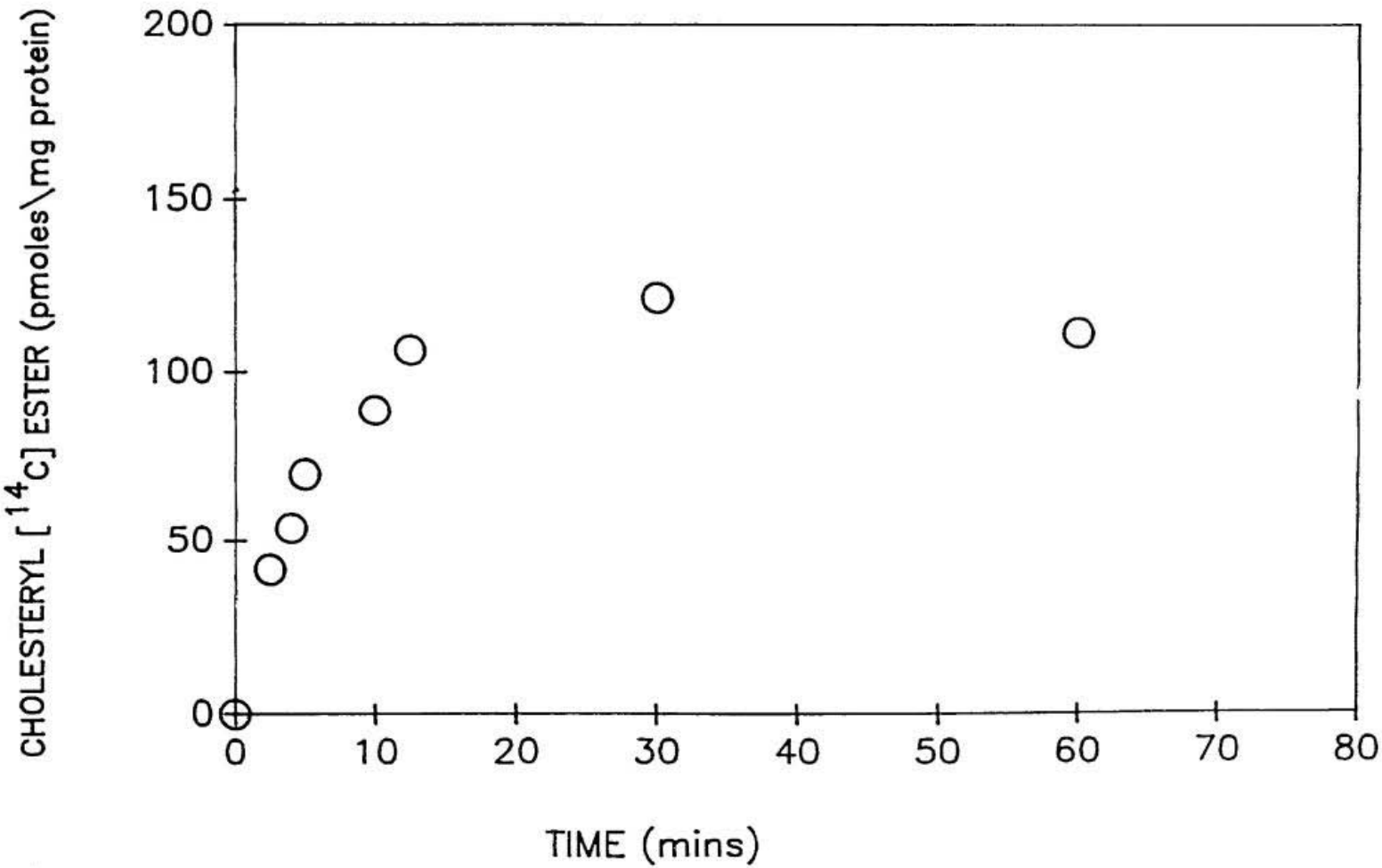
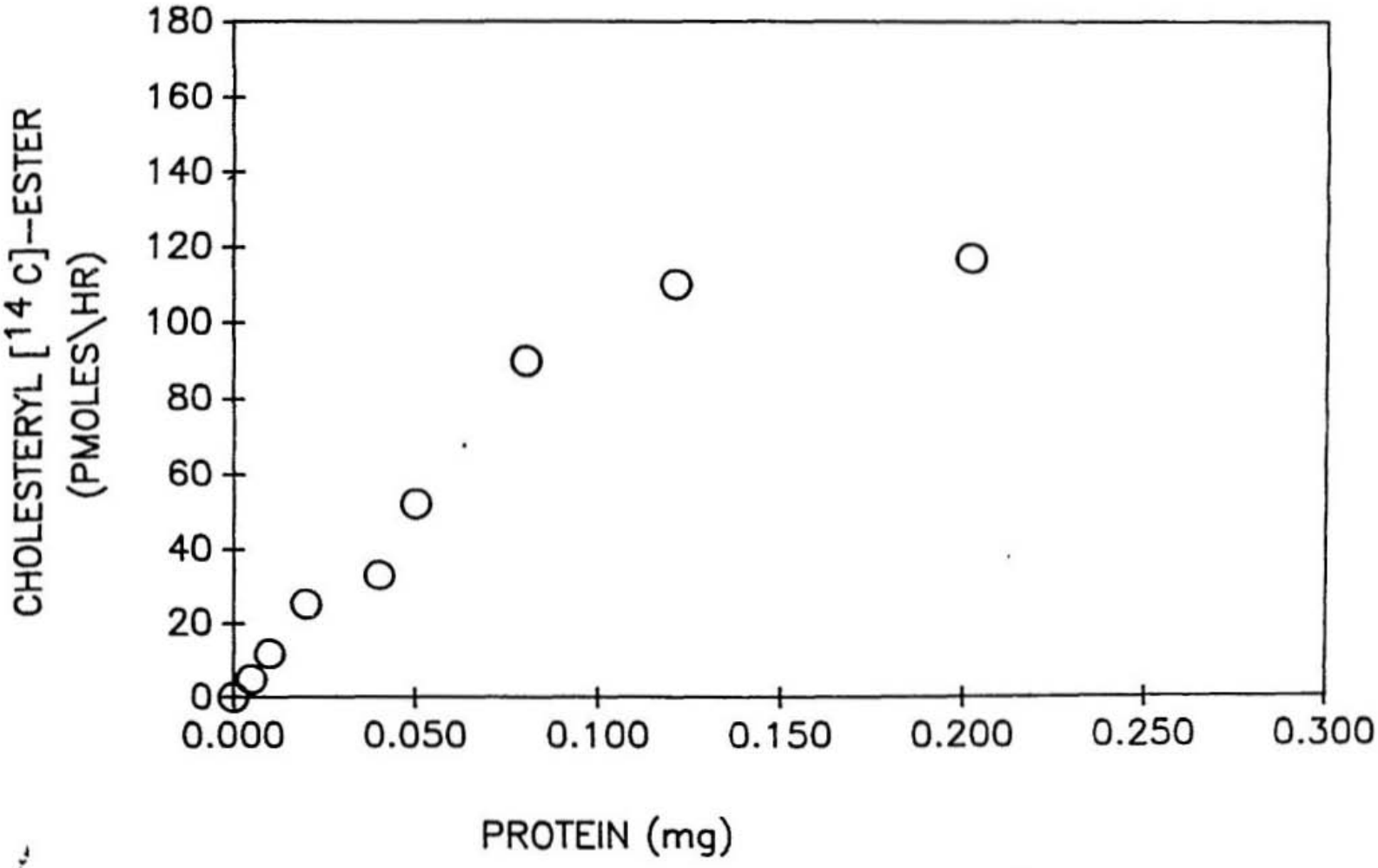

CHOLESTERYL [^{14}C] ESTER (pmoles\mg protein)
200
150
100
50
0
0
10
20
30
40
50
60
70
80
TIME (mins)

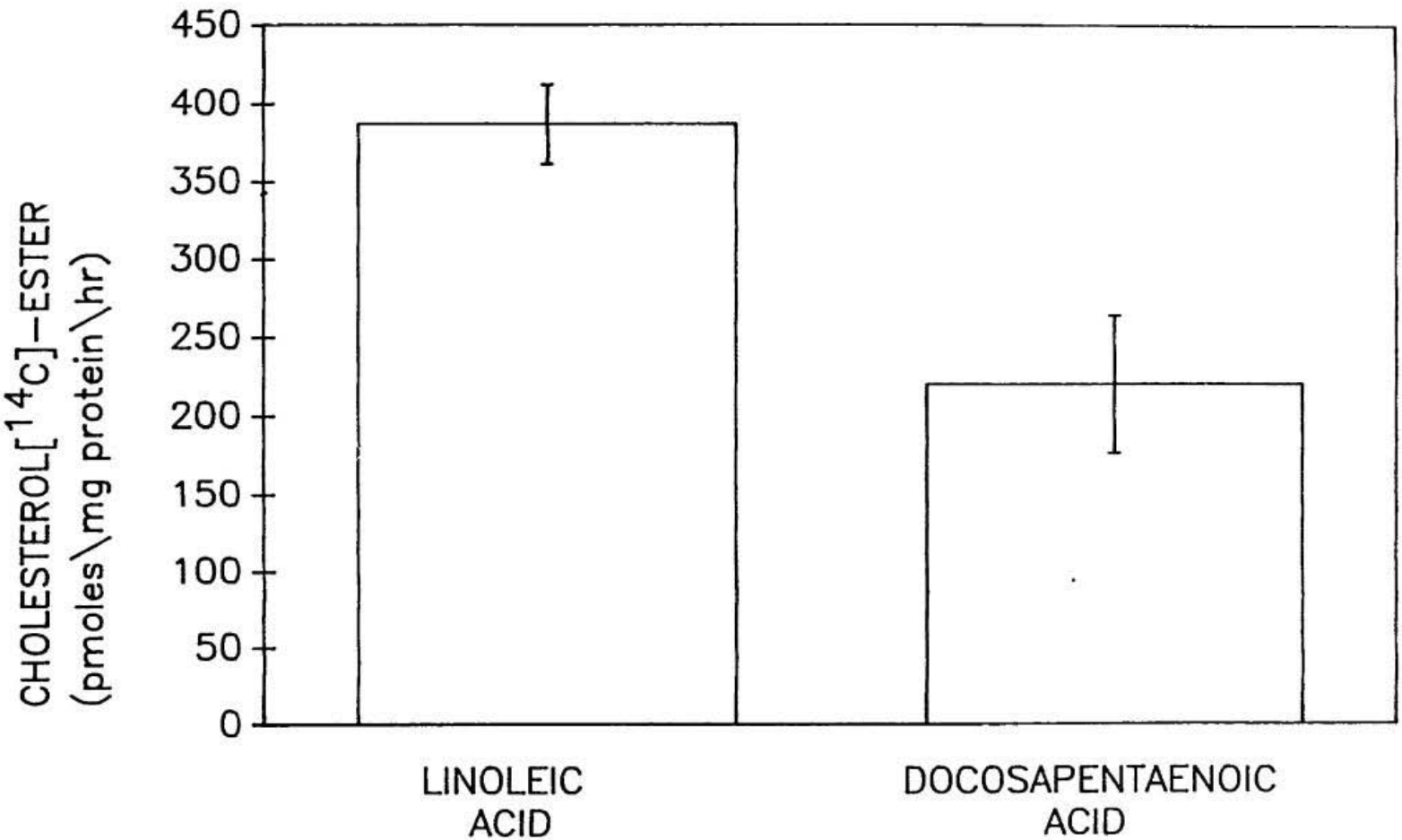
Figure 4: Linearity of incorporation of [^{14}C]-oleoyl CoA into cholesteryl esters with increasing protein concentration in whole cells.


CHOLESTERYL [14 C]--ESTER
(PMOLES\HR)
180
160
140
120
100
80
60
40
20
0
0.000
0.050
0.100
0.150
0.200
0.250
0.300
PROTEIN (mg)

3.2.3 Rate of Cholesterol Esterification in Human Fibroblasts Enriched with Polyunsaturated Fatty Acids

A decrease in cholesteryl ester content (Figure 1) in ω-3 PUFA enriched cells could have reflected a reduction in the rate of esterification of cholesterol. To measure cholesteryl esterification, the incorporation of radiolabelled oleoyl CoA into cholesteryl ester in cells enriched with different fatty acids was monitored. Figure 5 illustrates that the incorporation of [^{14}C]-oleoyl CoA into cholesteryl esters was 44.5% lower in lysates of human fibroblast that had been enriched with ω-3 PUFAs compared with lysates of cells enriched with ω-6 PUFAs (220 pmoles/mg protein/hr and 387 pmoles/mg protein/hr, respectively).

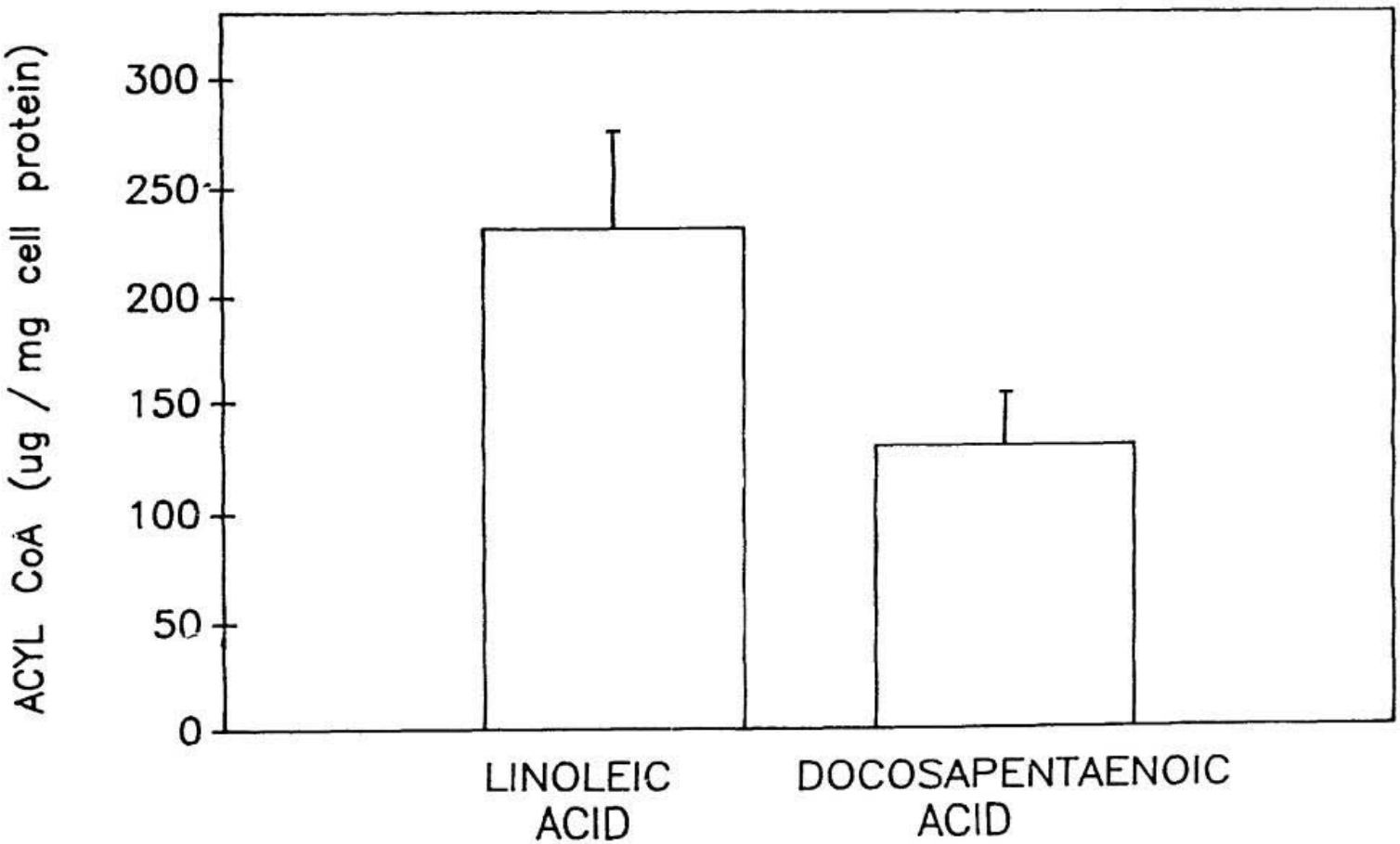
Figure 5 Cholesteryl esterification in fibroblasts enriched with linoleic acid or docosapentaenoic acid as measured by incorporation of radiolabelled oleoyl CoA into cholesteryl ester in whole cell lysates. Error bars are S.D. $p<0.01$ ($n = 4$).


CHOLESTEROL[^{14}C]-ESTER
(pmoles\mg protein\hr)
450
400
350
300
250
200
150
100
50
0
LINOLEIC ACID
DOCOSAPENTAENOIC ACID

3.3 The Effect of Polyunsaturated Fatty Acid Enrichment of Human Fibroblasts on Fatty Acyl CoA Pool Size

Cholesterol esterification by ACAT requires the presence of two substrates, cholesterol and fatty acyl CoA. It was shown previously that free cholesterol levels were unaffected by changes in the cell fatty acid composition (Figure 1). The size of the fatty acyl CoA pool was also measured in human fibroblasts after enrichment with either linoleic acid or docosapentaenoic acid. Figure 6 illustrates that ω-3 PUFA enriched cells had significantly lower levels of acyl CoA (130 ± 23.6 μg/mg protein) than ω-6 PUFA enriched cells (232 ± 44 μg/mg protein).

Figure 6: Acyl CoA pool size in human fibroblasts incubated with either linoleic acid (18:2, ω-6) supplemented medium or eicosapentaenoic acid supplemented medium (20:5, ω-3) $p<0.005$ ($n = 4$).


ACYL CoA (ug / mg cell protein)
300
250
200
150
100
50
0
LINOLEIC ACID
DOCOSAPENTAENOIC ACID

3.4 ACAT Activity in Human Fibroblast Microsomes

3.4.1 Assay Conditions

ACAT activity was measured in microsomes isolated from cells enriched with different polyunsaturated fatty acids to assess whether a reduction in the rate of cholesteryl esterification in ω-3 PUFA enriched cells reflected a reduction in ACAT activity. Microsomal ACAT activities were also measured using radiolabelled oleoyl CoA. Figure 7 shows that microsomal ACAT activity is linear up to at least thirty minutes. Increased cholesteryl esterification was observed with increasing amounts of microsomal protein, linear up to 30 μg of protein (Figure 8). Measurements of ACAT activity in microsomes with increasing time and increasing microsomal protein concentrations were done in microsomes isolated from cells that were not incubated with fatty acid supplemented medium. Therefore, in all subsequent experiments measuring ACAT activity in microsomes, 30 μg of microsomal protein was used and assay mixtures were incubated for 20 minutes.

Figure 7: ACAT activity in human fibroblast microsomes with increasing time

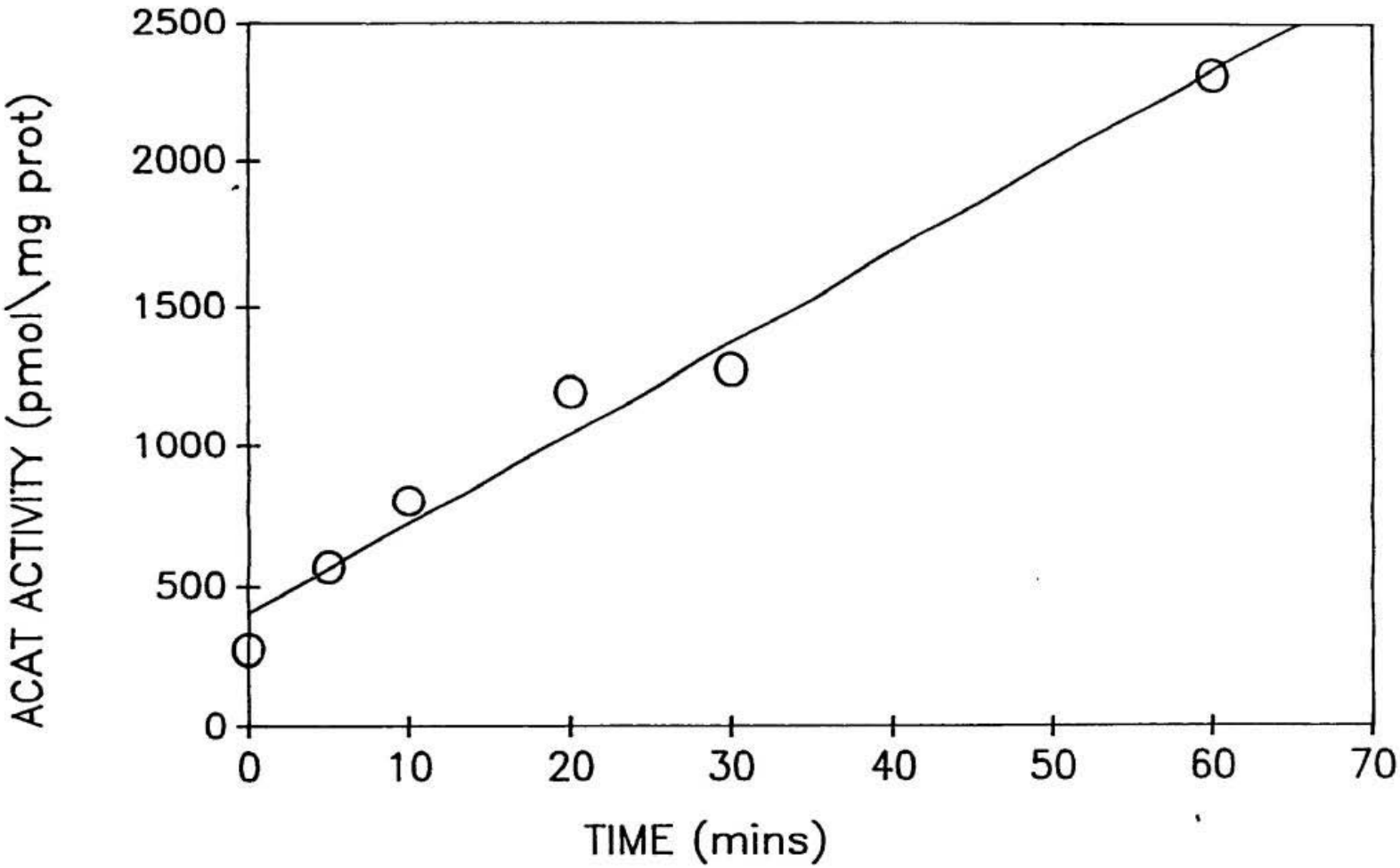
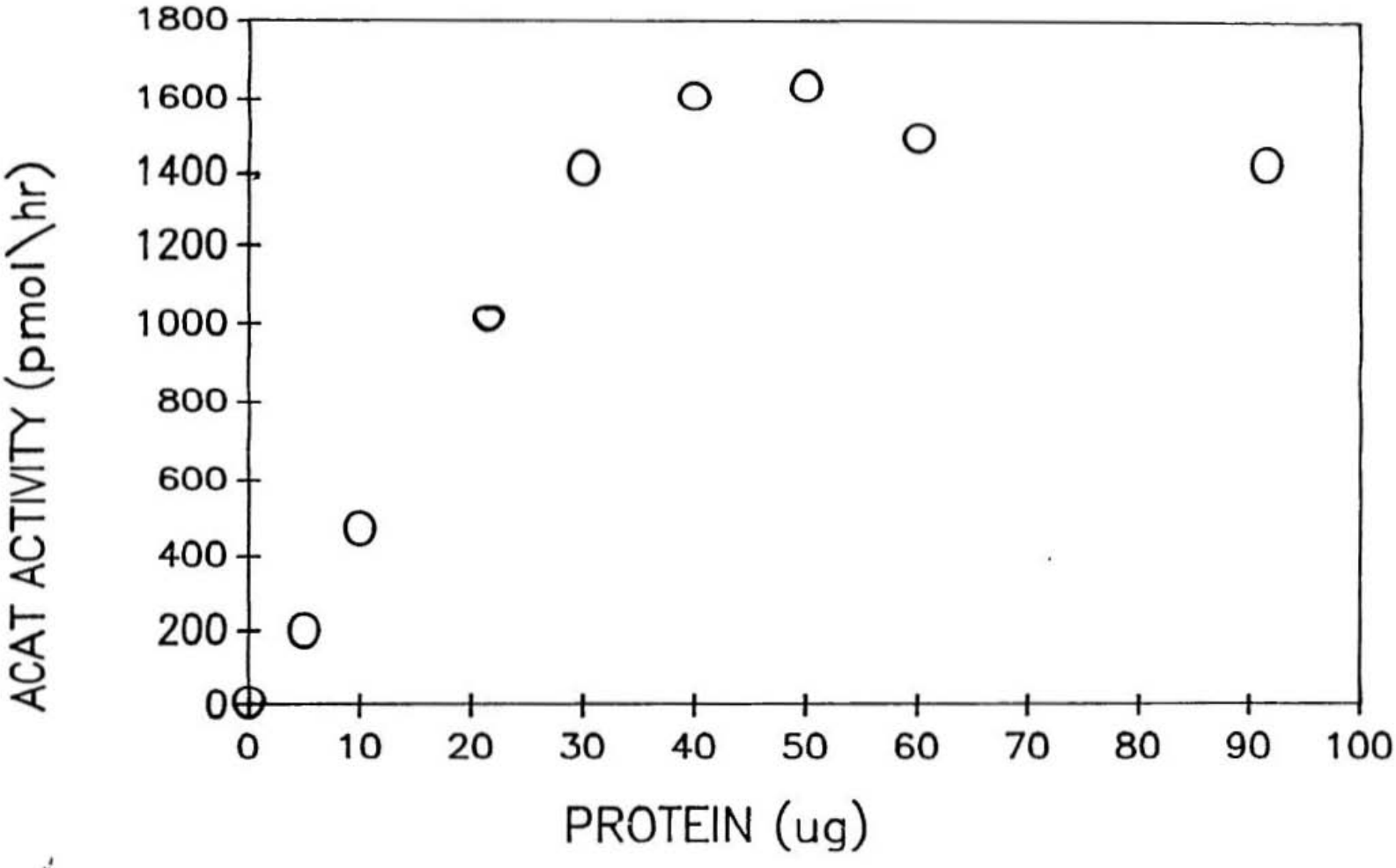

ACAT ACTIVITY (pmol\mg prot)
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
TIME (mins)

Figure 8: ACAT activity in microsomes with increasing protein concentration


ACAT ACTIVITY (pmol\hr)
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
90
100
PROTEIN (ug)

3.4.2 ACAT Activity in Human Fibroblast Microsomes Enriched with Different Polyunsaturated Fatty Acids

ACAT activities were measured in microsomes isolated from human fibroblasts enriched with either linoleic acid or docosapentaenoic acid. Figure 9 illustrates that ACAT was significantly reduced (by 37.7%) in microsomes enriched with ω-3 PUFAs compared with microsomes enriched with ω-6 PUFAs.

The free cholesterol levels were measured in microsomes from human fibroblasts enriched with different polyunsaturated fatty acids. Figure 10 clearly shows that cholesterol levels in microsomes enriched with either linoleic acid or docosapentaenoic acid enriched cells remained unchanged.

Figure 9: Acyl CoA:cholesterol acyl transferase (ACAT) activity in microsomes isolated from human fibroblasts enriched with linoleic acid or docosapentaenoic acid. Error bars are S.D. $p<0.005$ ($n = 4$).

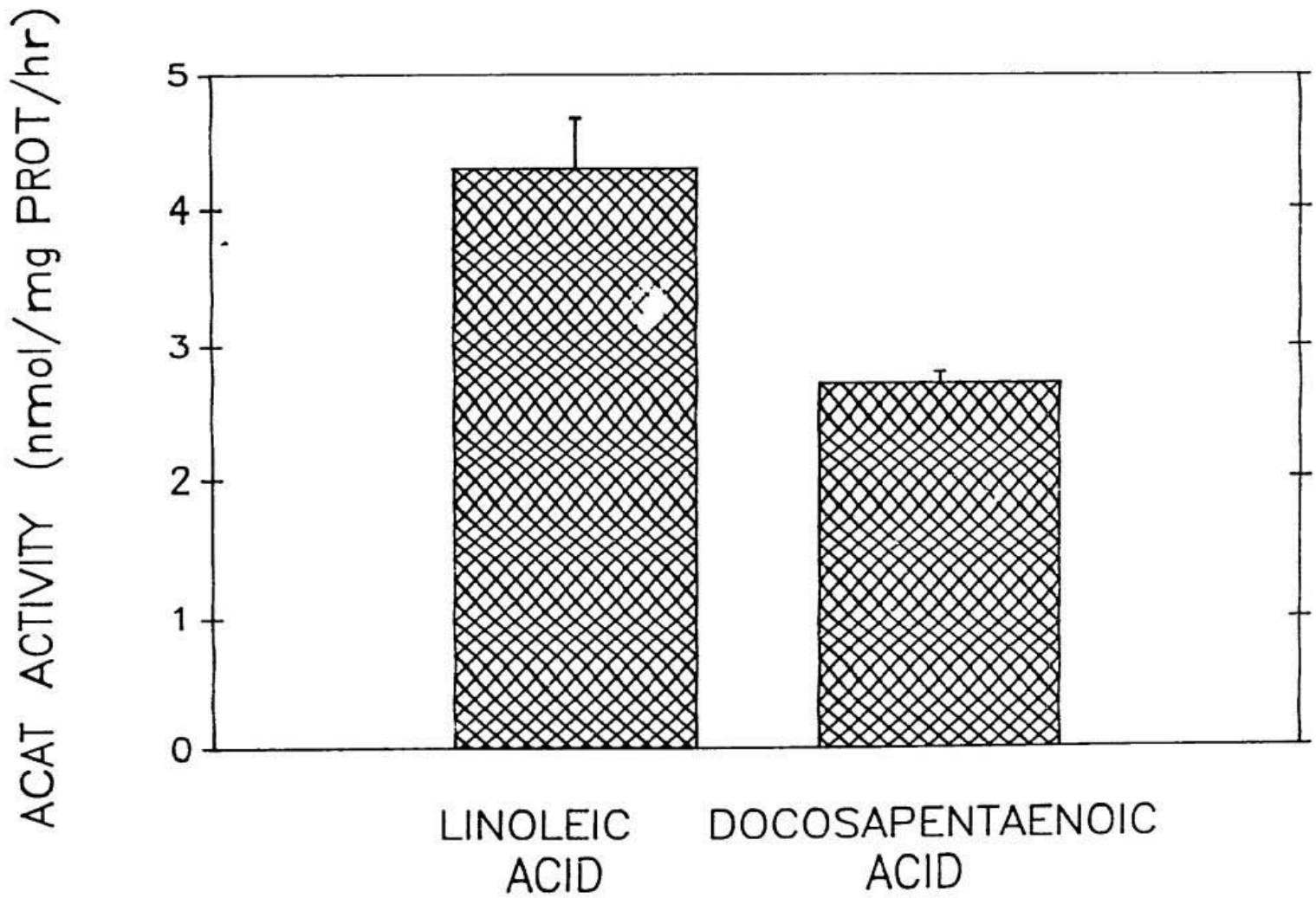
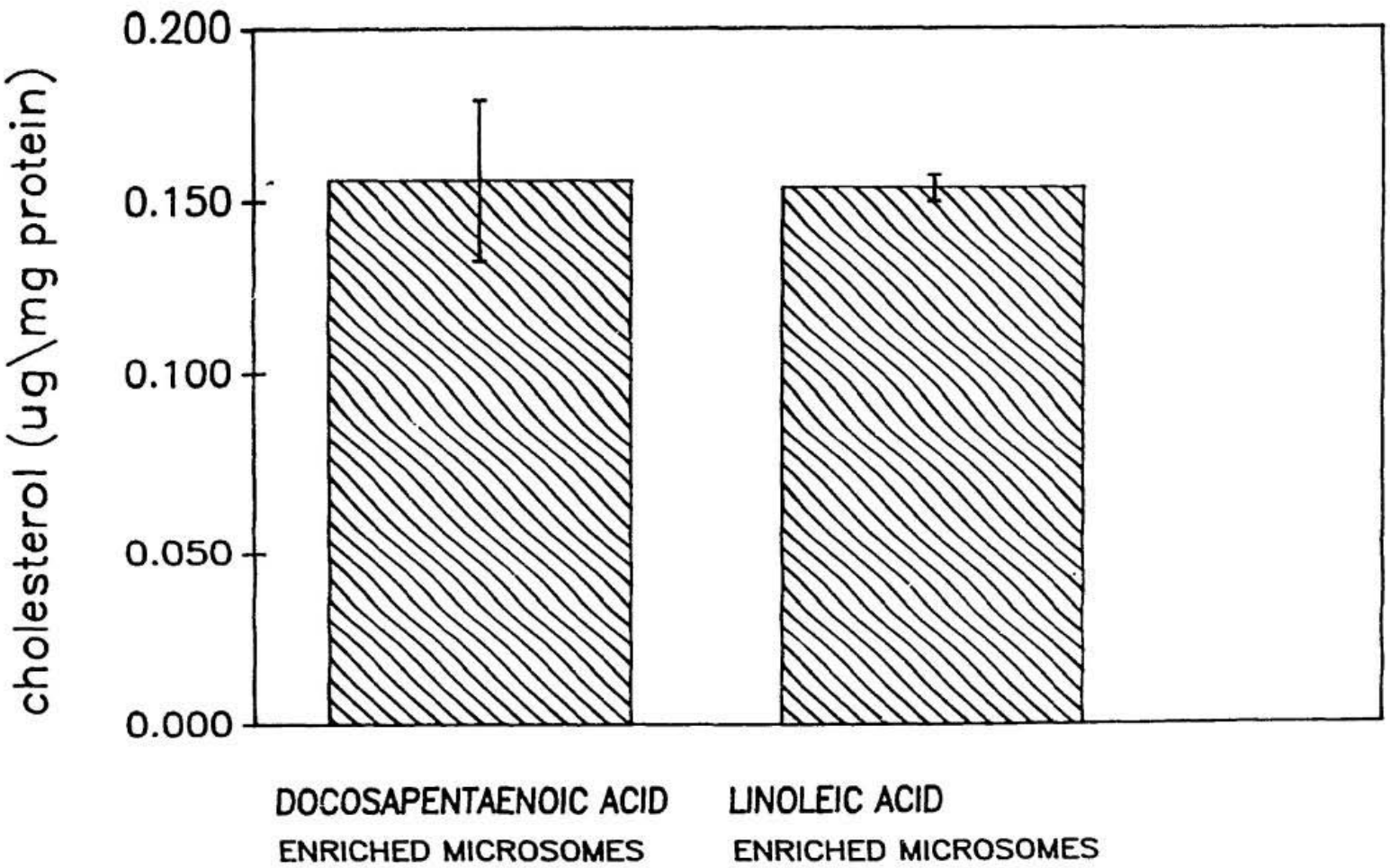

ACAT ACTIVITY (nmol/mg PROT/hr)
5
4
3
2
1
0
LINOLEIC ACID
DOCOSAPENTAENOIC ACID

Figure 10: Cholesterol content (unesterified) in human fibroblast microsomes enriched with docosapentaenoic acid or linoleic acid. Error bars represent S.D. (n = 3).

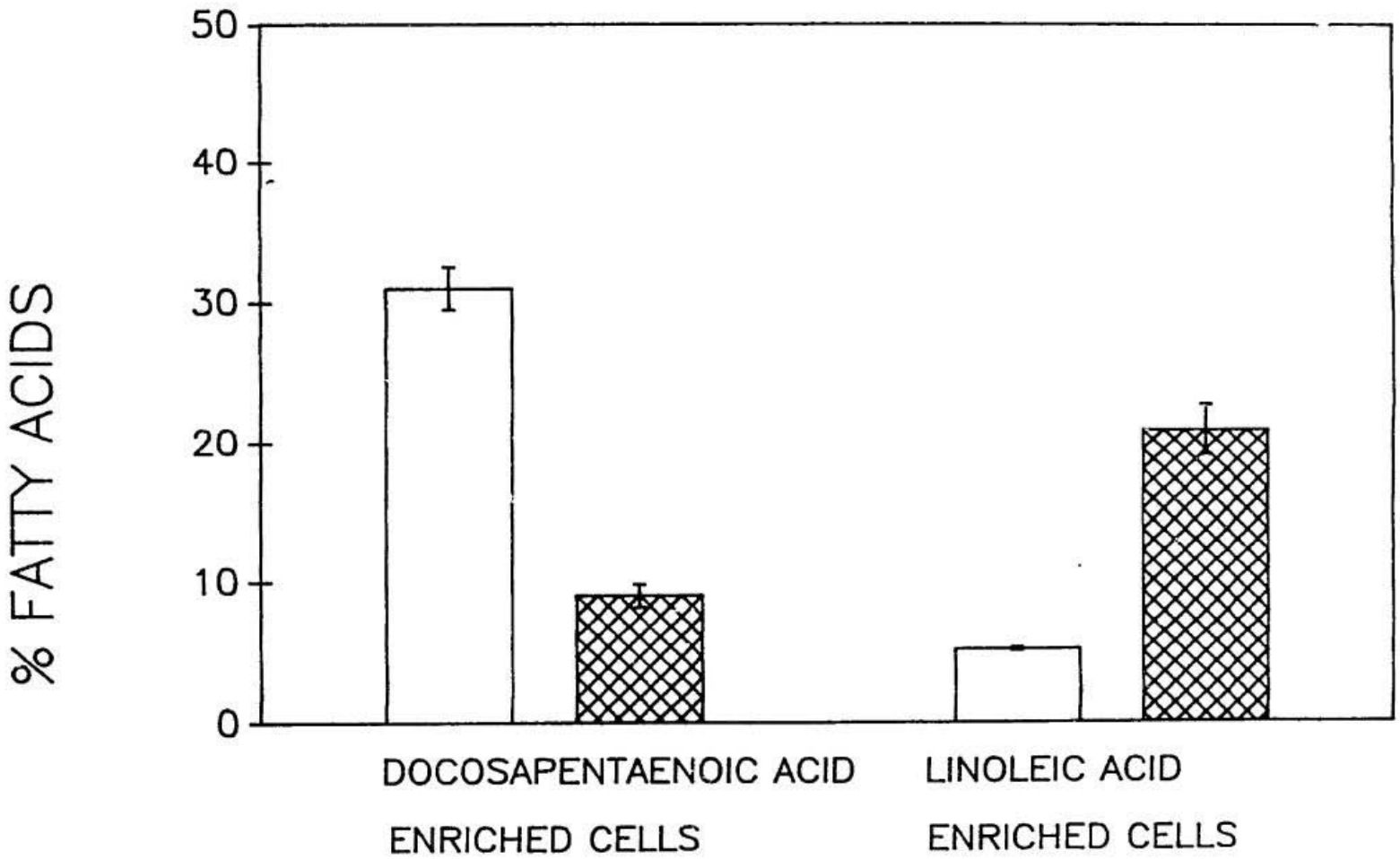

0.200
0.150
0.100
0.050
0.000
cholesterol (ug\mg protein)
DOCOSAPENTAENOIC ACID
ENRICHED MICROSOMES
LINOLEIC ACID
ENRICHED MICROSOMES

3.5 Cholesteryl Esterification in Macrophages

3.5.1 Polyunsaturated Fatty Acid Enrichment of J774 Macrophages

J774 mouse macrophages were incubated with different PUFA supplemented medium. Figure 11 summarizes the relative amounts of ω-3 and ω-6 PUFAs present in the phospholipid fraction of the cell membrane after incubation with medium supplemented with either linoleic acid or eicosapentaenoic acid. When cells were incubated with 60 μg/ml of linoleic acid, the relative content of ω-6 PUFAs present in the phospholipid fraction (20.9%) was higher than cells incubated with 60 μg/ml eicosapentaenoic acid (5.2%). The majority of the ω-6 PUFAs in cells incubated with linoleic acid was present as linoleate (15.8%). Cells incubated with eicosapentaenoic acid contained a higher relative percentage of ω-3 PUFAs in their phospholipid fractions (31%) than cells incubated with linoleic acid (9.0%). The majority of the ω-3 PUFAs found in cells incubated with eicosapentaenoic acid was present as docosapentaenoic acid (16.1%). The relative content of docosahexaenoic acid was similar in macrophages incubated with either linoleic acid or eicosapentaenoic acid.

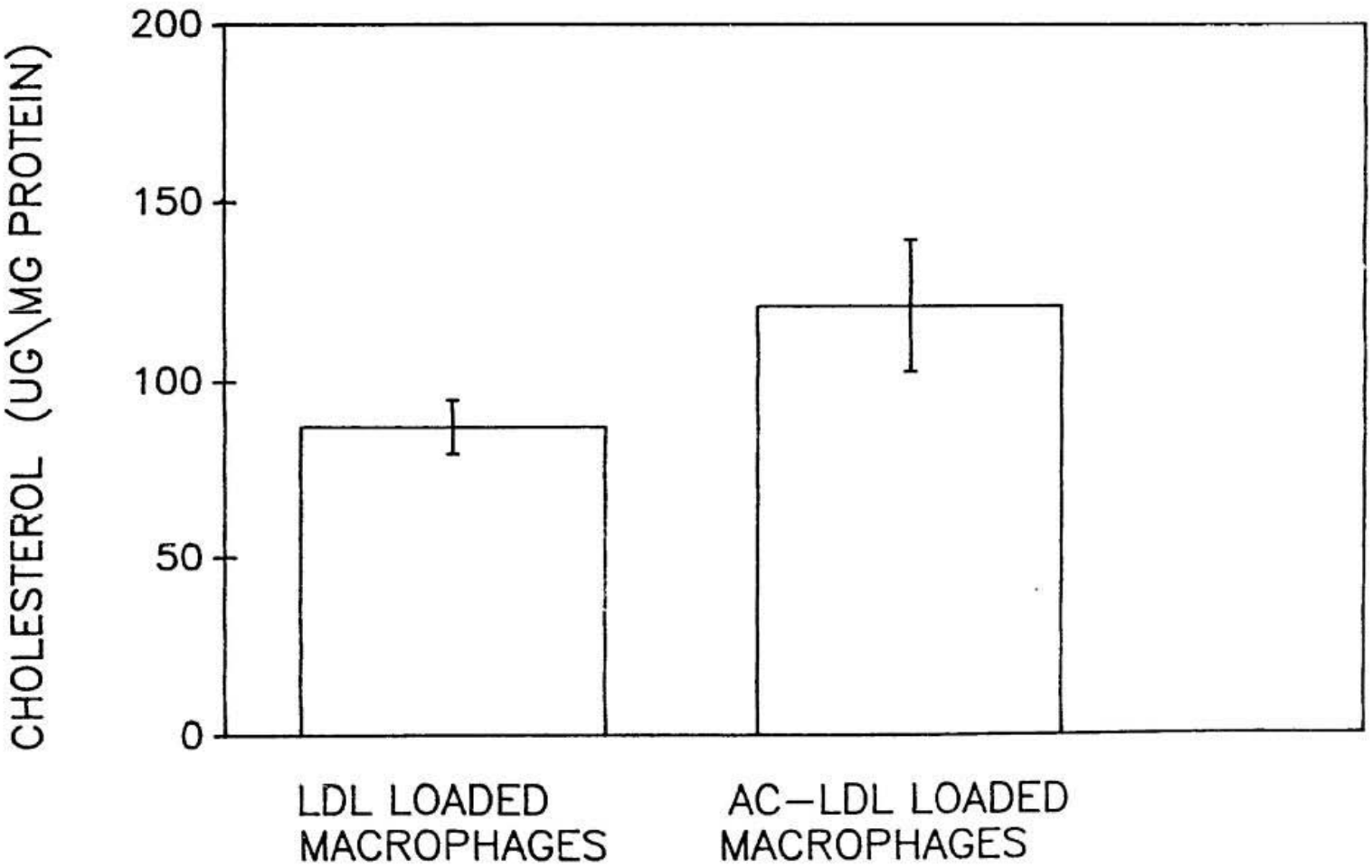
Figure 11: Relative percent of ω-3 PUFAs (open bar) and ω-6 PUFAs (crossed bar) in macrophages enriched with docosapentaenoic acid or linoleic acid. (n = 4.)


% FATTY ACIDS
50
40
30
20
10
0
DOCOSAPENTAENOIC ACID
ENRICHED CELLS
LINOLEIC ACID
ENRICHED CELLS

3.5.2 Stimulation of Cholesterol Esterification in Macrophages

Cholesterol in macrophages can be derived from native LDL entering the cell via the LDL receptor or modified form of LDL entering by the unregulated scavenger receptor (Steinberg, 1988). Acetylated LDL (a chemically modified form of native LDL) appears to be more efficient than native LDL in the stimulation of cholesteryl esterification. Figure 12 illustrates that macrophages contained significantly more cholesterol when they were incubated with 50 μg/ml acetylated LDL compared with macrophages incubated with 50 μg/ml native LDL.

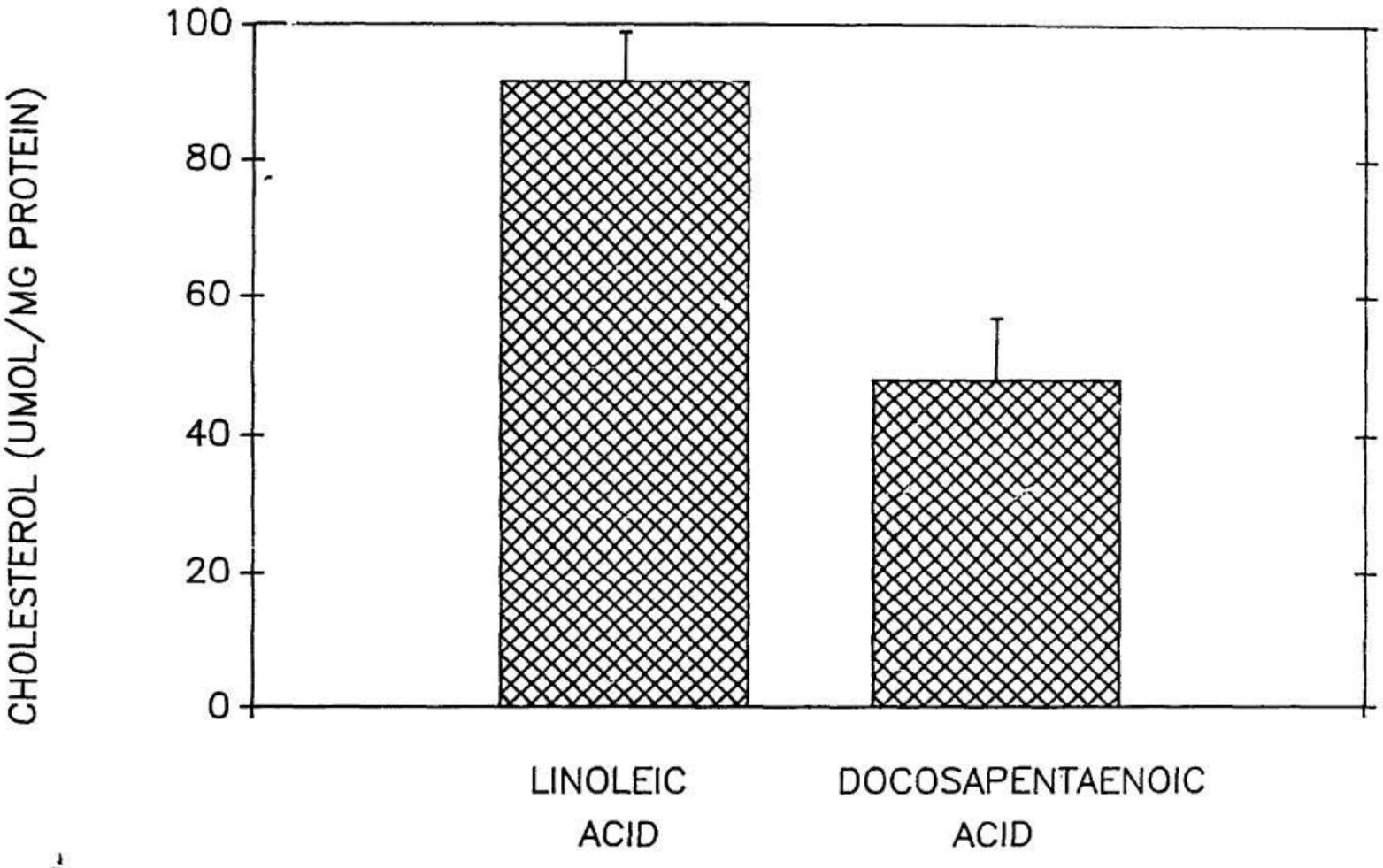
Figure 12: Cholesterol content of macrophages incubated with either LDL or acetylated LDL. Error bars represent S.D., $p<.01$ ($n = 4$).


CHOLESTEROL (UG\MG PROTEIN)
200
150
100
50
0
LDL LOADED MACROPHAGES
AC—LDL LOADED MACROPHAGES

3.5.3 The Effect of Polyunsaturated Fatty Acid Enrichment of Macrophages on Cholesterol Metabolism

The effect of PUFA enrichment on cholesterol was assessed in macrophages by determining the total cholesterol content. The amount of total cholesterol (both esterified and unesterified cholesterol) was significantly lower in macrophages enriched in ω-3 polyunsaturated fatty acids compared with macrophages enriched with ω-6 polyunsaturated fatty acids (91.8 ± 7.2 μg cholesterol/mg protein and 48.1 ± 8.8 μg cholesterol/mg protein, respectively) (Figure 13).

Figure 13: The cholesterol content in macrophages enriched with either linoleic acid or docosapentaenoic acid. Error bars represent S.D., $p<.001$ ($n = 6$).


CHOLESTEROL (UMOL/MG PROTEIN)
100
80
60
40
20
0
LINOLEIC ACID
DOCOSAPENTAENOIC ACID

3.6 The Effect of Polyunsaturated Fatty Acid Enrichment in Human Fibroblasts on Cholesterol Efflux

The efflux of radiolabelled cholesterol from human fibroblasts enriched with different fatty acids was measured over a period of 24 hours. The endogenous cholesterol pool in fibroblasts was labeled by adding [^{3}H]-acetate (1 μCi/ml) for 5 days. In the presence of HDL, the rate of efflux of radiolabelled cholesterol from cells enriched with ω-3 PUFAs was substantially faster than efflux from cells enriched with ω-6 fatty acids (Figure 14a). The efflux data could be fitted to a first order logarithmic decay (coefficients of 0.93 and 0.83 for ω-3 PUFA enriched cells and ω-6 PUFA enriched cells, respectively) (Figure 14b). The half times (T 1/2) for efflux were 16 $\pm$ 4.5 hr and 33.1 $\pm$ 9.2 hr for ω-3 and ω-6 PUFA enriched cells, respectively (Figure 15). Cholesterol efflux was not observed in cells enriched with either fatty acid in the absence of HDL (Figure 14a). To ensure that the difference in the rate of cholesterol efflux in different fatty acid enriched cells was due to changes in net efflux rather than increased cholesterol exchange between HDL and fibroblasts, the mass of cholesterol transferred from cells into medium was measured. Figure 16 shows that net transfer of cholesterol from cells into medium was greater when cells were enriched with ω-3 fatty acids (57% in 24 hours) compared with linoleic acid enriched cells (37% in 24 hours).

Figure 14a: Efflux of radiolabelled cholesterol from human fibroblasts incubated with linoleic acid (●) or docosapentaenoic acid (○) in the presence of HDL. No net eflux was observed in the absence of HDL with either linoleic acid enrichment (■) or docosapentaenoic acid enrichment (□) of human fibroblasts.

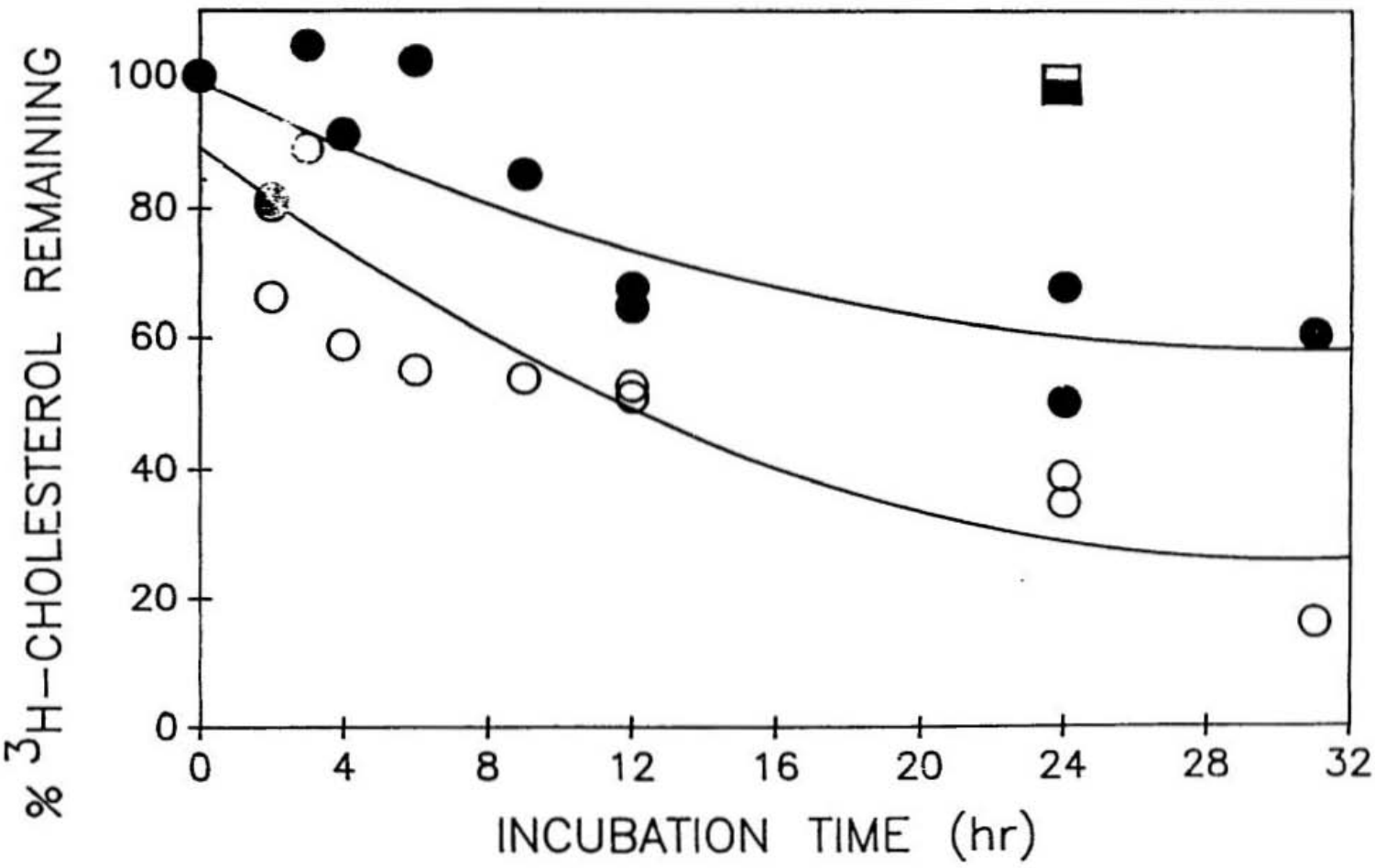

% ^{3}H–CHOLESTEROL REMAINING
100
80
60
40
20
0
0
4
8
12
16
20
24
28
32
INCUBATION TIME (hr)

Figure 14b: Efflux of radiolabelled cholesterol from human fibroblasts incubated with linoleic acid (●) or docosapentaenoic acid (O) in the presence of HDL. Data are presented as a logarithmic plots.

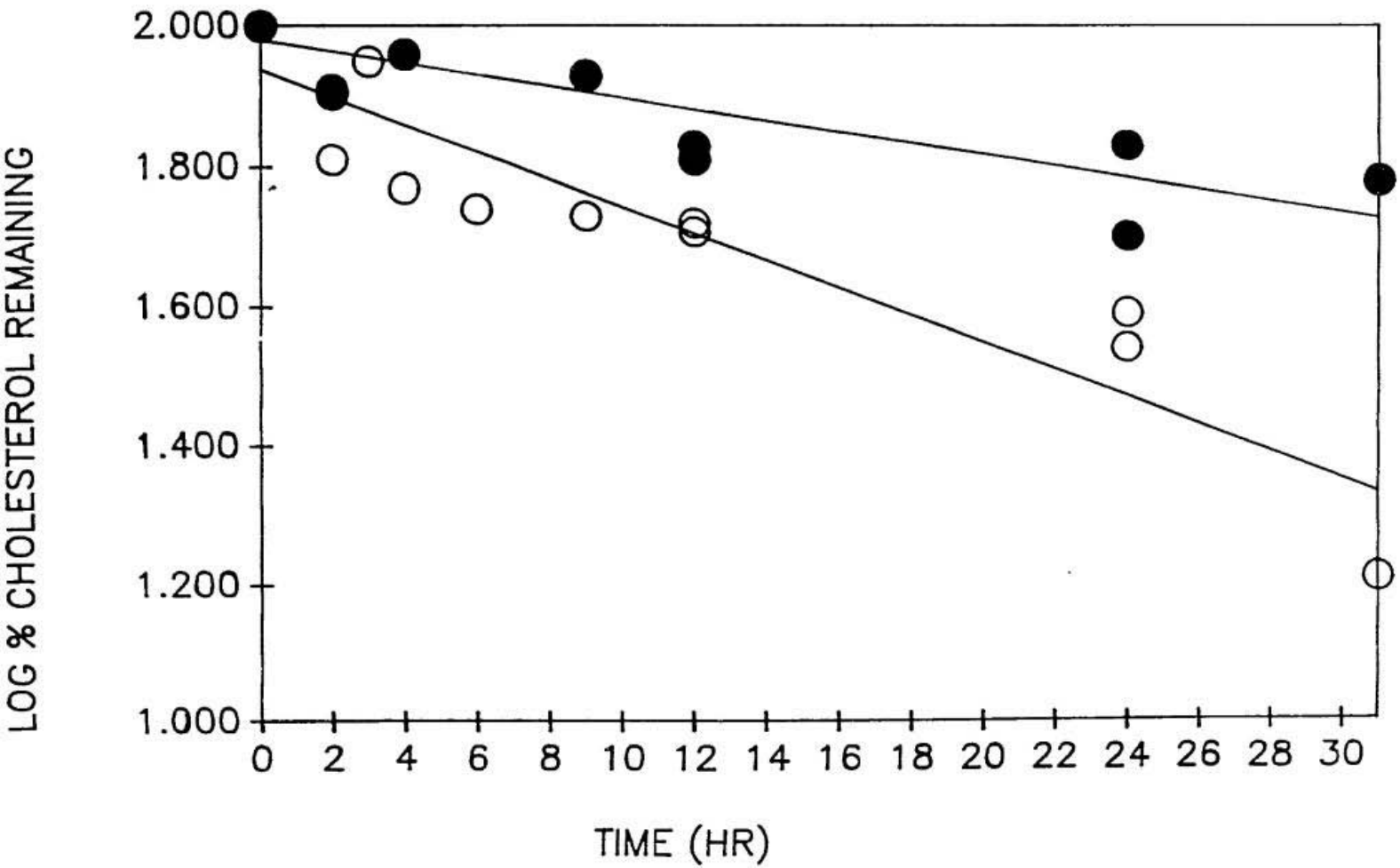

LOG % CHOLESTEROL REMAINING
2.000
1.800
1.600
1.400
1.200
1.000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (HR)

Figure 15: Half times (T 1/2) for cholesterol efflux from human fibroblasts enriched with linoleic acid or eicosapentaenoic acid. Based on radiolabelled experiments (closed bars) and on mass experiments (open bars). Error bars represent S.D. ($n = 3$), $p<0.05$.

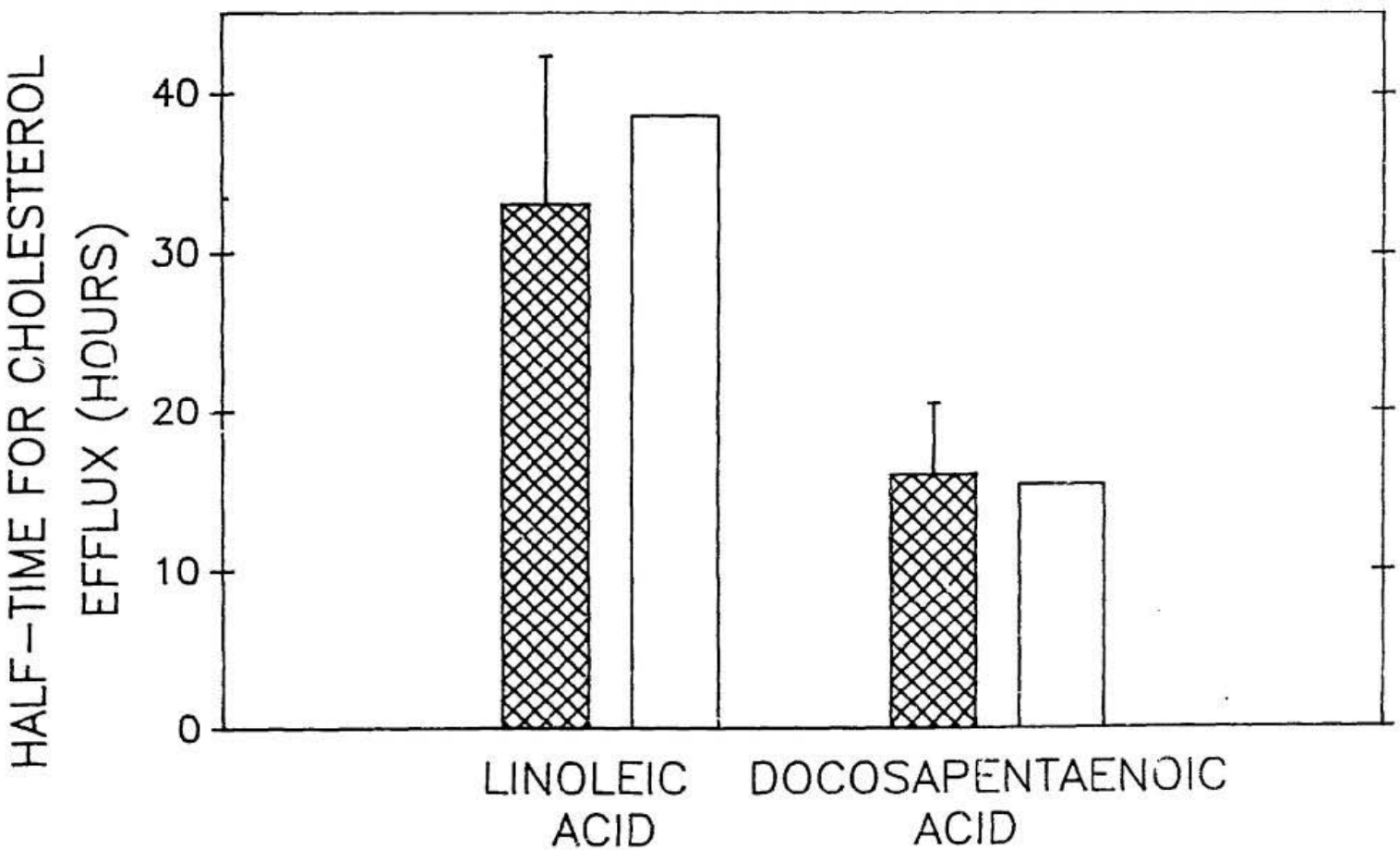
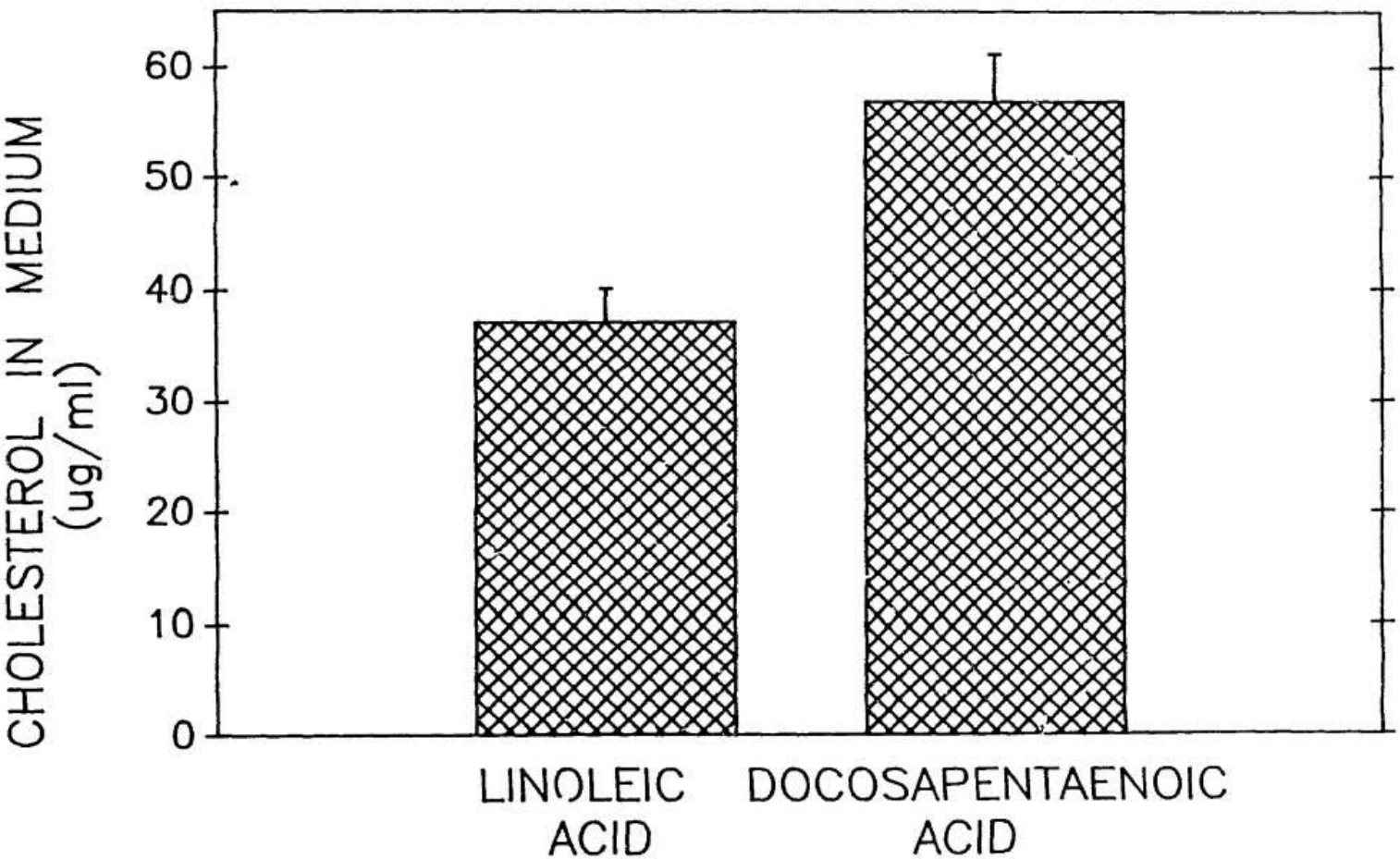

HALF–TIME FOR CHOLESTEROL EFFLUX (HOURS)
40
30
20
10
0
LINOLEIC ACID
DOCOSAPENTAENOIC ACID

Figure 16: HDL-mediated net release of cholesterol into the culture medium by fibroblasts enriched with linoleic acid or docosapentaenoic acid after 24 hours incubation with 250 μg/ml HDL. Error bars are S.D. (n = 4), $p<0.005$.


CHOLESTEROL IN MEDIUM (ug/ml)
60
50
40
30
20
10
0
LINOLEIC ACID
DOCOSAPENTAENOIC ACID

4. DISCUSSION

4.1 The Effect of Modification of Cellular Fatty Acids on Cholesterol Regulation

The major objective of this work was to study the impact of changes in the type of polyunsaturated fatty acids in cellular phospholipids on the regulation of cholesterol content in non-hepatic tissues. The planned approach involved the incubation of human skin fibroblasts and J774 macrophages with either linoleic acid (18:2, ω-6) or eicosapentanenoic acid (20:5, ω-3) by supplementing the culture medium with the sodium salts of these fatty acids. The incorporation of exogenous fatty acid into cellular phospholipids was monitored and the effect of the changes in fatty acid composition on two critical steps in cholesterol regulation, the esterification of free cholesterol and the removal of unesterified cholesterol from cells by HDL-mediated "reverse cholesterol transport", was examined.

4.1.1 Fibroblasts

When human fibroblasts were incubated with medium supplemented with 60 μg/ml of linoleic acid-sodium salt (18:2, ω-6), the cells readily incorporated the linoleate into their phospholipid. The level of enrichment of cell phospholipid with linoleic acid was comparable to the percentage of linoleate present in the supplemented medium (Table 1).

(Fatty acid analysis on the supplemented medium had been done previously by Chris Goodyear, Honours project.) Fibroblasts incubated with medium supplemented with 60 μg/ml of the sodium salt of eicosapentaenoic acid (20:5, ω-3) were also able to take up this fatty acid. However, the level of eicosapentanenoic acid in the phospholipid fractions from these cells was relatively low. Fatty acid analysis of the phospholipids showed a marked increase in the level of docosapentaenoic acid (22:5, ω-3) in the phospholipids from cells incubated with medium containing eicosapentaenoic acid. Docosapentaenoic acid is an immediate elongation product of eicosapentaenoic acid and the ratio of docosapentaenoic acid/eicosapentaenoic acid in the cells was about 10. The content of docosapentaenoic acid in the medium was negligible, suggesting that fibroblasts can convert eicosapentaenoic acid to docosapentaenoic acid. No significant increase in docosahexaenoic acid (22:6, ω-3) content in these cells was observed. The subsequent conversion of docosapentaenoic acid to docosahexaenoic acid requires a Δ4 desaturase. The data indicates that the human skin fibroblasts lack Δ4 desaturase activity. This is consistant with reports by others that have suggested that the Δ4 desaturase activity in fibroblasts is either low or absent entirely (Aeberhard et al., 1978; Dunbar and Bailey, 1975; Spector et al., 1981). It is also noteworthy that Δ4 desaturase was not detected in monocyte macrophages (Chapkin and Miller, 1990).

The effect of fatty acid modification on cholesterol metabolism in fibroblasts was initiallly assessed by measuring the mass of free (unesterified) cholesterol and cholesterol ester in lipid extract of these cells. While the free cholesteryl content was unchanged in fibroblasts by either ω-3 or ω-6 PUFA enrichment, the cholesteryl ester content was affected by the type of PUFAs used to enrich the fibroblasts (Figure 1). Human fibroblasts enriched with ω-3 PUFAs contained 60% less cholesteryl ester per mg protein than ω-6 PUFA enriched cells. This indicates that enrichment of the phospholipids in fibroblasts with docosapentaenoic acid or incubation with EPA supplemented medium can affect the level of cholesteryl esters in these cells. Decreased cholesterol ester levels could reflect decreased uptake of exogenous cholesterol, lower cholesterol biosynthesis, decreased esterification of cholesterol or enhanced removal of cholesterol from cells. However, the impact of changes in PUFA content on cholesterol esterification were of special interest.

The rate of cholesteryl esterification was measured using a radiolabelled substrate ([^{14}C]-oleoyl CoA). Figure 5 shows clearly that the rate incorporation of radiolabelled oleoyl CoA into cholesteryl ester was reduced by 44.5% when cellular phospholipids were enriched with ω-3 PUFAs compared with the incorporation in cells enriched with ω-6 PUFAs. These observations are consistent with the mass measurements

that showed a dramatic decrease in cholesteryl ester mass in ω-3 PUFA enriched cells (Figure 1). Similar results have been observed in several other studies that compare the effects of fatty acid enrichment on cholesterol metabolism. In CaCo-2, cells derived from a human colon adenocarcinoma, cholesterol esterification was 60% lower in cells incubated with eicosapentaenoic acid compared with cells incubated with palmitic acid (Murthy et al., 1988). Enrichment of the phospholipid fraction of hepatocyte membranes with ω-3 PUFAs has resulted in similar reductions in cholesterol esterification when compared with cells enriched with ω-6 PUFAs (Rustan et al., 1988).

As noted above, the content of unesterified cholesterol in fibroblasts enriched with ω-3 PUFAs was similar to the levels of unesterified cholesterol in ω-6 PUFA enriched fibroblasts (Figure 1). It is, therefore, unlikely that the differences in the rate of cholesteryl ester formation that were observed were the result of differences in the concentration of the unesterified cholesterol substrate. Even though most of the unesterified cholesterol is located within the cellular membranes, it is possible that there may be an intracellular pool(s) of cholesterol which may account for the results.

The second substrate for cholesterol esterification is the acyl CoA that provides the acyl chain to cholesterol. The fatty acyl CoA pool was measured in human fibroblasts to

determine whether it was affected by various fatty acid enrichment. Figure 6 illustrates that the fatty acyl CoA pool size was decreased in response to docosapentaenoic acid enrichment of human fibroblasts compared with linoleic acid enrichment. It is unlikely, however, that the decrease in the size of the acyl CoA pool in docosapentaenoic enriched cells would account for the lower cholesterol esterification rates in these cells. The rate of cholesterol esterification expressed in picomoles of cholesteryl [^{14}C]-oleate formed per milligram protein per hour would represent an even higher specific activity of the enzyme due to isotope dilution of the [^{14}C]-oleoyl CoA by the larger fatty acyl CoA pool in the ω-6 PUFA enriched cells compared with the ω-3 PUFA enriched cells. If isotope dilution is a factor, then my measurement of esterification using the medium concentration underestimates the differences between rates in cells enriched with the different fatty acids

My measurement of cellular cholesterol esterification was calculated by converting the dpms of cholesteryl [^{14}C]-oleate formed to picomoles of cholesteryl [^{14}C]-oleate without taking into account the endogenous fatty acyl CoA pool in human fibroblasts for a number of reasons. Cholesterol esterification expressed as pmoles of only radiolabelled cholesteryl [^{14}C]-oleate formed per mg protein per hr has been widely used when measuring ACAT activity in fibroblasts (Spence, 1989), hepatocytes (Rustein, 1988), and

intestinal cells (Murthy, 1988). Also, the amount of fatty acyl CoA present in the intracellular pool(s) required for esterification is unknown. The cellular distribution of fatty acyl CoA between the mitochondrial and cytosolic compartments has been determined in rat heart and was found to be located almost exclusively in the mitochondrial compartment (Neely, 1978). Since endogenous fatty acyl CoA present in the cytosolic fraction of human fibroblasts has not been determined, it would be inaccurate to calculate cholesterol esterification based on the total cellular fatty acyl CoA pool.

Calculations of cholesterol esterification based on the fatty acyl CoA pool in whole cell lysates of human fibroblasts enriched with ω-6 and ω-3 PUFAs were 37.7 and 8.23 nmoles/mg protein/hr, respectively. When calculated based only on the medium specific activity of [^{14}C]-oleoyl CoA, the cholesterol esterification in ω-6 and ω-3 PUFA enriched whole cell lysates were 387 and 220 pmoles/mg protein/hr, respectively, and microsomal preparations were 4.31 and 2.68 nmoles/mg protein/hr, respectively. Using only the extracellular medium [^{14}C]-oleoyl CoA concentration to calculate the rate of cholesteryl esterification appears to make more sense since the microsomal protein concentration is approximately about 10% that of total cellular protein. Therefore, it should follow that the ACAT activity in microsomes should be approximately 10% of the activity when

measured in the whole cell. Indeed this is in agreement only when cholesteryl esterification in whole cells is calculated based on exogenous medium oleoyl CoA.

4.1.2 Human Fibroblast Microsomes

The activity of acyl CoA:cholesterol acyltransferase (ACAT) was measured in microsomes isolated from cells enriched with ω-3 and ω-6 PUFAs. The fatty acid composition of the phospholipid fraction of microsomes isolated from fibroblasts was similar to the composition of total phospholipid from whole cells that were incubated with medium supplemented with either linoleic acid or eicosapentaenoic acid (Table 2). The fatty acid enrichment observed in microsomal membrane, therefore also appears to reflect the fatty acid composition of the incubation medium.

Microsomal phospholipids were further separated into two phospholipid classes, PE and PC. Results show (Table 3) that ω-3 PUFAs are preferentially incorporated in the PE fraction rather than the PC fraction of the microsomal phospholipids regardless of whether the microsomes were isolated from cells incubated with linoleic or eicosapentaenoic acid supplemented medium. However, the ω-6 PUFAs did not seem to favor one microsomal phospholipid class over the other when the cells were incubated with either linoleic acid or eicosapentaenoic acid supplemented medium. These results are similar to those observed by Murthy (1988), when CaCo-2 cells were incubated

with eicosapentaenoic acid supplemented medium, ω-3 PUFAs were preferentially incorporated into the PE fraction of cellular phospholipids. Similar results have also been reported in macrophages incubated with fatty acid supplemented medium (Davis, personal communication) and in blood platelets isolated from rats fed fish oil supplemented diets (Nordoy et al., 1985).

ACAT activity measured in microsomes enriched with ω-3 PUFAs was decreased by 37.7% compared with the activity in microsomes enriched with ω-6 PUFAs (Figure 9). The changes in ACAT activities observed in microsomes enriched with different PUFAs were consistent with the rate of cholesterol esterification that we observed in whole cell lysates. Thus, the ability of ω-3 PUFA enrichment to reduce cholesteryl ester mass and cholesteryl esterification in whole cells appears to be a direct consequence of decreased microsomal ACAT activity. Similar observations have been made in liver microsomes isolated from rats fed either fish oil or vegetable oil supplemented diets. ACAT activity was reported to be 40% lower in ω-3 PUFA enriched microsomes compared with microsomes enriched with ω-6 PUFAs (Field et al., 1987).

Microsomal ACAT measurements involve use of exogenous fatty acyl CoA. The fatty acyl CoA concentration (21 μM) was, therefore, identical in each reaction mixture containing microsomes from either ω-3 or ω-6 enriched cells. It is possible that the Km for oleoyl CoA in human fibroblasts

is close to the value that has been reported for macrophages and hepatocytes. For cholesterol esterification in hepatocytes and macrophages, the K_m's for oleoyl CoA are approximately 13.9 μM (McCloskay, 1988) and 22.7 μM (Field and Salome, 1982), respectively. It should be noted, however, that the K_m for oleoyl CoA for fibroblasts is unknown. It should also be noted that the microsomal (unesterified) cholesterol content was uneffected by either fatty acid enrichment (Figure 12). Therefore the differences in ACAT activities in microsomes isolated from ω-3 PUFA and ω-6 PUFA enriched cells may not be attributable to differences in substrate concentrations of cholesterol or fatty acyl CoA.

Exhaustive checks of the amount of contamination of the microsomal preparations was not carried out because of the difficulty of preparing sufficient amounts of microsomes from fibroblast cultures. Therefore, the activity of only one microsomal marker (NADPH-cytochrome c reductase) was assayed. The average specific activity of NADPH-cytochrome c reductase was 62.8 nmoles/mg protein and 57.7 nmoles/mg protein for linoleic acid and docosapentaenoic acid enriched microsomes, respectively. Therefore, differences in the amount of contamination in our microsomal preparation with other organelles did not appear to be responsible for the differences in ACAT activities observed in the two groups.

4.1.3 Macrophages

Electron micrographs of atherosclerotic lesions reveal the presence of cells with numerous cytoplasmic droplets. The droplets in these cells are primarily composed of cholesteryl esters and the cells containing them are usually called foam cells (McCloskey et al., 1987). Foam cells are derived from smooth muscle cells and resident monocyte-macrophages in the arterial initima as a consequence of the uptake of excess cholesterol (Steinberg, 1983). It is believed that the transformation of smooth muscle cells and macrophages is a key step in the initiation of atherosclerosis (Steinberg, 1983).

Modification of human fibroblasts with ω-3 PUFAs clearly decreased ACAT activity and hence decreased deposition of cholesteryl ester within these cells. If enrichment of macrophage phospholipids with ω-3 PUFAs also inhibits cholesteryl esterification, then deposition of esterified cholesterol would be decreased and hence foam cell development might be delayed.

One of the objectives of this thesis was to study the effect of ω-3 and ω-6 PUFA enrichment of the phospholipids of macrophages in culture on the cholesteryl ester content within these cells. Initial experiments were carried out to determine the extent to which the fatty acid composition of J774 macrophages could be modified by incubating them with medium supplemented with either linoleic acid or

eicosapentanenoic acid. Mass analysis of free (unesterified) and esterified cholesterol content of these cells were carried out to examine the effect of fatty acid enrichment on cholesterol metabolism.

Macrophages were incubated with 60 μg/ml of either linoleic acid or eicosapentaenoic acid. Enrichment of macrophages with ω-6 and ω-3 PUFAs was similar to the enrichment observed in fibroblasts when they were incubated with these fatty acids. Macrophages incorporated linoleic acid from the medium and again docosapentaenoic acid was incorporated at high levels when the medium was supplemented with eicosapentaenoic acid (see section 3.5.1). Similar results have been reported in mouse peritoneal macrophages by Chapkin et al. (Chapkin and Miller, 1990) when these cells were exposed to eicosapentaenoic acid. This would suggest that J774 and peritoneal macrophages, like fibroblasts, lack the Δ4 desaturase needed to convert docosapentaenoic acid to docohexaenoic acid.

The enrichment of J774 macrophages with ω-3 PUFAs decreased the cholesteryl ester content within these cells compared with cells enriched with ω-6 PUFAs. However, the free cholesterol content was unaffected by either enrichment. A similar reduction in cholesteryl ester mass was seen in fibroblasts enriched with ω-3 PUFAs suggesting that the effect of this fatty acid on cholesterol metabolism may be mediated through similar mechanisms in both cell types. The

implication of decreased cholesteryl ester deposition in macrophages in the arterial wall is especially relevent in the process of foam cell formation and hence atherosclerotic lesions. Recent experiments demonstrated that aorta from rabbits fed fish oil supplemented diets contained significantly less cholesteryl ester than rabbits fed vegetable oil diets (Davis, personal communication). Since the arterial wall contains cells such as monocyte-macrophages, it is not unreasonable to assume that decreased cholesteryl ester content observed in aortas isolated from rabbits fed ω-3 PUFAs may actually reflect decreased cholesterol esterification within the macrophages as a result of ω-3 PUFA modification. Similar observations have been made in aortas isolated from monkeys fed diets supplemented with fish oils and vegetable oils (Davis, 1987).

4.1.4 Molecular Mechanisms

4.1.4.1 Membrane Fluidity

Recent studies have indicated that the composition and fatty acyl chain mobility of a lipid bilayer can influence a number of membrane properties, such as the degree of exposure of surface proteins and the activity of membrane bound enzymes (Spector et al., 1979; Sandermann, 1978; Pechay et al., 1978). Since dietary unsaturated fatty acids are readily incorporated into the phospholipid fraction of cell membranes, the physical properties of these membranes may be

dramatically affected by changes in dietary fat (Spector et al., 1979).

Evidence suggests that increasing the unsaturated fatty acyl composition increases the "fluidity" of the membrane. Conversely increasing the saturated fatty acid content decreases membrane fluidity (Stryer, 1988). Some membrane enzymes appear to be sensitive to these changes in the microviscosity of the membrane lipids. Of special interest is the demonstration that the activity of a key enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), can be depressed when human fibroblast microsomes are enriched with saturated phosphatidylcholine (PC) and reactivated by unsaturated PC (Davis and Poznansky, 1987; George et al., 1990). A strong correlation between fibroblast microsome HMG-CoA activity and microsomal membrane ESR order parameters was observed suggesting that the impact of unsaturated PC on the enzyme was mediated by changes in fluidity. However, HMG-CoA reductase in rat liver microsomes does not appear to be affected by differences in membrane fluidity (George et al., 1990; Writz, 1990). Cholesterol biosynthesis is a critical function in liver but may not be so in non-hepatic tissue and this may be related to the different regulation of the same enzyme in different cell types (George et al., 1990). ACAT is also a key enzyme in cholesterol metabolism and has been suggested to be sensitive to the physical properties of the endoplasmic

reticulum (Mathur et al., 1982; Field and Salome, 1982). Norum et al. (1977) demonstrated that dietary cholesterol and/or fat could affect intestinal acyl CoA:cholesterol acyl transferase and Spector et al. (1980) showed that dietary polyunsaturated fat altered rat liver membrane fatty acids and stimulated ACAT activity more so than did saturated fat. Mathur et al. (1982) have shown that microsomes enriched with dipalmitoyl phosphatidylcholine exhibited 30-45% less ACAT activity as compared to microsomes enriched with dioleoyl phosphatidylcholine. The changes in ACAT activities with fatty acid enrichment were shown to parallel small changes in membrane fluidity as measured by ESR (Mathur et al., 1982). Based upon the dietary studies with rat liver microsomes, it has been suggested that dietary fatty acid enrichment effects on ACAT activity, might be mediated through changes in membrane fluidity (Spector et al., 1980).

The fluidity of biological membranes has been previously shown to be dependent on the composition of its phospholipids and their fatty acyl chains. Studies by Borell and others using differential scanning calorimetry have shown a correlation between membrane fluidity and fatty acid composition of phosphatidylcholine within a membrane (Borell, personal communication; Davis et al., 1980; Coolbear et al., 1983). Increased fluidity was observed with polyunsaturated fatty acids that possessed multiple double bonds compared with monosaturated fatty acids and polyunsaturated fatty acids with

1 or 2 double bonds, respectively.

4.1.4.2 Substrate Specificity

There have been several reports of changes in ACAT activity in the liver when the fatty acid composition is altered by enrichment of cells or microsomes (Field, 1982; Rustan, 1988). The molecular basis for decreased cholesterol esterification in cells modified with ω-3 PUFAs may be explained by substrate specificity. Drevon and coworkers suggested that the inhibition of ACAT in cultured parenchymal cells enriched with eicosapentanenoic acid is due to the ability of eicosapentenanoyl CoA to compete with other acyl CoAs for ACAT (Rustan et al., 1988). A high number of double bonds in ω-3 PUFAs may make the molecules more rigid than ω-6 PUFAs thereby reducing their access to the active site of ACAT (Rustan et al., 1988). However, it is unlikely that lower rates of cholesteryl esterification are due to the inability of ACAT to utilize ω-3 PUFAs as a substrate. It has been reported by others that dietary ω-3 fatty acids are incorporated into cholesteryl esters (Dyerberg, 1986). Therefore, if direct competition for binding of acyl CoAs is involved, the binding of EPA would have to follow a course of a competitive inhibitor and this appears unlikely. Drevon and coworkers have also suggested that these ω-3 PUFAs may interact with the active site of ACAT as well as if not better than other fatty acyl CoAs but are not metabolized as rapidly

as other fatty acyl CoAs. The conversion of radiolabelled oleoyl CoA to cholesteryl oleate would therefore be blocked by the presence of eicosapentaenoyl CoA and docosapentaenoyl CoA binding up the ACAT active site. This may be the mechanism of action of ω-3 PUFAs on ACAT activity.

4.1.4.3 Other Possible Mechanisms

The physical state of cholesteryl esters in foam cells could possibly influence the rate of hydrolysis and esterification and thus may be an important parameter in influencing the deposition and/or clearance of cholesteryl esters in atherosclerotic plaque (Snow, 1988). Examination of atherosclerotic lesions under polarizing light microscopy has suggested that lipids may be stored in either an ordered or disordered state. It appears that cholesteryl esters in an ordered state may be hydrolyzed more slowly than those in disordered liquid droplets. It is hypothesized therefore, that the physical state of lipid in atherosclerotic plaques may affect the flux of cholesterol between plaque and plasma. Although the mechanism remains to be understood, cholesterol esters comprised of ω-3 fatty acid may modulate ACAT activity by factors related to the physical state of the cholesterol ester droplets. It would follow from this that cholesteryl esters such as cholesteryl docosapentaenote and cholesteryl eicosapentaenote would be present in the cell in a more disordered state allowing a more rapid hydrolysis of these

particular esters. This may explain lower amounts of cholesteryl esters present in cells enriched in ω-3 PUFA. However, there is no evidence in the literature that suggest that cholesteryl esters comprised of ω-3 PUFAs are present in a disordered state nor that such esters modulate ACAT or cholesteryl ester hydrolase (CEH) activity.

Cytoplasmic cholesterol ester hydrolase activity may be increased as a result of ω-3 PUFA enrichment compared with ω-6 PUFA enrichment of human fibroblasts and therefore may explain the decreased cholesteryl ester mass in ω-3 PUFA enriched cells. However, this seems unlikely for a number of reasons. Even though we have not actually measured CEH activity, cytoplasmic cholesteryl ester hydrolase, unlike ACAT does not appear to be under the influence of strict metabolic regulation (Brown et al., 1980). Evidence suggests that there is a continous cycle of esterification and hydrolysis by ACAT and CEH respectively. However, it appears that if cells are deprived or loaded with cholesterol they respond appropriately by only stimulating or inhibiting ACAT. According to this theory then CEH may not be affected by membrane phospholipid composition. However, it is not entirely impossible to conceive that CEH activity might be modulated by the composition of the cholesteryl esters present in the cytoplasmic droplets of the cell. Cholesteryl esters such as cholesteryl eicosapentaenoate and cholesteryl docosapentaenoate may be more preferred substrates for CEH, therefore reducing

the size of the CE pool. Evidence has suggested that the CEH activity in aortas isolated form monkeys fed fish oil supplemented diets was reduced compared with the CEH activity in aortas isolated from monkeys fed vegetable oil diets (Davis et al., 1987). However, these results are very difficult to interpret since whole tissues were used in the measurements of CEH activity and substrate levels were not measured.

ACAT activity measured in microsomes isolated from fatty acid enriched cells most likely eliminates the interference of cholesteryl ester hydrolase located in the cytoplasm of the cell. The whole cell experiments show a similar reduction in ACAT activity upon ω-3 PUFA enrichment compared with the microsome experiments. Therefore, it is unlikely that CEH has any contribution to the results found in whole cell experiments. However, we cannot eliminate CEH interference since we have not measured its presence in either of the above experiments.

The regulation of ACAT may be mediated by the incorporation of ω-3 and ω-6 PUFAs preferentially into different phospholipid species in cellular membranes. It appears that the majority of the ω-3 fatty acids such as eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid are preferentially incorporated into phosphotidylethanolamine (PE) fraction of the phospholipid bilayer whereas ω-6 PUFAs such as linoleic acid was present in similar amounts in both phosphatidylcholine fractions and

phosphatidylethanolamine (Table 3). This trend where ω-3 PUFAs are preferentially incorporated into certain phospholipid fractions has also been observed in other studies (Murthy et al., 1988; Nordoy et al., 1985). Since ACAT is localized in the microsomal membrane, the type of fatty acid enrichment may change the microenvironment of ACAT.

In intact cells, the outer surface of the membrane is characterized by the presence of neutral phospholipids such as phosphatidylcholine and sphingomyelin whereas the inner leaflet of the bilayer contains phosphatidylethanolamine and other negatively charged phospholipids (Vance and Vance, 1985). ω-3 PUFAs preferentially incorporated into PE in the inner leaflet of the bilayer may change the microenvironment of the bilayer differently than fatty acids that are not incorporated into the PE fraction. These differences in the relative distribution between the two fatty acids may play a role in ACAT regulation. However, whether such changes in microenvironment of ACAT will affect its activity is only speculation.

The microenvironment of ACAT may also be influenced by the type of fatty acid incorporated in the phospholipid fraction and not by their position in the PL membrane. Fatty acids that vary in chain length, number and positions of their double bond may possibly affect the microenvironment of ACAT and hence possibly its activity.

Cell proliferation and growth have been shown to be

adversely affected by modification with unsaturated fatty acids (Spector et al., 1981). However, cell viability, cell number and total protein were similar in cells enriched with either eicosapentanenoic acid of linoleic acid (section 3.1 and 3.2.2). Differences in the rates of esterification between the two cell populations therefore are not related to the adverse effects of PUFA enrichment.

4.2 Cholesterol Efflux

4.2.1 The Effect of Fatty Acid Enrichment of Human Fibroblast on Reverse Cholesterol Transport

Maintenance of cellular cholesterol homeostasis is undoubtedly the result of a balance between two opposing mechanisms. LDL and endogenous cholesterol synthesis contribute to the intracellular pool of cholesterol whereas HDL functions to remove cholesterol (Steinberg, 1988). One of the key factors in protecting against the development of atherosclerotic lesions is the ability of HDL to promote removal of excess cholesterol from peripheral tissues, including those of the vasculature (Glomset, 1968). In humans, HDL appears to mediate the process of "reverse cholesterol transport", a process where excess cholesterol is transported to the liver for catabolism and excretion. The factors that regulate or influence the process of reverse cholesterol transport are unclear. Reduction in the rate of cholesterol esterification in cells has been reported to

enhance HDL-mediated cholesterol efflux from mouse peritoneal macrophages (Schmitz et al., 1985). The movement of cholesterol from the cholesterol ester pool to the plasma membrane may also influence the rate of cholesterol eflux (Daniels et al., 1981). Others have proposed that the rate limiting step in reverse cholesterol transport is the movement of sterol from the plasma membrane to the HDL particle (Johnson et al., 1990). Cholesterol efflux has also been shown to be dependent on the interaction of cholesterol with membrane phospholipids (Davis, 1984; Gold and Phillips, 1990). Therefore, an objective of this study was to investigate the effects of changes in phospholipid fatty acid composition in fibroblasts on cholesterol efflux.

Figure 14a illustrates that the efflux of ^{3}H-cholesterol from cells to HDL is significantly faster in cells enriched with ω-3 PUFAs compared with those enriched with ω-6 PUFAs (T 1/2 was 16.5 ± 4.5 and 33.1 ± 9.2 respectively, $p<0.005$). These experiments clearly show that the clearance of cholesterol from cells does not occur in the absence of HDL. Incubation of fibroblasts with MEM +5% LPDS did not stimulate cholesterol efflux (Figure 14a). To ensure that the differences in the rate of HDL mediated efflux of cholesterol from cells enriched with either linoleic acid or eicosapentanenoic acid were due to net efflux rather than to differences in cholesterol exchange rates between the cells and HDL, the mass of cholesterol transferred from the cells to

medium was also determined. Figure 16 illustrates that the net transfer of cholesterol mass from cells to medium is greater when cells were enriched with ω-3 PUFAs (57% in 24 hours) compared with ω-6 PUFA enriched cells (37% in 24 hours). The radiolabelled cholesterol disappearing from the cells in the presence of HDL was seen to reappear as cholesterol in the medium (Figure 16). From Figure 14a, the percentage of radiolabelled cholesterol present in the cells after incubation with HDL at 24 hours was approximately 37% and 60% from cells enriched with docosapentaenoic acid and linoleic acid, respectively. Mass measurements show a good correlation between release of label and net movement of cholesterol mass in cells enriched with either ω-3 PUFAs or ω-6 PUFAs. Therefore, it is reasonable to assume that enrichment of fibroblasts with ω-3 PUFAs results in an increase in "net" efflux of cholesterol.

4.2.2 Molecular Mechanisms

The molecular basis for differences in cholesterol efflux rates with cells enriched with linoleic acid or docasapentanenoic acid is unclear. Earlier observations (Figure 5) indicated an inhibition of cholesterol esterification resulted from ω-3 PUFA enrichment of human fibroblasts. Inhibition of cholesterol esterification would be expected to result in increased unesterified cholesterol content within the cell. However, Figure 1 clearly indicated

the absence of any change in the unesterified cholesterol content with either ω-6 or ω-3 PUFA enrichment. The absence of any change in the unesterified cholesterol pool may be a reflection of cells inability to tolerate changes in unesterified cholesterol to keep its membrane cholesterol:phospholipid ratio constant (Sabine, personal communication). Therefore, if the cells cannot esterify cholesterol, then the excess unesterified cholesterol has to be removed from them. The inhibition of cholesterol esterification by ω-3 PUFA enrichment could promote increased cholesterol efflux in the presence of a suitable acceptor (HDL) and thus maintain intracellular free cholesterol homeostasis, possibly by a simple mass action effects.

Enhanced efflux of cholesterol has been observed in mouse peritoneal macrophages treated with Sandoz 58-035, a specific inhibitor of ACAT (Bernard et al., 1990; Schmitz et al., 1985). Again no increase in cellular free cholesterol mass was observed when cells were incubated with HDL plus 58-035 or with HDL alone. ACAT inhibition in ω-3 PUFA enriched fibroblasts (see sections 3.2.3 and 3.4.2) could be responsible for enhanced cholesterol efflux to HDL. Therefore, it would appear that the clearance of unesterified cholesterol from cells by HDL may be linked to or dependent on ACAT activity.

The rate of efflux may be influenced by the translocation of cholesterol to the plasma membrane. Binding

of HDL to its receptor on cultured fibroblasts and aortic endothelial cells was previously shown to facilitate cholesterol efflux by the initiation of translocation of intracellular cholesterol to the plasma membrane (Aviram et al., 1989; Slotte et al., 1987). It appears that the transport of intracellular cholesterol to the plasma membrane is stimulated by the binding of HDL to its receptor. However, HDL binding does not appear to be required for the transport of cholesterol from the plasma membrane. These observations suggest a unique role of HDL in reverse cholesterol transport, since the enhancement of cholesterol translocation of cholesterol to the cell surface might enhance the ability of HDL to remove cholesterol from the internal pool(s) of cholesterol that are involved in the formation of cholesteryl esters. It is unclear how ω-3 PUFA enrichment of human fibroblasts may influence this process of cholesterol translocation. An enhanced efflux in these cells could reflect increased movement of lysosomal cholesterol to the plasma membrane. However, it should also be noted that neither inhibition of ACAT activity in Fu5AH cells by Sandoz 58-035 nor stimulation of ACAT activity by oleic acid supplemented medium increased the translocation of lysosomal cholesterol to the plasma membrane (Johnson et al., 1990). The lack of effect of increased or decreased ACAT activity on cholesterol efflux could imply that the pathway for the transport of lysosomal cholesterol to the plasma membrane may

not involve the passage of cholesterol through the rough endoplasmic reticulum, the subcellular location of ACAT (Johnson et al., 1990).

It has been suggested that different lipids interact differently with cholesterol and that such interactions influence both cholesterol exchange and net efflux of cholesterol (Davis et al., 1984). These authors reported that cholesterol exchange was much faster in Acholeplasma laidawii cells compared with Mycoplasma gallisepticum cells, suggesting that higher cholesterol exchange could be associated with a lower cholesterol to phospholipid ratio in A. laidawii cells. An enhanced efflux of cholesterol has also been shown for mycoplasma membranes that were enriched with 1-oleoyl-2-palmitoyl phosphatidyl compared with mycoplasma membranes enriched with dipalmitoylphosphatidylcholine (Rottem and Davis, 1986). It was suggested that the differences in the rate of cholesterol movement from these cells enriched with two different fatty acids could be associated with changes in the physical interaction between cholesterol and the different fatty acid phospholipids.

It is possible that alterations of the lipid structure of the fibroblast membranes may affect cholesterol efflux. Bellini (1984) demonstrated that the halftimes (t 1/2) for unesterified cholesterol efflux from Fu,AH rat hepatoma and WIRL-3C rat liver cells were 3.2 $\pm$ 0.6 and 14.3 $\pm$ 1.5 h, respectively. The same halftimes were observed for

plasma membrane vesicles isolated from the cells. He suggested that the differences in the structures of the plasma membranes of the two cell types accounted for the differences in cholesterol efflux from the cells. He suggested that local domain structures influenced by membrane proteins or the location, orientation and molecular motion of cholesterol molecules in different biological membranes may account for the different t 1/2 values. ω-3 and ω-6 PUFA enrichment of human fibroblasts may alter membrane structures such that cholesterol may desorb at different rates from various plasma membrane regions (Bellini et al., 1984).

From studies of model and cell membrane systems, the rate of cholesterol exchange or transfer is known to be sensitive to the degree of unsaturation of the phospholipid fatty acyl chains (Poznansky and Lange, 1975). Poznansky (1978) found that exchange of cholesterol between vesicles containing unsaturated fatty acids and red cell ghosts was much faster than exchange of cholesterol from vesicles containing saturated fatty acids. The exchange from dimyristoylphosphatidylcholine:cholesterol vesicles was the slowest, followed by dipalmitoylphosphatidylcholine and distearoyl phosphatidylcholine vesicles. There was a distinct correlation between chain length and exchange rates. Therefore, the nature of the fatty acid chains with respect to chain length and degree of unsaturation may be responsible for the different efflux rates observed in cells enriched with

either ω-3 or ω-6 PUFAs.

Recently Phillips (1990) has studied the effects of plasma membrane structure of red blood cells on cellular cholesterol efflux (Gold and Phillips, 1990). Results suggested that increasing the membrane spingomyelin (SM) to egg phosphatidylcholine (PC) ratio raised the t 1/2 for cholesterol exchange in both red blood cell plasma membrane and in simple SM/PC bilayers. The effect of PUFA enrichment of human fibroblasts on SM:PC ratios in the membrane was not studied and therefore, it was not possible to determine if increased SM:PC ratios in ω-3 PUFA enriched cells were responsible for enhanced efflux in these cells. However, a recent study by Schmitz (1990) indicated that ACAT inhibitors such as octimibate upregulated HDL binding and enhanced PC and SM synthesis in macrophages. He proposed a model for the role of phospholipids in intracellular cholesterol trafficking. For normal macrophages it was proposed that the availability of phospholipid at the site of cholesterol release from lysosomes critically determines whether cholesterol is resecreted or accumulated in cholesteryl ester containing lipid droplets. If SM synthesis is high, cholesterol is released from the lysosome, incorporated into lamellar bodies, which are then directed to the plasma membrane and can be released into the surrounding medium. It was suggested that the rate limiting step in the formation of cholesterol containing particles destined for secretion may be the level

of sphingomyelin biosynthesis. If inhibition of ACAT by octimibate increases sphingomyelin synthesis and hence intracellular trafficking of cholesterol then it may be possible that inhibition of ACAT by ω-3 PUFA enrichment may follow the same path and therefore lead to enhanced efflux.

4.3 Conclusion

Atherosclerosis is characterized by the deposition of lipid, primarily cholesterol and esterified cholesterol in the cells of the vascular initima (smooth muscle cells, monocyte macrophages and endothelial cells). The cholesteryl esters are stored as droplets in the cytoplasm of these cells and give rise to "foam cells", the major structural component of early atherosclerotic lesions (Brown and Goldstein, 1983). Recent investigations have suggested that foam cells originate largely from macrophages (Jerome and Lewis, 1987; Lewis et al., 1988). A model system has been proposed that suggests how the major histologic and biochemical features of foam cells can be reproduced in vitro and this development has led to the study of the metabolism of cytoplasmic cholesteryl ester droplets in macrophages (Brown and Goldstein, 1980). A morphologic change in macrophages has been associated with the fall of cholesteryl ester content within these cells as determined by mass measurement. Thus, it may be possible to inhibit the formation of foam cells if cholesterol esterification is reduced. Therefore, factors that can

regulate the esterification of cholesterol may be beneficial in delaying foam cell formation and hence the process of atherogenesis.

Polyunsaturated fatty acids in the diet have long been considered essential to the growth and proper nutrition of humans and animals (Lands, 1986). In recent years however, these fatty acids have been implicated in the prevention of atherogenesis (Goodnight et al., 1982). The plasma cholesterol lowering effects of dietary polyunsaturated fat has been demonstrated by many investigators (Connor et al., 1969; Grundy and Ahrens, 1970; Jackson, 1978; McNamara, 1987; Nestel, 1987). Gram for gram, saturated fat is twice as effective in raising the plasma cholesterol levels as polyunsaturated fat is in lowering it (Goodnight et al., 1982). As mentioned previously, the fascinating study done by Bang and Dyerberg (1972) linked the highly polyunsaturated fatty acids present in marine fish oils (ω-3 PUFAs) to low mortality from cardiovascular disease among Greenland Eskimos. This finding has stimulated further work to explore the mechanisms that might be involved in this relationship. Most of these studies focused on the effect of ω-3 PUFAs on plasma lipoprotein levels. Recently, however, there seems to be a great deal of uncertainty and inconsistancy in the literature on the effect of ω-3 PUFAs on plasma LDL and HDL levels (Goodnight et al., 1982; Harris, 1989, Nestel, 1990). On the other hand, VLDL levels have been shown to be reduced

significantly in animals fed fish oils (Goodnight,1982; Wong, 1985). Unfortunately, little is known about the influence of dietary ω-3 PUFAs on cholesterol absorption but cholesterol synthesis has been shown to be depressed in the intestine and in the liver microsomes isolated from rabbits (Field et al., 1987). Studies have shown that cholesterol esterification is reduced in human CaCO-2 cells, rat hepatocytes and rat liver microsomes when enriched with ω-3 PUFAs (Murthy et al., 1988; Rustan, 1988). However, there have also been other studies reporting that cholesterol esterification by ACAT is increased in rabbit liver and intestine when they were fed fish oil supplemented diets (Field et al., 1987). The effect of ω-3 PUFAs on ACAT activity in the liver may not be the same as their effects on nonhepatic tissues because unlike other tissues, the liver has a unique role in the synthesis and secretion of lipoproteins (Stryer, 1988). My results suggest that ω-3 PUFA enrichment of human fibroblasts and macrophages decreased cholesteryl esterification probably through inhibition of ACAT. If these findings actually represent the effect of ω-3 PUFAs on those cells in the arterial wall, then it is possible that dietary ω-3 PUFAs may be beneficial in delaying the process of atherosclerosis by inhibiting cholesterol esterification. Inhibiting cholesterol esterification may decrease lipid deposition and consequently plaque formation, fatty lesions and eventually reduce the atherogenic process.

ω-3 PUFAs may enhance the clearance of cholesterol from the arterial wall to further inhibit its deposition. The increased clearance of cholesterol in the presence of ω-3 PUFAs compared with ω-6 PUFAs (see section 4.2) seen in the human fibroblast system may also occur in arterial smooth muscle cells and macrophages. The cellular mechanisms involved in this clearance of cholesterol are not known but may be directly related to the inhibition of cholesterol esterification by ω-3 PUFA enrichment.

The study of the mechanisms and the regulation of cholesterol esterification and clearance in fibroblasts by ω-3 PUFAs could yield fundamental information regarding the process of atherogenesis and regression in the vascular intima. My research has focused on understanding the factors that may affect the initial step of atherosclerosis rather than understanding their effects on a well developed disease state. Controlling the regulation of cholesterol in the initial stages when it enters the arterial wall may be easier and subsequently more beneficial in the prevention of atheroma than trying to control cholesterol regulation once the disease has been well established. My findings suggest a possible mechanism of how ω-3 PUFAs may confer a protective effect against atherosclerosis and suggest a possible therapeutic role of dietary ω-3 PUFAs in the inhibition of atherogenesis and/or the accleration of regression of well developed plaque in humans.

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