EFFECTS OF HYPOLIPIDEMIC DRUGS ON CELLULAR CHOLESTEROL HOMEOSTASIS IN FAMILIAL HYPERCHOLESTEROLEMIA

BY

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TABLE OF CONTENTS

LIST OF FIGURES

Figure 13. Effect of Preincubation with LDL4 (Density 1.020-1.040 gm/cc) and LDL6 (Density 1.040-1.060 gm/cc) on Rates of Cholesterol Esterification by Cultured Human Fibroblasts

LIST OF TABLES

LIST OF TABLES

LIST OF ABBREVIATIONS

apo- apolipoprotein

FCR Fractional catabolic rate

FH Familial Hypercholesterolemia. This is a specific variety of type IIa hyperlipidemia which is transmitted in an autosomal dominant fashion. The patients studied in this thesis were all heterozygous for this condition.

HDL High density lipoprotein - Those lipoproteins isolated in the density range 1.060-1.21 g/cc

HDLc An abnormal cholesterol rich HDL present after cholesterol feeding

HMG-CoA reductase - 3-hydroxy-3-methyl glutaryl Coenzyme A reductase. This is the rate limiting enzyme in cholesterol synthesis. The direct end product of the enzyme is mevalonic acid.

IDL Intermediate density lipoprotein - Those lipoproteins isolated in the density range 1.006-1.019 g/cc

LDL Low density lipoproteins - A series of related lipoprotein particles isolated in the density range of 1.020-1.060 g/cc

LDL4 A lighter subfraction of LDL isolated in the density range of 1.020-1.040 g/cc

LDL6 A heavier subfraction of LDL isolated in the density range 1.040-1.060 g/cc

LPDS Lipoprotein depleted plasma

Specific degradation - The difference between the total amount of ^{125}I-LDL degraded at a given LDL concentration and the amount of ^{125}I-LDL degraded in the presence of excess unlabeled LDL.

Type IIa hypercholesterolemia - A phenotypic category of hyperlipidemia characterized by elevated plasma cholesterol levels with normal triglyceride levels. It is usually produced by elevation in the plasma LDL levels with VLDL and HDL remaining at normal levels.

VLDL	Very low density lipoprotein - Those lipoprotein particles isolated within the range 1.006-1.019 g/cc.

AN ABSTRACT OF THE THESIS OF

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Title: Effects of Hypolipidemic Drugs on Cellular Cholesterol Homeostasis in Familial Hypercholesterolemia

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Treatment of hypercholesterolemia with bile acid sequestrants has been shown to decrease levels of plasma low density lipoprotein (LDL) and to decrease rates of coronary mortality.

Measurements of cholesterol homeostasis in freshly isolated human mononuclear leukocytes showed an 80.5% increase in rates of LDL degradation and a 64.2% increase in rates of synthesis of cholesterol from acetate in response to colestipol treatment of the donor. Niacin or combined niacin/colestipol therapy yielded no significant changes in rates of cholesterol synthesis from acetate or degradation of LDL by freshly isolated human mononuclear cells.

To assess the effects of colestipol on the composition of LDL subfractions and their binding to normal fibroblast LDL receptors, 8 patients with FH were studied before and after stabilizing on 5-10g colestipol twice daily. LDL is not a homogeneous particle. Different subfractions of LDL have been shown to be metabolized at different rates. In both phases binding and composition studies were done using LDL fractions isolated in the density ranges 1.020-1.040 g/cc (LDL4) and 1.040-1.060 g/cc (LDL6). LDL4 free cholesterol (FC) content decreased 11.6% ($p<0.04$) and triglyceride (TG) content increased 49.8% ($p<0.04$). Concomitantly there were slight increases in the content of protein and phospholipid (PL), decreases in the ratios of FC/PL ($p<0.04$) and FC/cholesterol ester (CE) ($p<0.04$). LDL6 exhibited a 10.4% drop in FC ($p<0.05$) and decreases in the FC/PL ratio ($p<0.011$) and FC/CE ($p<0.05$). Neither fraction demonstrated a change in the distribution of phospholipid species. The composition changes were reflected in an increase in the LDL4 dissociation constant (Kd) from 0.6 x 10-5 ng/ml during the diet therapy phase to 2.2 x 10-5 ng/ml ($p<.01$) following treatment with colestipol. There was no change in the Kd of LDL6. There were no changes in the ability of the LDL fractions to suppress cholesterol synthesis or stimulate cholesterol esterification after incubation with normal human fibroblasts.

INTRODUCTION

Atherosclerotic heart disease is a problem of tremendous proportions. It is the major cause of disability and death in the western world[1]. It is a disease process which becomes symptomatic in the prime of life with great impact on the social and economic well-being of the individual and the family. The direct health care costs and lost wages due to coronary heart disease morbidity in the United States exceeds $60 billion a year. This is an estimate of only the direct cost of the medical payments, and time lost from work. It does not account for stresses upon the individual and family, nor does it address long term medication costs.

Several predisposing "risk factors" have been shown to be associated with the development of atherosclerosis and many genetic and environmental factors may contribute to its progression. Excess dietary intake of cholesterol and saturated fats, smoking, diabetes, hypertension and obesity are the major environmental or cultural factors thus far identified. Genetic factors relate to both the presence of one of a number of primary disorders of lipid metabolism (the dyslipoproteinemias)[2] as well as a positive family history for premature atherosclerosis, which is also an independent risk factor,even in the absence of an identified dyslipoproteinemia.

One of the more common and best understood inherited disorders of metabolism is familial hypercholesterolemia (FH). Familial hypercholesterolemia is an autosomal dominantly inherited disorder of lipid metabolism which occurs in its heterozygous form with an incidence of 1/500 in North America[3,4]. Thus, FH is a relatively common cause of severe hypercholesterolemia, and the only one which shows full phenotypic expression in childhood[5]. Homozygosity is much less common, with an incidence of about 1/1,000,000.

CHOLESTEROL AND LIPOPROTEIN METABOLISM

Cholesterol is an essential component of animal cell membranes, a precursor for steroid hormones, and a precursor of vitamin D. It may be derived either from the diet or from de novo synthesis. In animals fed a cholesterol-free diet, hepatic cholesterol synthesis increases, and together with cholesterol produced by the intestine and other tissues de-novo synthesis is able to provide enough cholesterol for the entire body. While almost all tissues can synthesize cholesterol, they produce only small amounts compared to the liver and, presumably because of hepatic production of large amounts of cholesterol, show minimal changes in synthetic rate with alterations in dietary cholesterol intake.

Regardless of the site of metabolic origin, cholesterol is transported from the liver or intestine to peripheral tissues as a component of lipoproteins. The major transport

lipoproteins in man are chylomicrons, VLDL and LDL whose major structural protein is apolipoprotein B (apoB). The major sources of cholesterol, the liver and the intestine, are the sole sites for apoB synthesis. A schematic representation of lipoprotein metabolism is shown in Figure 1.

Dietary fats and cholesterol are taken up from the gut lumen into enterocytes and packaged into large triglyceride-rich particles called chylomicrons. These particles contain one form of apoB called B48. These nascent chylomicrons enter the lacteals of the gut wall, and make their way through the lymphatic drainage to enter the circulatory system at the superior vena cava. Once in the circulation, these particles begin to undergo metabolism. In the blood-stream, they acquire surface apoC and apoE by exchange from HDL. One of the C apoproteins (apo CII) is a cofactor for the action of lipoprotein lipase on triglycerides contained within the chylomicron.

Apoprotein E acts as the recognition protein for the hepatic uptake of the chylomicron remnant particles by a specific receptor. Intravascular catabolism of chylomicrons with loss of triglyceride to peripheral cells and to storage in adipocytes leads to the production of chylomicron remnants which are then taken up by the liver; thus dietary and reabsorbed biliary cholesterol, initially incorporated into chylomicrons, are ultimately taken up by the liver.

The liver repackages this dietary cholesterol and fat along with endogenously derived cholesterol into VLDL. VLDL contains an apoB subspecies known as apoB100 as its intrinsic structural protein, as well as extrinsic apoC and apoE when initially secreted by the liver. Intravascular catabolism of VLDL by lipoprotein lipase with loss of triglyceride and some of the apoC[6] leads to the production of IDL and eventually, with further loss of triglyceride and apoproteins C and E, to LDL. LDL is the metabolic endproduct of this cascade and has a longer residence time in the circulation than VLDL. The LDL is then taken up by cells via specific receptor mediated endocytosis. Several tissue culture studies have documented and characterized the cell surface receptors for apoB which mediate LDL uptake[8,14,15,18].

CELLULAR CHOLESTEROL AND LDL METABOLISM

The first step in the cellular metabolism of LDL involves the binding of the lipoprotein particle to a specific receptor on the surface of the cell membrane. This receptor is of the classic type, being highly specific, exhibiting high affinity, and being saturable.

Several studies have characterized the LDL receptor molecule and its synthesis. It is an acidic glycoprotein with a molecular weight of 164,000 daltons and an isoelectric point of pH 4.6[7]. It is initially synthesized

as a 120,000 dalton protein which gains an additional 40,000 daltons of carbohydrate during post-translational modification in the Golgi apparatus. This increase in molecular weight is not solely due to carbohydrate addition[8]. Each receptor can bind four particles of LDL (Kd 3 nM). There is no evidence for cooperativity among the binding sites[9]. Only one class of binding site is implied by these studies since the deactivation of the binding sites in response to radiation damage is linear[10]. The LDL receptor is also able to bind apoE-rich lipoproteins such as ß-VLDL (a cholesterol and apoE-rich lipoprotein synthesized in response to cholesterol feeding), IDL, and HDLc (an apoE rich particle synthesized during cholesterol feeding).

There are reports which suggest tissue variability in the LDL receptor. Studies of a human hepatic LDL receptor[11] have shown differences in the Kd (11 nM vs 3 nM), relative EDTA insensitivity, temperature sensitivity, and size (270,000-330,000 kilodaltons vs 130,00 kilodaltons) compared to the fibroblast receptor. This hepatic receptor was also demonstrated in a patient lacking the fibroblast receptor. This would indicate that there may be an expressed hepatic receptor for LDL which is distinct on both a biochemical and genetic basis from the classic fibroblast receptor. The physiologic significance of this receptor is unclear as most FH patients who lack functional fibroblast LDL receptor also seem to lack the hepatic receptor.

The size of the receptor, and thus the length of the DNA needed to code the protein, combined with the presence of many Alu sequences in both introns and exons[12], make the receptor protein susceptible to mutation through insertions and deletions at these sites, as well as allowing potential duplication of all or part of the gene in another region. In addition to the binding site for LDL, there are at least two other functional regions of the protein. The first is a membrane specific portion which is responsible for retaining the receptor on the cell surface, rather than having it secreted[12], and the other which is responsible for the localization of the receptor into specialized areas of the cell surface called coated pits[13]. Residence of the LDL receptor within the coated pit is required for the receptor-LDL complex to be internalized. This has been documented by the identification of a group of patients whose cells can bind LDL with a normal affinity, but in which the receptors are distributed randomly across the cell surface rather than in coated pits. In this instance LDL binds to the receptors normally, but cannot be internalized[14,15]. In at least one patient this is due to a mutation which truncates the cytoplasmic domain of the receptor[13].

After LDL binds to the receptor in the coated pit, the complex is internalized via endocytosis, becoming part of a coated vesicle. Internalization of the coated pit is dependent on a normal intracellular concentration of potassium and does not occur following the depletion of

intracellular potassium pools[16]. Following internalization, the LDL is dissociated from its receptor and the coated vesicle loses its coating of clathrins which are recycled. Dissociation of the receptor-LDL complex and release of the receptor from the endosome occurs before fusion of the endocytic vesicle with the lysosome[17]. The internalized LDL particle maintains its integrity until the endocytic vesicle fuses with a lysosome[18,19]. The LDL receptor is recycled to the cell surface[20] by pathways which are not yet clear, but are thought to involve at least two distinct types of vesicles[21].

Once in the lysosome, enzymes hydrolyze the LDL apoprotein to constituent amino acids and oligopeptides[22] releasing the cholesterol ester and triglyceride contained in the core. Cholesterol ester is broken down by a lysosomal acid hydrolase allowing the free cholesterol to enter the cytosol and become available for metabolism [18,19,23]. The surface phospholipids are acted upon by phospholipases. Instead of being broken down, portions of the phospholipid may be used in cellular membrane synthesis. Triglyceride is degraded by triglyceride lipases which release the fatty acids for use by the cell. The free fatty acids are rapidly metabolized, either being used for energy production, or reesterified into cholesterol ester, triglyceride, or phospholipid. Not all of the LDL which is internalized ends up being degraded. Under certain circumstances, the intracellular processing may get

prematurely terminated, and the partially processed LDL may be released (a process termed retroendocytosis) back into plasma[24]. The metabolic fate and significance of these altered LDL particles is unknown.

In all cells, the cholesterol incorporated via the LDL receptor pathway regulates at least two microsomal enzymes: it suppresses 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG CoA reductase) which decreases endogenous cholesterol synthesis[19,25] and it activates acyl Coenzyme A: cholesterol acyl transferase, thereby facilitating the storage of cholesterol in an ester form[26,27]. When a cell accumulates sufficient cholesterol for its needs, synthesis of the receptor is suppressed[28], and the number of cell surface receptors decreases. This in turn, decreases the cellular uptake of cholesterol and prevents the accumulation of excessive amounts of cholesterol in either the free or esterified form[28]. A segment of the LDL receptor gene containing a sterol responsive regulatory region containing both positive and negative transcriptional elements has recently been identified[29], thus explaining the direct end-product repression of LDL receptor synthesis which has been shown by many previous studies[22,23,25,28].

Cellular regulation of cholesterol synthesis has been extensively studied in both hepatocytes and fibroblasts. Hepatic cholesterol synthesis is regulated by dietary cholesterol through feedback inhibition of HMG CoA

reductase, an NADPH-dependent enzyme which irreversibly converts HMG CoA to mevalonic acid. There appear to be two mechanisms for regulation of the enzyme. In the rat, within 12 hours after cholesterol feeding, there is decreased enzyme activity without loss of immune reactive enzyme. This short term regulation of HMG CoA reductase activity is accomplished by phosphorylation to temporarily inactivate the enzyme, and dephosphorylation of the enzyme[30,31] to restore activity. After 24 hours of cholesterol feeding there is decreased immunochemically detectable enzyme, apparently because of decreased enzyme synthesis[32,33]. A sterol sensitive regulatory element has also been demonstrated in the HMG CoA reductase gene[34].

In a similar fashion, factors which increase cholesterol removal from the liver result in an increase in the rate of cholesterol synthesis. The removal of bile acids either through an external fistula, ileal bypass, or bile acid sequestrant treatment will increase bile acid synthesis[2,35], apparently through increased 7-alpha-hydroxylase activity[36]. This, in turn, causes a secondary increase in hepatic cholesterol synthesis[2,31,32]. The recently identified sterol responsive regulatory elements probably play a large role in the fine tuning of this regulation.

The concept that changes in cholesterol synthesis in the hepatocyte may be paralleled by similar changes in non-

hepatic cells has been addressed in several different model systems. Young and Rodwell[37] showed that factors which altered HMG CoA reductase activity in rat hepatocytes caused parallel changes in leukocytes. Increases in dietary cholesterol, which are known to affect hepatic cholesterol synthesis, have also been shown to decrease HMG CoA reductase and LDL receptor activity in freshly isolated mononuclear cells from normal subjects[38].

A well characterized example of a defect in the regulation of cholesterol synthesis in man is the disorder familial hypercholesterolemia (FH). As described by Brown and Goldstein in 1974[22], this disorder is caused by a series of defects in the LDL receptor gene which result in a partial or total loss of functional LDL receptors from the surface of the cell. The metabolic defect in FH is expressed in vitro by freshly removed tissues as well as by lymphoid cells and fibroblasts in culture. In normal mononuclear leukocytes as in the fibroblast, HMG CoA reductase is suppressed and cholesterol esterification is increased by LDL[39]. Cells isolated from FH homozygotes, as would be expected, do not respond this way to LDL. Ho and co-workers[40] have shown that these metabolic effects in cultured mononuclear cells are due to LDL binding to high affinity receptors. Observations that the receptors are missing from mononuclear cells isolated from FH homozygotes and decreased in cells isolated from FH heterozygotes have been reported by Fogelman, et al[41].

It has been demonstrated that there are differences in rates of cholesterol synthesis in freshly isolated mononuclear cells from patients receiving treatment for one of several forms of hyperlipidemia. McNamara and coworkers[42] showed that cholestyramine treatment increases rates of in vitro cholesterol synthesis while clofibrate treatment did not. Although these results are consistent with the data on the effects of cholestyramine and colestipol on whole body cholesterol synthesis, the patient population in this study was heterogeneous and had multiple etiologies responsible for their hypercholesterolemia.

These results are similar to those of Lees and Lees[43] and Sundberg and Illingworth[44] who showed increased rates of cholesterol synthesis and increased LDL receptor mediated degradation of ^{125}I-LDL in mononuclear cells freshly isolated from patients with well characterized heterozygous FH who were being treated with colestipol. These studies reinforce the view that human mononuclear cells provide an accessible cell line in which the influences of hyperlipidemic and hypolipidemic states on cholesterol homeostasis and LDL receptor activity may be examined in vitro.

LDL APO-B STRUCTURE AND FUNCTION

Apolipoprotein B subspecies (apoB48 and apoB100) are the major structural proteins of chylomicrons (apoB48), and

VLDL, and LDL (apoB100). They have a role which extends far beyond maintaining structural stability. ApoB100 also provides the recognition signal needed for receptor mediated uptake of LDL by cells. The two major forms known as B100 and B48 were identified originally as two different proteins which both reacted to a monoclonal antibody to apoB. Typical of most secreted proteins, apoB100 is highly glycosylated[45]. Classic structural studies have been difficult because of the large size (about 400 kilodaltons) and insolubility of the protein following delipidation. Recent investigations have utilized the techniques of molecular biology to derive a sequence for apoB100 and apoB48 and to allow probing of the chain and mapping of active sites and functional domains.

Mapping of both B100 and B48 has been done using several groups of monoclonal antibodies directed at apoB[46]. These studies have shown a linear array of binding sites which do not overlap. Some of these sites block the LDL binding site, but most do not. Those towards the N-terminus of B100 also cross react with B48, however those on the C-terminus do not[47].

Further mapping of peptides derived from the cleavage of apoB by thrombin or Staphylococcus aureus derived protease led to the isolation and sequencing of two 24 amino acid peptides[48]. This sequence information was used to generate cDNA probes used by Mehrabian, et al[49] who probed

both a human chromosomal library and HepG2 clones. This identified a 20 Kb mRNA in HepG2, liver, and gut cells, and a 9 Kb mRNA in gut cells. The derived protein sequence is not homologous to other known proteins or apoproteins and contains no amphipathic alpha helix. In-situ hybridization of cDNA probes for this gene[50] mapped it to $2p^{ter}$. Unlike the gene for apoE, the apoB gene is not linked to the LDL receptor or any of the other apoprotein loci located on chromosomes 1, 11 and 19. The gene is highly polymorphic[51,52] and exhibits restriction fragment length polymorphisms with many different enzymes.

Further studies by Huang et al., using a HepG2 derived library confirmed the size of hepatic apoB mRNA as 20-22 Kb[53]. They mapped their probe to the C terminus and 500 base pairs of 3' flanking sequences of the apoB-100 gene. Because their probe hybridized only to the 22 Kb hepatic mRNA and did not hybridize to gut mRNA this provided evidence that apoB-48 was not related to the C-terminus of apoB-100, but was probably related to the N-terminal sequence.

Two independent research groups have recently published the complete sequence for apoB-100 which was identified from overlapping cDNA clones[54,55]. They both describe a very large (4536-4563 amino acid residue) primary structure. Over half of the residues implied by the cDNA sequence have been confirmed by direct sequencing of tryptic peptides[53].

Structural studies of the protein sequence derived from the cDNA shows that the distribution of trypsin-accessible and inaccessible amino acids is non-random. It allows grouping into five structural regions: an N-terminal globular region; two lipid binding domains; the receptor recognition region; and the C-terminal. Twenty potential glycosylation sites have been identified: thirteen were shown by direct determination to have linked carbohydrate. There are 25 cysteine residues present in the entire molecule, but 12 are located in the first 500 amino acids adjacent to the N-terminal. In contrast, there is only one in the entire central 1500 amino acids of the molecule. Approximately 80% of the cysteine residues are shown to be involved in intramolecular disulfide linkages, though there is at least one free sulfhydryl residue[54].

Computer analysis of the sequence[54] shows a large number of long (70-320 residue) sequences with high degrees of internal homology and many short sequences of internal homology. This argues that much of the primary sequence may derive largely from internal duplications. There are no true amphipathic alpha helices, though there are many regions of alpha type structure. The lipid binding function provided by amphipathic alpha helices may be taken by many regions of ß-sheet which, in apoB, have the structure of $(\text{-hydrophobe-PRO-hydrophile-PRO-hydrophobe-})_n$ where n may be 14 or more[55]. These sequence findings are suggestive of a protein which winds in and out of the lipid micelle.

Supporting evidence for this winding intrinsic structure is provided by non-random distribution of trypsin sensitive regions of the protein, and a previous study[56] which showed only one molecule of apoB per lipoprotein particle.

The location of the putative LDL receptor binding domain has been implied from sequence homology to that of apoE[57] which can bind to the same cellular receptor. The location was confirmed by combining a synthetic peptide (containing the sequence of residues 3345-3381) with lipoprotein particles which had been treated with protease to remove the native binding site and showing that binding was restored[53].

The receptor binding of apoB is dependent on lysyl- and arginyl- residues present in apoB. Modification of these amino acids, whether the charge is eliminated or preserved, decreases or eliminates receptor-dependent uptake of the particle[58,59]. On the receptor side, the putative binding domain is made up of a 40 amino acid, cysteine-rich sequence which is repeated 7 times[60]. Deletion of one of these sequences has been shown to abolish the binding of LDL[61] but not the binding of apoE-containing lipoproteins which normally are completely cross-competitive, thus indicating that the affinity and specificity of the receptor is a result of a very complex interaction.

PATHOPHYSIOLOGY OF FAMILIAL HYPERCHOLESTEROLEMIA

Despite the fact that FH has been recognized as a clinical entity for over one hundred years, the connection between the cutaneous manifestations (xanthoma and xanthelasma) and the primary health problem (premature coronary artery disease), has only been recognized during the last 50 years [62,63,159]. The accelerated progression of coronary heart disease, which occurs along with myocardial infarction at a rate 8 to 25 times that in normolipidemic subjects [1,4,64], decreases the average life expectancy by 20 to 25 years.

The biochemical defect in FH involves a decrease in the relative number of high affinity receptors for low density lipoprotein (LDL) expressed on the surface of cells from affected individuals[65]. In persons heterozygous for the disorder there is a 50% reduction in the number of functional high affinity receptors for LDL. This loss of function may take the form of aberrant synthesis of the protein[61], as secretion of the receptor from the cell[66], or as a defect in the localization of the receptor into functional membrane domains[67].

In the rare individual who is homozygous for FH, there is a virtual absence of functional high affinity receptors for LDL expressed on the surface of cells. This is because of the inheritance of an abnormal gene from each parent. The incidence of homozygosity is estimated at 1 per million

in North America. Drug treatment is possible for patients with heterozygous FH, but the inherent lack of LDL receptors with the resultant severe hypercholesterolemia in homozygous FH, necessitates other forms of intervention including liver transplantation[68] and repetitive plasmapheresis.

The defect in FH has been demonstrated to be at the receptor level, rather than at another step in the metabolic pathway for cholesterol. In cultured fibroblasts from FH homozygotes who lack functional LDL receptors, the defect in regulation of cholesterol metabolism can be overcome if a non-lipoprotein form of cholesterol is used which allows cellular uptake without LDL receptor mediation[23,25,26,69]. If fibroblasts cultured from patients with homozygous FH are exposed to cholesterol dissolved in ethanol, the rate limiting enzyme in cholesterol synthesis, HMG CoA reductase is suppressed[26] as occurs in normal cells. Therefore, the intracellular mechanisms for metabolic regulation of cholesterol biosynthesis are available and functional if the sterol can be delivered into the cell.

There is substantial evidence to indicate that in FH there is heterogeneity at the gene level. A number of distinct abnormalities have been characterized in the synthesis and processing of the LDL receptor protein within the cell[8]. In most of the patients with homozygous FH there is no demonstrable high affinity LDL binding to their cells, yet in many there is immunologically reactive LDL receptor

protein on the surface of the cell. In some homozygotes, however, there is no demonstrable LDL receptor protein on the cell surface, either by LDL binding studies or by reaction with a monoclonal antibody prepared against purified LDL receptor protein[70].

There is also an "internalization defective" form of FH in which there is a normal amount of LDL binding to the surface of the cell. This bound LDL is not able to enter the cell normally, however, because the receptors are randomly located across the cell rather than being concentrated in the coated pits. The normal cycle for the LDL receptor is synthesis in the endoplasmic reticulum, and insertion in the cell surface membrane followed by localization in special domains of the cell surface known as coated pits. In normal cells 62% of the LDL receptors are found in coated pits. In internalization defective heterozygotes and homozygotes only 40% and 12% respectively are properly located [71].

Although LDL can bind to the receptor when it is at any location on the cell surface, internalization can only take place by endocytosis in which the coated pits are invaginated. All of these genotypic variants of FH identified by specialized techniques give a common presentation- negligible LDL receptor function. In view of the similar phenotype seen in heterozygous patients with FH, genotypic variation does not impact therapeutic decisions

but may contribute to the variations in both lipid levels and response to diet and drug therapy.

DRUGS USED IN THE TREATMENT OF HYPERCHOLESTEROLEMIA

The ability to successfully treat elevated plasma cholesterol levels with effective hypolipidemic agents is relatively recent. Although studies of efficacy and toxicity have been done, much more needs to be learned about the mechanisms of the various agents at the tissue and cellular level, and about the natural history of FH following long term hypolipidemic therapy.

There have been questions raised as to whether lowering elevated plasma cholesterol would have a significant impact upon coronary heart disease, especially in light of the cost of lipid lowering drugs. The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) was a seven and one half year, prospective, multicenter, randomized, double-blind study which tested the efficacy of lowering plasma cholesterol levels with a bile acid sequestrant in terms of reducing the rate of coronary heart disease. The study population consisted of 3806 asymptomatic middle aged men with primary hypercholesterolemia who had LDL cholesterol levels of greater than 190 mg/dl with total triglyceride levels under 300 mg/dl (Frederickson class IIa and IIb). Patients with type III hyperlipidemia were excluded. Both

treatment and control groups followed a 300 mg/day cholesterol diet.

The LRC-CPPT treatment group had a 20% decrease in LDL cholesterol levels compared to the 12.6% decrease in the control group. The treatment group had a 19% reduction in nonfatal myocardial infarction and a 24% decrease in coronary heart disease death. There was also a 20-24% decrease in new onset angina and positive exercise stress tests in the treatment group, indicating that treatment of hypercholesterolemia has a major impact upon the severity of coronary heart disease [72,73]. There have been other recent studies which used combined drug regimens which also documented regression of xanthomas[74], and the lack of progression of coronary artery lesions demonstrated by angiography[75,76].

BILE ACID SEQUESTRANTS

Cholestyramine and colestipol are bile acid sequestrants. They both bind bile acids within the intestinal lumen, thereby preventing their reabsorption in the ileum[77]. The result is an increased excretion of bile acids in the feces. This causes a compensatory increase in hepatic bile acid synthesis from cholesterol[78] which depletes the hepatic cholesterol pool and results in an increased number of hepatic LDL receptors and an enhanced rate of LDL catabolism[79], as well as a compensatory increase

in hepatic cholesterol synthesis. There is also an increase in the production of VLDL by the liver as a linked response to the increase in cholesterol synthesis. The major side effects of the bile acid sequestrants are constipation, which may be severe, interference with absorption of fat soluble vitamins, and poor patient compliance because of the poor organoleptic properties of the product.

It has been proposed that because of the inherently decreased number of LDL receptors, patients with FH require an increased level of plasma LDL to deliver cholesterol to peripheral tissues and suppress cholesterol synthesis[80]. However, it is not known whether the therapeutic lowering of plasma cholesterol results in increased synthesis of cholesterol within non-hepatic cells, such as the arterial wall, thereby possibly negating the effectiveness of the hypolipidemic therapy. It has been shown that there is an increase in cholesterol synthesis in mononuclear cells with hypolipidemic regimens involving colestipol[42,43,44], which may be suppressed by the concomitant use of niacin[44]. The mechanism for this increase is not known. Treatment of patients having heterozygous FH, using a combination of niacin and colestipol will reduce plasma LDL and total cholesterol levels by 30-50%[81,74].

NIACIN

The impact of niacin on plasma cholesterol levels was first described in 1955[82]. Niacin seems to depress plasma LDL levels by reducing free fatty acid mobilization from adipocytes, with a resultant secondary suppression of hepatic cholesterol synthesis[83], thus decreasing the synthetic rates of VLDL and LDL[84]. However, at the cellular level, the precise mechanism is not known. This drug is a first line choice in the treatment of combined hyperlipidemia, and may be very effective against primary hypercholesterolemia (especially when combined with a bile acid sequestrant). The utility is limited by its side effects which include severe flushing (thought to be prostaglandin mediated), gastrointestinal intolerance, and elevations of plasma uric acid, glucose, and liver function tests. When tolerated as a single agent, niacin can result in decreases in LDL cholesterol of 15 to 30%[85,86].

HMG CoA REDUCTASE INHIBITORS

Lovastatin (mevinolin), simvastatin, pravastatin and mevastatin (compactin) are members of a new class of drugs which competitively inhibit the synthesis of cholesterol at the level of HMG CoA reductase. At this metabolic level, all of the products are water soluble and do not have known toxicity. Lovastatin is the member of this family most extensively tested in humans. It is effective, lowering LDL

cholesterol levels by about 35-40% in patients with FH[87] and Familial Combined Hyperlipidemia[88]. It increases LDL catabolic rates[89] and increases hepatic LDL receptors[89,90]. Lovastatin is one of the most powerful cholesterol-lowering agents which has been studied. The known short term side effects include gastrointestinal intolerance, myositis, and elevations of the plasma levels of hepatic transaminases and creatine phosphokinase. The long term side effects are not known. It has been undergoing human trials for 5 years in the United States and has recently been released from investigational status.

PERIPHERAL CELL RESPONSES TO LIPID LOWERING DRUGS

Applying the model for cholesterol metabolic regulation outlined above to peripheral cells would predict that since plasma concentrations of LDL are far above those required to saturate the receptor (about 40-fold excess) there should be little detectable LDL receptor activity in freshly isolated human mononuclear cells, and minimal response of the mononuclear cells to hypolipidemic regimens. This is not the case. Many workers have shown low levels of LDL receptor activity (receptor mediated degradation of LDL) and low rates of cholesterol synthesis in freshly isolated human cells[40,91,92,93] in normal subjects as well as patients with hyperlipidemia. It has also been demonstrated that these cells respond to increased dietary cholesterol in much the

same way as hepatic cells, i.e. by decreasing LDL receptor activity and HMG CoA reductase activity[38].

If, as these studies suggest, changes in the rates of cholesterol synthesis and LDL receptor activity in hepatocytes are paralleled by similar changes in freshly isolated mononuclear cells, then the predicted effects on LDL receptor activity and rates of cholesterol synthesis following treatment with colestipol would be an increase in hepatic cholesterol synthesis with an increase in LDL receptor activity. Since colestipol is not absorbed into the body, the changes expected would be increases in the rate of cholesterol synthesis and LDL receptor activity secondary to the decreases in plasma levels of LDL. A combined drug regimen using both niacin and colestipol should yield an increase in the LDL receptor activity and an increase in the rate of cholesterol synthesis by the mononuclear cell.

What has been observed in previous studies[42,44] with freshly isolated mononuclear cells is the expected increase in the rate of cholesterol synthesis with colestipol therapy, a decrease in the cholesterol synthesis rate with niacin administration, and an increase in LDL receptor activity following colestipol treatment. However, there was no increase in LDL receptor activity with niacin treatment, nor with the combination of niacin and colestipol[44]. This disparity between the predicted events and those found (i.e.

only observing increases in LDL receptor activity associated with an increased rate of cholesterol synthesis) would imply a linkage between the increase in cholesterol synthesis and the increase in LDL receptor activity. The recently identified sterol-dependent regulatory regions adjacent to the genes for the LDL receptor and for HMG CoA reductase, intracellular pools of free and/or esterified cholesterol may provide this link. This is an area which requires further investigation.

One hypothesis to explain the increased rates of cholesterol synthesis and LDL receptor activity seen in freshly isolated mononuclear leukocytes from FH patients treated with colestipol is that they may be secondary to differences in the composition of LDL from patients with FH compared to normal LDL. The LDL isolated from untreated patients with FH has been shown to have a higher ratio of sphingomyelin to lecithin than LDL isolated from normal subjects[100], and to possess a higher cholesterol to protein ratio. Therapeutic intervention using cholestyramine or colestipol has been shown to alter these ratios towards a more normal composition[100,101].

The cause for these alterations in LDL composition found in patients with FH is thought to be due in part to an accumulation of sphingomyelin and cholesterol in LDL due to an increase in the time that LDL resides in the plasma because of the clearance defect found in FH. This is

supported by the increase in the catabolic rate for ^{125}I-LDL found after treatment with bile acid sequestrants.

Further evidence to support this view comes from studies of liposomes containing sphingomyelin and octyldecylamine[94]. When these liposomes, which contain no cholesterol, were incubated with normal fibroblasts, the binding of ^{125}I-LDL was less than 30% of control within two hours of incubation, and 10% of control levels after 10 hours. The incorporation of ^{14}C-acetate into cholesterol was increased 2.3 times over control at 24 hours and 8.1 times control values at 48 hours. These effects were reversed by a further 24 hour incubation in the same medium without liposomes. The effect is specific to sphingomyelin because incubations of liposomes made from lecithin and octyldecylamine had less than one third of the effect on LDL binding of the sphingomyelin liposomes. These effects are not due to toxicity of the sphingomyelin because cell proliferation continued at normal rates for over 48 hours and phospholipid incorporation of ^{14}C palmitic acid was normal or increased[94].

STUDIES INVOLVING CHANGE IN LDL COMPOSITION

The heterogeneity of plasma LDL has been described by many researchers[95,96,97]. When examined on an ultracentrifugal gradient of density 1.006-1.063 g/cc, the LDL isolated from a given individual may have a relatively

narrow density distribution (monodisperse) or it may be separable into several distinct bands spread over a broad density range (polydisperse)[98]. The dispersion of LDL in a density range is related to increased plasma triglyceride levels rather than elevated plasma cholesterol levels and is due to an increase of triglyceride-rich particles within the density range of 1.006-1.020 g/cc.

The composition of a `total LDL' isolated by heparin-manganese precipitation of the density >1.006 plasma fraction was analyzed by Kuchinskiene and Carlson[99]. They showed decreases in the estimated number of molecules per particle of triglyceride and `soluble proteins' (apoC and apoE species) when going from VLDL to LDL. The number of apoB and cholesterol ester molecules remained constant. These changes were felt to be due to decrease in particle size. There were no sex differences in composition.

In patients with FH, total LDL isolated by heparin-manganese precipitation of the density >1.006 g/cc plasma proteins had a greater cholesterol-phospholipid ratio in men than in women and a greater amount of sphingomyelin relative to lecithin[100]. These studies did not estimate the relative composition of the LDL particle, nor was there an attempt to investigate the effects of modification of LDL levels by diet and drug therapy. After plasmapheresis the cholesterol-phospholipid ratio and lecithin-sphingomyelin ratio decreased towards normal.

Witztum, et al[101], showed a decrease in the cholesterol-apoB ratio in 18 patients with Type II hyperlipidemia who were treated with colestipol[101]. LDL was isolated by precipitation of the density 1.006 gm/cc infranate following ultracentrifugation. The patients were a varied clinical group including eight who were obese (>120% of their ideal body weight) and seven who were hypertriglyceridemic (>180 mg/dl); patients with known secondary causes of hyperlipidemia were excluded.

There have been several previous investigations into the distribution and composition of LDL subfractions, isolated by different centrifugation techniques. Teng, et al[102] separated LDL in the 1.019-1.063 gm/cc density range using a discontinuous KBr gradient in a swinging bucket rotor, centrifuging to achieve equilibrium banding. They separated the top two bands visually and analyzed the isolated lipoproteins.

They showed that the light fraction had an increased content of cholesterol relative to protein when compared to the heavy fraction in the same population, and that LDL isolated from FH patients had a higher cholesterol-protein ratio in the light fraction but a lower cholesterol-protein ratio in the heavier fraction compared to normals. Composition analysis showed a decrease in the light fraction triglyceride and protein as well as an increase in the

cholesterol ester content compared to fractions isolated from normal individuals.

TURNOVER STUDIES

Studies have measured rates of LDL synthesis and catabolism using simultaneous injections of ^{125}I-LDL and cyclohexanedione modified ^{131}I-LDL (which abolishes receptor mediated catabolism of the LDL particle by blocking the lysine residues needed for apoB to bind to the LDL receptor)[103]. In patients with heterozygous FH, 22% of the LDL was cleared through receptor-dependent pathways, while in subjects without FH, the receptor-mediated clearance was 40%. These studies also showed variable amounts of overproduction of LDL apoB in FH.

Treatment of patients with heterozygous FH with the drug cholestyramine doubles the rate of clearance of LDL through the receptor-dependent path, without changing the rate of removal via the receptor-independent path[79]. This shows that patients with heterozygous FH can upregulate the "normal" or specific clearance path for LDL, and that it is via this mechanism that the bile acid sequestrants reduce plasma LDL concentrations.

Untreated patients with heterozygous FH show a deviation from the usual precursor-product relationship of VLDL-IDL-light LDL-heavy LDL found in normal subjects[104]. There was a markedly decreased fractional catabolic rate for

LDL which is related to slightly increased rate of synthesis of LDL apoB and to the decreased receptor mediated clearance of LDL in FH. Simultaneous LDL subfraction turnover measurements have demonstrated decreased conversion of light LDL to heavy LDL as well as independent synthesis of heavy LDL. This second pathway for LDL synthesis has been confirmed by studies using ^{75}Se-selenomethionine incorporation[105] which showed that in patients with FH, 46% of LDL is derived from IDL and 54% from direct synthesis, whereas in normal subjects 86% of LDL is derived from IDL.

HYPOTHESIS

1) That colestipol, a non-absorbed bile acid binding resin, increases LDL receptor activity and rates of cholesterol synthesis in freshly isolated mononuclear leukocytes from patients with heterozygous familial hypercholesterolemia (FH) by altering the composition of circulating LDL.

2) That these LDL particles (with an altered composition) display different binding characteristics to the LDL receptor as compared with LDL particles isolated from untreated patients having FH.

3) That the LDL isolated from colestipol-treated patients with FH may have differing metabolic effects and interactions with the cell than LDL isolated from untreated patients with FH.

4) That nicotinic acid, an agent which reduces LDL synthesis and which is as effective a hypolipidemic agent as colestipol, does not produce the same effects on cholesterol homeostasis and LDL receptor activity in freshly isolated mononuclear cells, or the same LDL composition changes as occur with colestipol. This implies that the LDL composition shifts and increases in mononuclear cell cholesterol synthesis and LDL receptor activity found following colestipol treatment are not related to the

magnitude of plasma LDL reduction but are related to the mechanism by which LDL levels are lowered.

EVALUATION OF HYPOTHESIS

The working hypothesis, which is designed to explain the previously documented[44] increases in rates of cholesterol synthesis and high affinity LDL receptor activity seen in freshly isolated mononuclear cells obtained from FH patients during treatment with colestipol, is that these increases are the result of changes in the composition of LDL or a subfraction of LDL. Such changes in composition might result in reduced LDL uptake by the cells or alternatively might promote efflux of free cholesterol from the cells to plasma, thereby depleting the cellular free cholesterol pool and stimulating compensatory increases in LDL uptake and cholesterol biosynthesis.

The hypothesis was evaluated in several ways:

1) A longitudinal study of LDL receptor activity and rates of cholesterol synthesis in freshly isolated mononuclear cells from patients with heterozygous FH on a low cholesterol diet treated additionally with colestipol, nicotinic acid, and/or the combination of colestipol and nicotinic acid was performed. This was done in order to confirm and reinforce the data from the previous cross-sectional studies of Lees and Lees[43], and Sundberg and

Illingworth[44] which reported differences between the therapeutic regimens.

2) LDL is not a single molecular species, but is a continuum of related particles which may be separated into several subclasses with different sizes and, potentially, compositions. The relative distribution and composition of LDL within two subclasses was measured by isolating LDL within the density ranges of 1.019-1.040 gm/cc and 1.040-1.060 gm/cc. The composition of each LDL fraction was expressed as both the absolute and the relative contribution of protein, triglyceride, free cholesterol, esterified cholesterol, and phospholipid to the lipoprotein. The amounts of the individual phospholipids present were measured because of reports citing changes in the behavior of cells and/or membranes which have been selectively enriched in these components[94]. Derived measures of composition were the ratios of free cholesterol-phospholipid, total cholesterol-phospholipid, cholesterol-protein, and lecithin-sphingomyelin. Another derived relationship used was the ratio of surface components (free cholesterol, protein, and phospholipid) to core components (esterified cholesterol and triglyceride).

These studies were done in both a cross-sectional (initial studies of total LDL composition) and longitudinal manner (all succeeding studies) in patients with well characterized heterozygous FH in the untreated state, and

after treatment with colestipol. Patients with FH treated with nicotinic acid, the combination of colestipol and nicotinic acid, and normal volunteers with no identified lipid abnormality were used as comparison groups.

3) Since small changes in LDL composition were expected (using routine chemical analyses), the interactions of LDL subfractions isolated before and after colestipol therapy with cultured normal human fibroblasts were studied. The kinetics of LDL subfraction binding, internalization, and degradation as well as the ability of the LDL subfractions isolated before and after colestipol therapy to suppress cholesterol synthesis and stimulate cholesterol esterification were measured in cultured normal human skin fibroblasts.

squalene, phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, phosphatidylserine, sphingomyelin, and phosphatidylinositol) or as part of the incubation media (unlabeled oleic acid, fatty acid-free human serum albumin) were purchased from Sigma, and were of the highest purity available. Other chemicals were of analytic reagent grade or better. Scintillation fluid was made using scintillation grade toluene (J.T. Baker) and Omniflour^{tm} premixed primary and secondary flours from New England Nuclear. Solvents were glass distilled and came from Burdick and Jackson. Radioisotopes including 2-^{14}C acetate, ^{3}H-oleic acid, and ^{125}I were purchased from Amersham.

Beckman model L8-70,L8-55 and L5-40 ultracentrifuges with type 50.3 Ti and 60 Ti rotors were used for lipoprotein isolation and LPDS preparation. A Beckman LS9000 liquid scintillation counter was used for counting ^{3}H and ^{14}C samples. A Packard Instruments gamma counter with a 3 inch crystal was used for counting ^{125}I.

Thin layer chromatography supplies including silica gel G, and silica gel H were obtained from E. Merck and Co. The silica gel H was prewashed in chloroform-methanol 1:1 to remove any organic phosphates before use for quantitative phospholipid isolation. Whatman K6 precoated thin layer chromatography plates were obtained through Analabs. All thin layer chromatography plates were activated in an oven at 120 C for 1 hour directly before use.

PATIENT POPULATION

Familial hypercholesterolemia was defined as:

a. Persistent plasma cholesterol greater than 300 mg/dl with triglycerides lower than 150 mg/dl on a normal diet.

b. The presence of a type II-a phenotype in at least one first degree relative with an inheritance pattern consistent with autosomal dominant.

c. The presence of tendonous xanthoma in the patient or at least one first degree relative.

d. The absence of multiple phenotypes in other family members.

e. The presence of hypercholesterolemia greater than 250 mg/dl in children within the family.

Not all patients were able to meet all the criteria. When this occurred, criteria a, b and d,in combination with c or e were considered diagnostic. Subjects were drawn from adult patients being followed at the OHSU Lipid Disorders Clinic, as well as new adult patients referred for evaluation. Patients on estrogens or patients with additional disorders such as diabetes, which are known to affect lipid metabolism or patients with excessive alcohol intake were excluded from the study. Because hypothyroidism may produce a secondary hypercholesterolemia which is a phenocopy of FH[106], thyroid function was checked to ensure euthyroid status before the patient was studied.

OUTLINE OF PATIENT STUDIES

A series of six studies were performed in order to evaluate the metabolic effects of colestipol therapy on the rates of mononuclear cell cholesterol synthesis and receptor mediated LDL degradation, as well as a potential mechanism for these effects.

The following studies were performed:

1) an examination of the effect of colestipol and/or niacin treatment on rates of cholesterol synthesis and receptor mediated degradation of LDL in freshly isolated mononuclear leukocytes from FH patients;
2) an evaluation of the effect of colestipol and/or niacin treatment upon total LDL composition in these patients;
3) a study of the effect of colestipol on the composition of total LDL,
4) studies of the effect of colestipol on LDL subfraction composition;
5) an examination of the effect of colestipol treatment of FH patients on the binding, internalization, and degradation of LDL subfractions by normal human skin fibroblasts grown in tissue culture and
6) studies on the effects of colestipol treatment in FH patients on the ability of isolated LDL subfractions to regulate the synthesis and storage of cholesterol by normal human skin fibroblasts.

All of the studies were longitudinal, with the exception of number two which was cross-sectional. All patients were instructed on a diet containing less than 300 mg cholesterol per day and less than 30% fat. Compliance was estimated by interview with a dietitian.

The cross-sectional study involved 65 patients with well characterized FH who were seen in the OHSU Lipid Disorders Clinic, but did not participate in the longitudinal studies presented here. Reasons for not participating included declining to be involved, living too far from Portland to make frequent (every one to three months) return visits, clinical indications for the use of a different lipid lowering medication (such as gemfibrozil, clofibrate, probucol, or lovastatin), intolerance of colestipol or niacin, non-compliance with medications either by self-report or in the clinical judgment of the examining physician, or intercurrent major medical event such as surgery or myocardial infarct.

The longitudinal studies involved a total of forty-three patients with heterozygous FH as defined above, who were examined in a prospective fashion during baseline periods and during at least one of three drug treatment periods. These periods consisted of colestipol or niacin as single agents, and the combination of colestipol with niacin. The patients in this group did not participate in

all portions of the longitudinal studies for the same reasons outlined above.

The longitudinal studies were prospective but not randomized. Colestipol dosage adjustments as well as the addition or substitution of niacin was done based on clinical response to colestipol treatment and/or tolerance of side effects. Both the patient and physician were aware of the drug(s) being used. Compliance was estimated by patient report and by the opinion of the examining physician. Usual drug dosage was five to ten grams of colestipol given orally twice daily. Nicotinic acid (3 to 6 grams per day in divided doses) was added or substituted, based upon clinical indications, if needed to achieve therapeutic goals. Optimal treatment goals were a total cholesterol of less than 200-220 mg/dl and/or a LDL cholesterol of less than 130-150 mg/dl. These goals were not achieved in all patients with these medications despite attempts at optimizing drug dosages and schedules. Selection bias was avoided by selecting patients based on the drugs used (and documentation of compliance) but not on the basis of their clinical response (or lack of response).

During the prospective study of the effects of colestipol and niacin on mononuclear cell cholesterol homeostasis, the study subjects had blood drawn for analysis of plasma cholesterol, triglyceride, low and high density lipoprotein levels, freshly isolated mononuclear cell LDL

receptor activity, and mononuclear cell sterol synthesis measurements in order to establish baseline values prior to being started on hypolipidemic drugs. These studies were repeated following stabilization on the prescribed lipid lowering regimen for a minimum of six weeks to a maximum of twelve weeks. All patients were able to tolerate the medications prescribed. A subgroup of 24 patients who had definite FH who had no evidence for other concurrent primary lipid disorders participated in this study.

The longitudinal evaluation of the effects of colestipol on total LDL composition involved 8 patients studied before and after two to three months of treatment. They had blood samples drawn for determination of plasma lipid and lipoprotein determinations, LDL composition, and LDL phospholipid composition both at baseline and after 8 to 12 weeks of colestipol treatment.

Another 15 patients with well characterized FH were enrolled in studies of LDL subfraction (light and heavy LDL) composition before and after 6 to 12 weeks of colestipol treatment. At each visit, these patients had blood samples drawn for plasma lipid and lipoprotein determinations, LDL subfraction composition, LDL subfraction phospholipid composition. Two patients were unable to make return visits during the study period and were dropped from the study, and one patient was unable to tolerate colestipol.

Of the twelve patients remaining, 1 patient was dropped from the study because further clinical evaluation suggested that he had a concurrent second primary lipid disorder and thus data from 11 patients were analyzed in the study. Four patients did not have sufficient blood drawn to enable the performance of studies other than LDL subfraction composition. Three patients had additional studies involving measurements of LDL subfraction binding, internalization and degradation by normal human fibroblasts. One patient had studies on the effects of LDL subfractions on fibroblast cholesterol homeostasis, and 4 patients participated in both the studies.

No binding affinity studies were done on LDL isolated from patients with FH who were being treated with niacin as a single agent because it is not commonly used alone in the treatment of FH and no patients were being treated with niacin alone during this portion of the study.

Since most of the study designs were longitudinal, each patient served as his or her own control. Samples from normal subjects were obtained from laboratory personnel as well as other volunteers with no identified lipid metabolic disorder. The studies were not blinded as to treatment being used. The purpose of the study was explained and informed consent obtained in all cases. This study was approved by the OHSU Human Research Committee.

PLASMA LIPIDS AND LIPOPROTEINS

Venous blood was drawn into tubes containing 1 mg/ml EDTA as an anticoagulant and antioxidant for the lipid and lipoprotein studies[107] or 14 units/ml lithium heparin for the mononuclear white blood cell studies. The volumes of blood drawn varied with the study. 10cc was needed for total LDL composition, 20cc for LDL subfraction composition, 35cc for mononuclear cell studies, and 45cc for LDL subfraction composition and the fibroblast binding affinity and cholesterol homeostasis studies. Plasma was separated by centrifugation. Total plasma cholesterol and triglyceride were determined using the Auto-Analyzer II[108].

LDL ISOLATION

Low density lipoprotein was isolated by sequential flotation ultracentrifugation as two fractions in the density ranges 1.019-1.040 g/cc and 1.040-1.055 g/cc for LDL subfraction composition analysis, the fibroblast binding affinity studies, and the fibroblast cholesterol homeostasis studies. LDL was isolated as a single fraction 1.019-1.060 g/cc for use in mononuclear cell LDL receptor activity studies and the total LDL composition studies.

The lipoproteins were isolated using a Beckman type 60Ti or Type 50.3Ti rotor in a Beckman Instruments L8-70, L8-55, or L5-40 ultracentrifuge. EDTA anticoagulated plasma isolated as described above had 18.53 mg/ml solid KBr

added to increase the density to 1.019 gm/cc. After centrifugation at 180,000g for 15 hours at 4 C, the supernatant containing VLDL and IDL was removed with a pipet and discarded. The density of the infranate was raised to 1.040 g/cc by the addition of 39.5 mg/cc solid KBr^{109}. The sample was then centrifuged 18 hours at 180,000g. The 1.020-1.040 gm/cc supernatant was removed as light LDL (LDL4). The infranate density was adjusted to 1.060 g/cc using 29.15 gm/cc solid KBr and centrifuged as above. The 1.040-1.055 gm/cc supernatant was removed as heavy LDL (LDL6). Total LDL for mononuclear cell receptor activity studies was isolated as a single band in a similar manner. After removing the VLDL and IDL as described above, the density of the infranate was adjusted to 1.060 g/cc using 61.53 mg/cc solid KBr and a single spin for 18 hours at 180,000g.

All of the LDL subfractions were then refloated once at their isolation density and dialysed against PBS pH 7.4. The isolation density was confirmed using a Metler/Paar calculating density meter calibrated at 20 C. Recovered light and heavy subfraction densities averaged 1.038 $\pm$ .001 g/cc and 1.055 $\pm$ .008 g/cc respectively.

The lipoprotein depleted serum used in tissue culture was derived from pooled normal human serum. Prior to use, the pool was screened and found to be negative for hepatitis B surface antigen. The density was raised to 1.21 g/cc by

adding 366.45 mg/cc solid KBr. The serum was then centrifuged at 180,000g for 40 hours at 4 C. The infranate was dialysed against six changes of 15 volumes each of 0.9% NaCl, 1 mM K_2EDTA, 5mM sodium phosphate, pH 7.4 at 4 C. After dialysis, the serum was reconstituted to its original volume with normal saline. The LPDS was then heat inactivated at 56 C for one hour. Precipitates were removed by centrifugation at 1500g. The LPDS was then sterilized by filtration through a 0.8u pre-filter, a 0.45u filter and then a 0.2u filter. The LPDS was then frozen at -20 C until use. It was again filtered through a 0.45u filter immediately before use to remove any freezing related precipitates.

LIPOPROTEIN LABELING

The isolated lipoproteins were labeled by the method of McFarlane[110]. This was done at pH 10 at which the incorporation of iodine into lipids was minimal. Iodine monochloride was added to give a molar ratio of iodine atoms to protein of less than one. Unbound iodine was removed by dialysis against four to six changes of 500 to 1000 volumes each of 0.9% NaCl, 1mM EDTA, 5 mM sodium phosphate pH 7.4. The purified ^{125}I-LDL was filtered through a 0.45u filter into a sterile vial with aliquots taken for counting, lipid and protein determinations. Studies show that less than 1% of the label was trichloroacetic acid soluble, and less than 3-5% was lipid bound, mostly in the lecithin and

triglyceride fractions. The efficiency of protein labeling was 10-25%.

LIPOPROTEIN COMPOSITION STUDIES

Total cholesterol and triglyceride were determined using the Auto-Analyzer II[108]. The LDL fractions were analyzed for total protein by the method of Hartree[111] using purified bovine serum albumin as a standard. Lipid extraction was performed using the technique of Folch, et al[112]. This method is reported as extracting >99% of the lipid present. All glassware was chloroform and distilled water rinsed before use to remove any lipids or inorganic phosphate present.

One volume of lipoprotein was extracted with 8 volumes of chloroform and 4 volumes of methanol. These were mixed and refrigerated at 4 C overnight. The system was broken into 2 phases by the addition of 5 volumes of water. The aqueous phase was aspirated off and the chloroform phase was washed with an additional 5 volumes of water. After the majority of the water was aspirated off, the bottom chloroform phase was transferred to a clean dry glass tube and dried under nitrogen.

The lipid residue was redissolved in chloroform and aliquots taken for the determination of phospholipid phosphorous, phospholipid composition, and amounts of free and esterified cholesterol. Phospholipid phosphorous was

measured on a washed total lipid extract[112] by the method of Bartlett[113]. Free and esterified cholesterol were measured on an aliquot of the extract using gas-liquid chromatography as described below.

PHOSPHOLIPID CLASSES

Approximately 200 ug of phospholipid from the LDL fraction extract was spotted on a Whatman K6 silica gel plate pretreated by migrating in chloroform-methanol 2:1 and heat activating at 100 C to avoid humidity effects. Samples were spotted in lanes 2-9 with standards containing phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine placed in lanes 1 and 10. The TLC plates were developed in chloroform-methanol-petroleum ether-acetic acid-and water (40:20:30:10:1.8 by volume)[114]. After drying under nitrogen, the plates were visualized with iodine vapor. The bands were marked and the iodine evaporated. The bands were scraped into pre-washed tubes and analyzed by a modification of the Bartlett technique for phosphorus for evaluation of the relative phospholipid composition. Recovery from the plates averaged 95.3% (range 85 to 103%).

FREE AND ESTERIFIED CHOLESTEROL MEASUREMENT

An aliquot representing an estimated 200 ug of total cholesterol was taken for analysis. 50 ug each cholestane

and stigmasterol were added as internal standards. The sample was dried under nitrogen. The trimethylsilyl-ether derivative was formed by the addition of 200 ul of a mixture of dimethylformamide: hexamethyldisilazine: trimethylchlorosilane (40:40:1 by volume).

An aliquot of the sample was injected onto a Hewlett Packard 5370 gas-liquid chromatograph with a fused silica capillary SE-30 column and hydrogen flame ionization detectors to determine the amount of free cholesterol. The column temperature was 250 C. Nitrogen was used as the carrier gas. The relative retention time for cholesterol compared to cholestane is 1.65. The relative area response is 0.964. The sample remaining after the injection was saponified for 2 hours at 37 C in 2% KOH in 95% ethanol. The sample was extracted twice with hexane, dried under nitrogen, the trimethylsilyl ether reformed, and the sample reinjected to measure the total cholesterol present. The amount of esterified cholesterol was the difference between the free and total cholesterol.

The method was standardized against a separation of the free and esterified cholesterol by thin layer chromatography on silica gel G plates using a solvent system of hexane: diethyl ether: chloroform: acetic acid (80:10:10:1 by volume). ^{3}H-cholesterol and cholesterol-1-^{14}C-oleate were used as internal standards to monitor recovery from the thin layer chromatography plates. The plates were imaged with

rhodamine spray and the cholesterol extracted with 3 changes of 5cc ethyl ether. The cholesterol was quantitated by a modification of the method of Abell, et al[115,116]. The recovery of the gas-liquid chromatography procedure using paired replicate analyses of lipoprotein extracts and standard mixtures of free and esterified cholesterol was 99% (range 97-103%) compared to thin-layer chromatography after correction for recovery of the internal standards. The recovery figure is valid for cholesterol content up to at least 240 ug/sample, and at a full range of proportions of free to esterified cholesterol (0%, 5%, 10%, 20%, 25%, 40%, 50%, 60%, 75%, 80%, 95%, 100% esterified cholesterol).

MONONUCLEAR CELL CHOLESTEROL SYNTHESIS STUDIES

The buffy coat was isolated from 30-40 ml heparinized whole blood by centrifugation at 1500 x g and diluted with RPMI-1640 at 37 C. Three volumes of the cell suspension were layered over 2 volumes of Ficol-paque and centrifuged for 30 minutes at 1500 x g at room temperature. The cells at the upper interface were collected with a plastic pipet, resuspended in warm RPMI-1640, and pelleted at 1500 x g. This washing was repeated once. The cell pellet was resuspended in 1cc warm RPMI-1640. Aliquots were taken for assessing the number of granulocytes by Wright's staining[117] and to determine the relative numbers of monocytes and lymphocytes by staining smears for alpha-napthyl-acetate esterase activity[118]. These preparations yielded >99%

mononuclear cells which were approximately 70% lymphocytes and 30% monocytes as determined by examination of smears stained for non-specific esterase. Eighty per cent of LDL receptor activity is associated with the monocytes, 20% with lymphocytes. Cells were counted on a Coulter model S automated cell counter and their viability assessed by trypan blue exclusion. Viability averaged >95%.

Duplicate aliquots of mononuclear cells were incubated in a medium of 30% RPMI-1640 and 70% autologous serum containing 1.5 micromoles/ml 2-^{14}C-acetate and 25 micromoles/ml phosphate buffer pH 7.4. After a 5 hour incubation at 37 C in a shaking waterbath, the incubation was stopped by the addition of 10cc chloroform: methanol 1:1 (by volume). The tube was mixed to ensure complete extraction. The tube was centrifuged and the solvent was removed to a clean glass tube. The pellet was re-extracted with 1cc distilled water and 10 cc chloroform: methanol (1:1 by volume). The extracts were pooled and the system broken into two phases by the addition of 8cc distilled water. This modified Bligh-Dyer extraction[119] is reported to yield >94.5% extraction of the lipid in each extraction. Incorporation of 2-^{14}C-acetate into cholesterol, lanosterol, and squalene were determined after separation of the lipid extract by thin layer chromatography[120].

LDL RECEPTOR STUDIES IN MONONUCLEAR CELLS

Low density lipoprotein receptor activity was measured as the degradation of ^{125}I-LDL by freshly isolated mononuclear cells obtained during each study period. Because the cells were assayed within 2 hours of isolation from plasma, this measurement was believed to reflect the in-vivo LDL receptor activity from each patient at that time and not to be influenced by changes in LDL receptors which occur after a more prolonged time allows changes in the cellular milieu. Mononuclear cells were isolated from heparinized venous blood as described above. Aliquots of the cell suspension were incubated in 2cc RPMI-1640 containing 30% human lipoprotein depleted serum (LPDS), 100 u/ml penicillin, 100 ug/ml streptomycin, 2mM/L glutamine and 25ug/ml ^{125}I-LDL in triplicate wells. An additional 500 ug/ml unlabeled LDL was added to triplicate control wells to allow the specific and non-specific catabolism of ^{125}I-LDL to be determined. Blank values were based on ^{125}I-LDL degradation in control incubations performed in the absence of cells.

The cells and blanks were incubated for 4 hours at 37 C in a humidified incubator with 95% air, 5% CO2. The incubation plates were placed on ice to stop the incubation and were maintained on ice for the remainder of the procedures. The cells and the medium were removed from the well with a plastic pipet and the well was rinsed with an

additional 1cc of ice-cold RPMI-1640. The rinse was pooled with the medium and cells. The cells and medium were separated by centrifugation at 1500 x g at 4 C.

An aliquot of the medium was precipitated with 500 ul 50% trichloroacetic acid after the addition of normal serum as a carrier. After centrifugation to separate the precipitated protein, the free iodide in an aliquot of the supernatant was oxidized to iodine by addition of hydrogen peroxide. The free iodine was removed by washing with chloroform. The samples were counted on a gamma counter to measure the remaining ^{125}I as degraded LDL. Specific high affinity degradation was calculated as the nanograms of ^{125}I-LDL degraded by cells in the presence of 25 ug/ml ^{125}I-LDL (total degradation) minus that in the presence of a 20 fold excess unlabeled LDL (non-specific degradation); both values were corrected for control incubations performed in the absence of cells.

FIBROBLAST LDL RECEPTOR AND CHOLESTEROL SYNTHESIS STUDIES

Human skin fibroblasts were grown from foreskin explants derived from 3 normal human subjects. Preliminary receptor studies were performed on cells cultured from each explant to assure that there were no identified LDL receptor abnormalities before use of the cell lines in the studies presented in this thesis.

Stock flasks of fibroblasts were grown and maintained in McCoys 5a medium supplemented with 100 u/ml penicillin, 100 ug/ml streptomycin, 100 ug/ml amphoteracin B, MEM non-essential amino acids and 10% human serum, hereafter referred to as medium A. The medium A was replaced biweekly and the cells were passed when confluent.

For subsequent assays, the cells were subcultured into 6 well plates seeded at an initial density of 20-35,000 cells/well (2.5 cm^2 wells) six days before the experiment. The medium A was replaced on the day following passage and again 3 days following passage. Four days after passage the cells were about 70% confluent. The medium was changed to medium A as described above with the substitution of 10% LPDS in place of the human serum (medium B). The day prior to the experiment the medium was replaced with fresh medium B. All the cells used in a given experiment were derived from the same explant and were between the fourth and twelfth passage at the time they were used for the LDL binding studies. Four passages were the earliest practical time to have sufficient numbers of cells for an experiment and maintain the cell line. The upper limit was selected arbitrarily because some enzyme systems show decreased activity with age although I am unaware of this being documented for the LDL receptor in vitro.

LDL BINDING STUDIES

This was done to assess the differences, if any, between LDL subfractions isolated from patients with FH before and during treatment with colestipol. The method used was that of Chait, et al[121], which is a modification of the technique originally described by Bierman, et al[122]. The method is described briefly below. The assay was started by replacing the culture medium B on the fibroblasts with fresh medium B containing graded amounts of ^{125}I-LDL ranging from 2.5 ug/ml to 100 ug/ml. Control wells had an average 20 fold excess of unlabeled LDL (usually 500-1000 ug/ml). Blank wells were set up in an identical fashion at each concentration of LDL, but contained no cells. The plates were incubated for 5 hours in 5% CO2/ 95% air at 37 C in a humidified incubator.

After incubation, the cells were placed on ice and maintained at 4 C for the remainder of the procedure. The medium was removed and retained for measurement of LDL degradation as described later. Each flask was rinsed five times with phosphate buffered saline pH 7.4 (PBS) containing 2% albumin at 4 C, discarding the washes. Each flask was then washed with plain PBS at 4 C. The last of this wash was removed by suction aspiration, and 0.05% trypsin in PBS was added. The cells were then incubated for 10 minutes at 37 C to release LDL which had been bound to the surface but not internalized. The cells and supernatant were transferred

number and non-specific binding. Both LIGAND and EBDA were obtained through Elsevier-BIOSOFT and were run under DOS 2.1 on an IBM PC-XT.

LDL COMPETITION STUDIES

This series of experiments measured the relative ability of the light and heavy LDL fractions isolated from FH patients who were taking colestipol to compete with ^{125}I-LDL fractions isolated from untreated FH patients for LDL receptor binding sites on cultured human fibroblasts. This was done by incubating cells with 7.5 ug/ml 'control' ^{125}I-LDL and amounts of unlabeled FH LDL ranging from 2 ug/ml to 100 ug/ml to provide saturating amounts of LDL at the upper end of the concentration curve.

Normal human fibroblasts grown to near confluency were incubated 48 hours in McCoys medium supplemented with 10% LPDS, penicillin, and streptomycin. The LPDS was included to provide a near identical incubation medium which was nearly lipoprotein free and therefore derepress the LDL receptors and stimulate cholesterol synthesis. In order to measure the binding characteristics of the different LDL species, the medium was then changed to a similar medium containing graded amounts of LDL isolated in the range 1.019-1.040 gm/cc or 1.040-1.055 gm/cc from each of 2 patients with FH on one of the treatments specified above. The cells were incubated for an additional 5 hours to

measure the binding, internalization and degradation of ^{125}I-LDL as described above.

Results were plotted as percent of total binding versus the logarithm of the concentration of added LDL subfraction. The IC50 is that concentration of unlabeled ligand which will displace 50% of the binding of the labeled ligand to a given binding site. This concentration of LDL was determined by linear regression of the percent of the total labeled lipoprotein binding against the logarithm of the concentration of added unlabeled LDL subfraction.

INFLUENCE OF LDL SUBFRACTIONS ON THE CELLULAR SYNTHESIS AND ESTERIFICATION OF CHOLESTEROL

Confluent monolayers of normal skin fibroblasts grown in medium A were derepressed by a 24 hour pre-incubation in medium B. Fresh medium B containing graded amounts of LDL subfraction ranging from 2.5 ug/ml to 200ug/ml was added to the fibroblasts. The plates were then incubated for 16 hours. 1500 micromoles/ml 2-^{14}C-acetate and 9.9 mM ^{3}H-oleic acid complexed with 12% albumin^{125} were added and the plates were incubated for 4 hours. The medium was removed, the cells were dislodged from the plate by incubation with 0.05% trypsin and the cells pelleted by centrifugation.

The incorporation of 2-^{14}C acetate into cholesterol was measured by extracting the cell pellet with 1:1 chloroform-methanol, saponifying an aliquot of the extract, and

separating the non-saponifiable fraction into cholesterol, lanosterol, and squalene by thin layer chromatography[120]. Incorporation of ^{3}H-oleic acid into cholesterol ester was measured in an aliquot of the cell extract following thin layer chromatography to resolve the lipid classes[125]. The cell pellet was dissolved overnight in 1N NaOH in a capped tube and assayed for protein by the method of Lowry[123].

STATISTICAL ANALYSIS

Analysis of the differences between plasma lipid and lipoprotein levels were by paired t test, as were the mononuclear cell acetate incorporation studies. Comparisons of total LDL, LDL4 and LDL6 subfraction compositions in FH patients treated with the combination of colestipol with niacin versus untreated patients with FH were done using a t test for two means. The results of the mononuclear cell LDL degradation studies were bimodal and did not show a normal distribution. Therefore, they were analyzed using the Wilcoxon rank sum[126], a non-parametric test. Goodness of fit for the lines generated in the LDL subfraction binding affinity studies were tested using a F statistic generated by LIGAND.

RESULTS

CHANGES IN PLASMA LIPID AND LIPOPROTEIN CONCENTRATIONS WITH DRUG THERAPY

The effects of two hypolipidemic drugs, colestipol and niacin, alone and in combination on plasma lipid and lipoprotein concentrations were evaluated prospectively in 32 patients with heterozygous FH (Table 1). All of the lipid lowering treatments resulted in significant decreases in plasma total cholesterol which ranged from 12.2% to 27.7%. The use of colestipol (mean dose 18 gm/day, range 10-30 grams/day) as a single agent in 23 patients resulted in a drop in total plasma cholesterol of 12.2% ($p<0.01$) and a decrease of 19.1% ($p<.001$) in LDL cholesterol without a significant change in HDL cholesterol. Niacin (mean dose 3 gm/day, range 2-5.5 grams/day)was used as a single agent in nine patients and resulted in a decrease in total plasma cholesterol of 24.0% ($p<0.01$) with a decrease in LDL of 31.5% ($p<0.001$). The combination of niacin (mean 3 gm/day) with colestipol (mean dose 20 gm/day) was evaluated in eleven patients and this regimen resulted in a 27.7% drop in total cholesterol ($p<0.01$) and a 41.2% fall in LDL cholesterol ($p<0.001$).

Total plasma triglycerides were increased 35.4% ($p<.05$) by the use of colestipol as a single agent, whereas niacin lowered plasma triglycerides by 26.6%. The latter change

was not significant because of the small number of patients, the great variation in initial plasma triglyceride levels and individual differences in responses to therapy. The combination of colestipol with niacin decreased triglyceride levels by 42.7% ($p<.05$). Treatments containing niacin also tended to increase plasma HDL levels. These results are consistent with the results of previous clinical trials which prospectively established the clinical efficacy of colestipol, niacin and the combination of colestipol with niacin[72,73,74,75,76,77,79,81,86,87].

VALIDATION OF MONONUCLEAR CELL PROCEDURES

Studies were done using fresh mononuclear leukocytes isolated from a normal volunteer to validate the assay conditions for measuring in-vitro rates of cholesterol synthesis by measurement of the incorporation of acetate into sterols. The incubation medium was autologous plasma buffered to pH 7.4 in order to most closely approximate in vivo conditions. The incorporation of 2-^{14}C-acetate into sterols is shown in Figure 2. The curves show a precursor-product relationship with squalene peaking at 4 hours, lanosterol (and other methyl sterols) at 4 hours, and cholesterol at 5 hours.

The effect of variations in substrate concentration is shown in Figure 3. Incorporation of 2-^{14}C-acetate into sterols increased over the range 0 to 0.8 micromoles/ml

acetate then levelled off or decreased slightly. 1.5 micromoles/ml was chosen as a midrange value for the experiments presented in this thesis.

LDL degradation in freshly isolated mononuclear cells was found to be linear over a range of 1 to 20 million cells per milliliter (Figure 4). With time, degradation of ^{125}I-LDL was found to be linear during the time period of two to six hours (Figure 5). As the calcium concentration was increased from 0.3 mM to 1.8 mM, the degradation of ^{125}I-LDL decreased, being maximal at 0.3 mM (Figure 6). 0.3 mM calcium is the basal amount present in the incubation medium. It was not possible to check lower concentrations. The possible effect of calcium concentration on LDL binding was tested because the LDL receptor has been reported to have calcium dependant binding in vitro[7]. The effect of substrate concentration on ^{125}I-LDL degradation was biphasic (Figure 7), although overall degradation increased as the LDL concentration was increased up to 200 ug/ml. The biphasic nature is consistent with two concurrent binding processes which have previously been described for cellular LDL uptake and metabolism[22,23]. One process is a high affinity, low capacity receptor mediated process and the other is a low affinity, high capacity bulk uptake process[22,23,28].

MONONUCLEAR CELL STUDIES

To expand and confirm the earlier studies of Sundberg and Illingworth[44], the rate of cholesterol synthesis from 2-^{14}C-acetate and the rate of high affinity degradation of ^{125}I-LDL were examined in freshly isolated mononuclear cells from 11 patients with FH who were studied prospectively on diet alone and restudied on single drug therapy with colestipol, 7 patients studied on diet and niacin, and 6 patients on diet and the combination of colestipol and niacin. The data from these 24 patients are shown in Table 2.

Despite comparable decreases in plasma cholesterol, the effects of drug treatment upon measures of cholesterol homeostasis were different. Colestipol as a single agent resulted in a 64% increase in 2-^{14}C-acetate incorporation into cholesterol in-vitro ($p<.025$), as well as an 80% increase in the high affinity degradation of ^{125}I-LDL by freshly isolated mononuclear leukocytes ($p<.005$). The other drug regimens did not result in a significant change in either of these parameters.

LDL COMPOSITION

The initial study to investigate the role of LDL as an intermediary in the responses of mononuclear cells to colestipol treatment was a cross sectional study of the composition of LDL. The relative contribution of each of

the major components of low density lipoprotein were measured in total LDL (1.020-1.060 gm/cc) in 65 patients with well characterized FH as described in the methods. The values obtained before treatment reflect patients instructed in a lower fat, low cholesterol diet (<300 mg/day cholesterol, <30% fat, with a ratio of saturated fat to polyunsaturated fat of 1.0). The composition of total LDL isolated from these patients is summarized in Table 3.

The amount of free cholesterol present in total LDL was decreased with drug therapy. Treatment with colestipol, but not the combination of colestipol with niacin, lowered the percentage of free cholesterol in LDL from 8.09% to 7.20% ($p<.05$). The post-treatment value was indistinguishable from the value for free cholesterol content of 7.46% found in total LDL isolated from normal volunteers. The proportion of triglyceride in LDL was increased following treatment with colestipol as a single agent (3.70% to 5.20% $p<.05$). The content of protein and phospholipid components were not changed in their relative amounts present in total LDL. Colestipol treatment decreased the ratio of free cholesterol to phospholipid, an index of surface composition, from 0.242 to 0.211 ($P<0.02$). The ratios of free to esterified cholesterol decreased from 0.39 to 0.36 and the ratio of total cholesterol to protein increased from 1.472 to 1.567. These differences were not statistically significant.

Eight patients with well characterized FH participated in the longitudinal study of the effects of colestipol treatment on total LDL composition. As seen in Table 4a, LDL protein was lowered by 24% (2152 ug/ml to 1632 ug/ml, $p<0.008$ by Wilcoxon rank sum). This was accompanied by a 20.7% decrease in LDL free cholesterol (821 ug/ml to 651 ug/ml, $p<.04$) and a 14.6% drop in LDL cholesterol ester (2090 ug/ml to 1786 ug/ml) with no change in LDL phospholipid content and an increase in triglyceride from 460 ug/ml to 581 ug/ml. The changes in cholesterol ester and triglyceride content were not of statistical significance ($p<0.10$ for both), possibly because of the variation in individual response and the small number of patients studied.

The data were analyzed again after correcting for protein content (Table 4a). The difference in free cholesterol content disappeared after correcting for protein. The amount of total cholesterol per unit protein increased 12% ($p<0.04$) probably due to a 12% increase in cholesterol ester content ($p<0.10$), although the difference in cholesterol ester was not statistically significant. There was also a tendency towards ($p<0.10$) an increase in the phospholipid content per unit protein. The content of triglyceride per unit protein increased 43% ($p<.008$). These results, although roughly corrected for changes in particle number, do not differentiate between the other issues of change in particle size or change in composition.

Krauss, et al[97], and Teng, et al[102], have previously studied the composition of human lipoproteins, and have also expressed their data in terms of weight percent of each of the components of the lipoprotein particle in order to evaluate particle composition. The results of the current study are presented in this fashion in Table 4b. When addressed in terms of the contribution that each component made to the total particle, free cholesterol decreased by 11.6% ($P<0.039$ by Wilcoxon rank sum), while triglyceride increased 49.8% ($p<0.039$). The contributions of cholesterol ester, protein, and phospholipid were not changed significantly. The ratio of FC/PL decreased from .244 to .208 ($p<0.039$), and the ratio of free to esterified cholesterol decreased 13.3% ($p<0.039$). A ratio of the sum of the surface component weights to core component weights was derived to address the issue of size changes versus composition changes. This surface/core ratio decreased from 1.667 to 1.505 ($p<0.10$) but the change was not significant.

There were too few patients available for longitudinal study on niacin as a single agent or the combination of colestipol with niacin to allow extensive statistical comparisons of the LDL composition in those groups.

Despite the relative frequency with which niacin is used as an additional agent to lower plasma cholesterol in patients with FH, it was infrequently used as a single agent during the course of this study. In the time between the

completion of the mononuclear cell studies reported here and the initiation of the LDL composition studies, most of the patients with FH who had been on niacin alone were switched to other therapy.

DISTRIBUTION AND COMPOSITION OF LIGHT (LDL4) AND HEAVY (LDL6) SUBFRACTIONS OF LDL

The concentrations and chemical composition of the two LDL subfractions were measured in plasma samples obtained from twelve FH patients at baseline, and after being treated with colestipol to determine if these subfractions were influenced selectively. The results are presented in Tables 5a and 5b for LDL4 and 6a and 6b for LDL6.

As is shown in Table 5a, The absolute amount of LDL4 free cholesterol decreased by 18.3% from 276 ug/ml in samples isolated before treatment to 226 ug/ml ($p<0.005$ by Wilcoxon rank sum) in samples of LDL4 isolated after colestipol treatment. The absolute amount of LDL4 protein present in untreated FH patients was 687 ug/ml before treatment. Following treatment with colestipol, the absolute amount of LDL4 protein decreased to 625 ug/ml ($p<0.021$ by Wilcoxon rank sum). The amounts of cholesterol ester and phospholipid present did not change significantly. Triglyceride increased, on average, by 29.6%, but this did not reach statistical significance because of the great variability in individual response.

After correcting to constant protein content the results were similar. The ratio of free cholesterol to protein decreased 11.2% ($p<0.05$) and the ratio of triglyceride to protein increased 30.5% ($p<0.05$). There were no changes in the ratios of cholesterol ester to protein, total cholesterol to protein, or phospholipid to protein.

These results, expressed as weight percent to show the relative contribution of each component to the particle are presented in Table 5b. The relative content of free cholesterol decreased by 10.4% ($p<0.046$ by Wilcoxon rank sum) with no change in the remainder of the components. There was a tendency towards an increase in triglyceride, but it did not achieve statistical significance. There were decreases in the ratios of free to esterified cholesterol (7.3%, $p<0.026$), free cholesterol to phospholipid (12.6%, $p<0.011$), and total cholesterol to phospholipid ($p<0.046$). There was no change in the ratio of surface to core components.

Results of the analyses of LDL6 are shown in Table 6a. The level of LDL6 free cholesterol was 553 ug/ml before treatment and 339 ug/ml after treatment with colestipol (38.6%, $P<0.0046$ by Wilcoxon rank sum). The amount of LDL6 protein in plasma isolated from FH patients decreased from 1416 µg/ml at baseline to 1047 ug/ml after treatment with colestipol (26.1%, $p<0.006$ by Wilcoxon rank sum). The

absolute amounts of cholesterol ester and phospholipid present decreased 27.3% ($p<0.006$) and 23.2% ($p<0.0046$) respectively. There was no difference in the amount of LDL6 triglyceride present. When corrected for protein content changes, most of the differences disappeared. There was a 14.7% decrease in the ratio of free cholesterol to protein ($p<0.006$). There were no differences in the ratios of cholesterol ester, total cholesterol, triglyceride, or phospholipid to protein.

The relative composition of LDL6 is shown in Table 6b. The content of free cholesterol decreased 15.3%. There were no significant changes in the content of cholesterol ester, protein, or phospholipid. There was a tendency towards an increase in triglyceride content (24%, $p<0.10$) but it did not achieve statistical significance. There were decreases in the ratio of free cholesterol to phospholipid (17.4%, $p<0.005$) and to cholesterol ester (13.0%, $p<0.005$). There were no significant changes in the ratio of surface to core components or in total cholesterol to phospholipid.

The decrease in LDL6 protein was three times that of LDL4 (26.1% for LDL6 vs 9.9% for LDL4) following treatment with colestipol. There is other supporting evidence. Eighteen percent of the decrease in total LDL cholesterol is attributable to a decline in LDL4 concentrations and 82% to a change in LDL 6. A similar distribution was noted in the decline in protein (14% from LDL4 and 86% from LDL6). The

ratios of LDL4 to LDL6 in terms of protein increases from 0.485 to 0.597, while in terms of cholesterol the ratio changes from 0.502 to 0.600.

PHOSPHOLIPID COMPOSITION

The relative proportions of the individual phospholipids in LDL were measured in samples of total, light and heavy subfractions of LDL isolated from FH patients on no treatment, after colestipol treatment and normal subjects. A previous report[100] documented increases in the content of sphingomyelin in LDL isolated from untreated FH patients: this was believed to be due to prolonged residence times of LDL in plasma.

The relative distribution of the individual phospholipids within the total LDL and within the LDL4 and LDL6 subfractions as influenced by colestipol is shown in Tables 7a, 7b, and 7c. Total LDL isolated from FH patients on colestipol showed a 14% ($p<0.046$ by Wilcoxon rank sum) decrease in the percentage of the total phospholipid present as sphingomyelin when compared to LDL isolated from untreated FH patients. This is mostly accounted for by the 7.7% decrease in sphingomyelin in the LDL4 subfraction ($p<.05$ by Wilcoxon rank sum). Not all patients had both total and LDL subfraction phospholipid composition measured because of limited amounts of clinical material available.

There was no significant change in the content of sphingomyelin in LDL6.

There were no significant changes in any of the other phospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, or lysophosphatidylcholine) in any of the LDL subclasses tested. Compared to normal subjects, the patients with FH had lower levels of phosphatidylinositol and higher levels of lysophosphatidylcholine.

CELLULAR BINDING AND METABOLISM OF LDL SUBFRACTIONS

LDL was isolated from 8 patients with FH on diet intervention only and repeated after stabilizing on 5-10g oral colestipol therapy twice daily to assess the effects of colestipol on the binding affinity of LDL subfractions to LDL receptors on cultured normal human fibroblasts. In both drug treatment and control phases, the binding and composition studies were performed using LDL fractions isolated in the density ranges 1.020-1.040 g/cc (LDL4) and 1.040-1.055 g/cc (LDL6). The kinetics of LDL binding, as well as internalization and degradation were measured to determine whether the composition changes which were seen were biologically significant. The receptor mediated components of each of these parameters was measured by incubating graded amounts of individual ^{125}I-LDL subfractions with cultured human fibroblasts in the absence

and presence of a 20 fold excess of unlabeled total human LDL as described in the methods section. Pilot studies showed no difference between the use of total LDL or specific LDL subfractions as the unlabeled competitor for LDL binding.

The IC50 was measured to define the ability of the LDL subfractions isolated from treated patients to compete with the comparable subfraction isolated from untreated patients or from normal subjects for binding to the LDL receptor. This was done by incubating normal fibroblasts in 10% LPDS for 48 hours to increase the expression of LDL receptors, then with graded amounts of unlabeled LDL4 or LDL6 (isolated from FH patients before and after treatment with colestipol, as well as normal subjects) and 7.5 ug/ml ^{125}I-LDL subfraction isolated from patients with untreated FH.

The results of the LDL4 binding studies are shown in Figure 8a. Specific high affinity binding of ^{125}I-LDL occurred up to a LDL4 concentration of 25 ug/ml for samples of LDL4 isolated during dietary treatment, or 50 ug/ml for samples of LDL4 isolated during colestipol treatment. At these concentrations saturation of the LDL4 binding sites occurred. Binding site saturation did not occur at any tested concentration of LDL4 isolated from normal subjects. There was insufficient material available from normal controls to carry the binding curves to higher concentrations than 50 ug/ml. There was a decrease in the

slope of the binding curve for ^{125}I-LDL4 isolated from FH patients following treatment with colestipol.

The binding inhibition curve for LDL4 is shown in Figure 10a. The changes in slope seen in the LDL binding curves (Figure 8a) are reflected in shifts of the estimated IC50. LDL4 isolated before treatment had an estimated IC50 of 7.1 ug/ml when competing against LDL4 also isolated before drug treatment. Following colestipol treatment the IC50 increased to 12.5 ug/ml. For comparison purposes, the IC50 of normal LDL4 was estimated as 13.0 ug/ml. An estimated IC50 is given because complete inhibition of binding for labeled LDL4 was not achieved. There were insufficient data to address the statistical significance of these differences, however, the magnitudes of the differences in IC50 for LDL4 are the same as the magnitudes of the differences in Kd as noted in Table 8. Competition at higher concentrations of unlabeled ligand could not be performed because of limited amounts of lipoprotein which could be isolated.

There were no significant changes in ^{125}I-LDL internalization (Figure 8b). Changes in the slopes of the internalization curves occurred at the same concentrations of LDL4 at which saturation of binding occurred. A change in slope was not seen in LDL4 isolated from normal subjects, because of the inability to provide saturating concentrations of normal LDL4 to the LDL receptor.

High affinity receptor-mediated degradation of ^{125}I-LDL4 isolated from patients decreased following colestipol treatment (Figure 8c). This is expected in that degradation should be proportional to binding, and LDL4 isolated from FH patients prior to treatment had higher rates of receptor dependent binding and receptor-mediated degradation than LDL4 isolated after colestipol treatment. A similar relationship between decreased LDL4 binding and degradation is seen with LDL4 isolated from normal subjects. Thus the differences in high affinity LDL4 degradation seen in Figure 8c reflected the changes in binding affinity of the lipoproteins seen in Figure 8a.

The binding, internalization and degradation curves for LDL6 are shown in Figures 9a, 9b and 9c. There were slight differences in the binding curves for LDL6 (Figure 9a). The LDL6 isolated from FH patients on diet only exhibited slightly higher binding to normal fibroblasts than LDL6 isolated during colestipol treatment. The binding, internalization and degradation of LDL6 increased progressively up to a concentration of 15 ug/ml in the case of LDL6 isolated during dietary treatment, and up to 25 ug/ml LDL6 in the case of LDL6 isolated during colestipol treatment. After that point binding continued to increase but at a much lower rate, implying that saturation of the high affinity binding site had probably occurred.

Internalization of ^{125}I-LDL6 was concentration dependent and increased with increasing concentrations of LDL6 in the medium as seen in Figure 9b. An inflection point for the curve occurred at 15 ug/ml for LDL6 isolated both during dietary intervention and during colestipol treatment. The internalization curves are virtually identical in shape and magnitude.

Figure 9c shows that there were no differences between the curves for high affinity receptor mediated degradation of LDL6 isolated from patients with FH before or after colestipol treatment, or from normal subjects. This is consistent with the lack of difference seen in the binding and internalization curves.

The competition curves for ^{125}I-LDL6 isolated before initiating colestipol therapy with unlabeled LDL6 following therapy or that isolated from normal individuals is shown in Figure 10b. The estimated IC 50 values for LDL6 fractions were: Diet treatment (65 ug/ml); Colestipol therapy (51 ug/ml); normal (29 ug/ml). There were insufficient data to address the statistical significance of these differences.

RECEPTOR BINDING ANALYSIS

Lipoprotein binding parameters were estimated in a linear manner using equilibrium binding dissociation analysis (the EBDA package). Final estimates of Kd and goodness of fit of the derived curves were arrived at by

pooling all the data from patients within an experimental group (six pairs each of pre- and post- treatment LDL4 and LDL6 as well as 2 normal LDL4 and LDL6 samples) and fitting the resulting curves using LIGAND which is an iterative non-linear curve fitting routine. The model was constrained to a fixed maximal binding and zero nonspecific binding as described in the methods.

The individual results for the six patients and the mean values for normal lipoproteins are shown in Table 8. The composition changes previously described were reflected in an increase in the LDL4 Kd from 0.58×10^{-5} ng/ml to 2.18×10^{-5} ng/ml ($p<0.01$ by F statistic). There was a decrease in the Kd of LDL6 from 1.75×10^{-5} ng/ml before treatment to 0.92×10^{-5} ng/ml following colestipol treatment. The values calculated for normal subjects are 2.42×10^{-5} ng/ml for LDL4 and 2.24×10^{-5} for LDL6. Scatchard plots for the data are shown in Figures 11a, 11b, 11c, and 11d. In Figure 11a the differences between the mean lines for LDL4 from normal subjects and LDL4 from FH patients before and after treatment with colestipol are shown. The data for LDL6 shown in Figure 11b have no significant differences between any of the groups either by F statistic or by paired t test of the data. The plots for a representative patient LDL4 and LDL6 sample are shown in Figures 11c and 11d respectively. The Kd and estimated binding site number are given in the legends.

THE INFLUENCE OF LDL SUBFRACTIONS ON CHOLESTEROL SYNTHESIS IN CULTURED HUMAN FIBROBLASTS

The ability of the LDL fractions to suppress rates of incorporation of 2-^{14}C-acetate into cholesterol was measured in cultured normal human fibroblasts. The cells were derepressed for 24 hours in 10% LPDS, incubated 16 hours in LPDS containing graded amounts of individual LDL subfractions and then incubated for a further 3 hours after the addition of 2-^{14}C-acetate as described in the methods. The curves for suppression of acetate incorporation by incubation with LDL subfractions are shown in Figures 12a (LDL4) and 12b (LDL6). Each curve is labeled by initials as to the patient from whom the LDL subfraction was isolated and whether the sample was obtained at baseline (solid line) or during colestipol treatment (dashed line). The curves for mean data from each patient group are shown in Figures 12c (LDL4) and 12d (LDL6). Maximal suppression of cholesterol synthesis was achieved at LDL cholesterol concentrations under 25 ug/ml (representing receptor saturating concentrations of LDL protein). There were no differences between the curves for LDL4 and LDL6 when they are plotted as a function of cholesterol content.

THE INFLUENCE OF LDL SUBFRACTIONS ON CHOLESTEROL ESTERIFICATION IN CULTURED HUMAN FIBROBLASTS

These studies measured the ability of the LDL subfractions isolated from FH patients on no medication and

during treatment with colestipol to deliver cholesterol to cells and stimulate esterification of the internalized cholesterol. Cultured normal human skin fibroblasts were incubated with 10% LPDS for 24 hours then incubated with graded amounts of LDL4 or LDL6 for 16 hours. The medium was changed to one containing 10% LPDS, the specific lipoprotein subfraction, and 9.9 mM ^{3}H-oleic acid complexed with albumin. The cells were incubated an additional 3 hours to allow incorporation of the label into cholesterol ester. The results for individual patients are shown in figures 13a (LDL4) and 13b (LDL6), while the mean lines for the groups of patients before and after treatment are shown in Figures 13c(LDL4) and 13d(LDL6). The stimulation of cholesterol esterification showed its maximal rate of increase over the range of 0 to 34 ug/ml LDL4 cholesterol and 0 to 40 ug/ml LDL6 cholesterol. This amount of cholesterol is representative of the amount of LDL protein needed to saturate the LDL receptor.

The different LDL preparations showed a great deal of intra-individual variation. When LDL4 and LDL6 stimulation of oleic acid incorporation into cholesterol esters was plotted against the total amount of LDL subfraction cholesterol added to the medium, the differences between individuals was larger than the differences between the LDL4 and LDL6 subfractions. There was no consistent difference between the amount of ^{3}H-oleate incorporated into cholesterol ester by cells incubated with LDL subfractions

isolated from untreated FH patients or subfractions isolated after treatment with colestipol. Those LDL particles with the highest content of esterified cholesterol were the most able to stimulate the incorporation of oleic acid into cholesterol ester.

DISCUSSION

Severe hypercholesterolemia in FH is the result of the 50% decrease in the number of functional LDL receptors. The causal relationship between the decreased receptor number and the elevated plasma cholesterol found in FH is supported by studies showing a decreased rate of catabolism of ^{125}I LDL in patients with FH[127,128,129]. This decrease in LDL catabolic rate is present with a normal to increased synthesis rate for apoB[127,128,129]. Other primary genetic causes of hypercholesterolemia show normal catabolic rates for LDL apoB and normal levels of LDL receptors. The primary defect in combined familial hyperlipidemia, for example, is believed to be an overproduction of LDL and VLDL, rather than a clearance defect[130,131].

Treatment for hypercholesterolemia may be approached from several directions. The methods generally fall into one of three categories: 1) interventions which decrease LDL synthesis (directly by dietary cholesterol and fat restriction or agents like lovastatin, indirectly by agents such as niacin, or "surgically" by maneuvers such as liver transplantation); 2) those which increase catabolism (using the bile acid sequestrants or charcoal[132], or surgically by distal ileal bypass); and 3) physical removal by plasmapheresis[133] or by hemoperfusion[134]. Appropriate selection among these agents for patient care requires an understanding of the mechanisms and the effects of each of

these agents in vivo. FH is a useful model to elucidate some of the mechanisms for drug effects.

The first line choice for the treatment of primary hypercholesterolemia are the bile acid binding resins colestipol and cholestyramine. These agents bind bile acids in the gut lumen and prevent their reabsorption in the terminal ileum. This diversion of bile acids induces increased hepatic synthesis of bile acids from cholesterol[135,136]. This, in turn, decreases the hepatic pool of cholesterol and will increase the rate of hepatic cholesterol synthesis[137]. Colestipol has been shown to promote LDL catabolism by increasing the number of hepatic LDL receptors in dogs[138] and in rabbits[139]. Turnover studies with ^{125}I-LDL following cholestyramine treatment show increased catabolic rates[140]. Turnover of cyclohexanedione-modified LDL is unchanged, thus implying that the major effect is via increased receptor mediated uptake of LDL[79].

Bile acid sequestrant therapy usually provides a 10 to 30 percent decrease in LDL cholesterol and apoB levels. These changes may occur with small increases in VLDL levels, but usually without any change in HDL levels[135,136,141]. In the current study, the effects of the hypolipidemic drug colestipol on plasma lipid and lipoprotein concentrations were evaluated prospectively in 23 patients with heterozygous FH. Preliminary studies involving niacin and

the combination of colestipol with niacin were done in an additional 9 patients with heterozygous FH as comparison groups to attempt to separate drug specific effects from general effects related to lowering of plasma LDL cholesterol.

The use of colestipol as a single agent resulted in a decrease in total plasma cholesterol and in LDL cholesterol without a significant change in HDL cholesterol (Table 1). Total plasma triglycerides were increased by the use of colestipol as a single agent. All of the studies to date, including the data presented in this thesis, have shown great individual variation in the response to treatment with bile acid sequestrants in patients with FH. One possible explanation for this is inherent allelic variation in the LDL receptor and/or its response to induction by bile acid sequestrant therapy. Other contributors to this variation would be inherent differences in the ability to increase the synthesis of bile acids, cholesterol, LDL, or possible differences in non-receptor mediated pathways of LDL degradation which may affect the hepatic response to the drain on the cholesterol pool.

Niacin is also an effective hypolipidemic agent[142]. While the primary effect seems to be through inhibition of free fatty acid mobilization from adipocytes, an inhibition of cholesterol synthesis has been shown when niacin has been used as a single agent[143]. The precise cellular mechanism

for these effects are not known. When parameters of LDL metabolism were studied in patients with type IIa hyperlipidemia and compared with normal controls, both the FCR and metabolic half-life of LDL apoB were unchanged, however the net LDL synthetic rate was reduced[140]. Comparable studies on VLDL metabolism in a group of patients with heterogeneous causes of hyperlipidemia showed a 21% decrease in VLDL synthesis in the group as a whole following the administration of niacin[84]. There were no significant changes in cholesterol excretion or sterol balance.

Niacin treatment of the patients with FH reported in this thesis caused a decrease in total plasma cholesterol of 24.0% with LDL decreasing by 31.5%. Niacin lowered plasma triglycerides by 26.6% (Table 1). This change was not significant because of the small number of patients, the great variation in initial plasma triglyceride levels and the individual differences in response to therapy. Treatment of patients with niacin also increased plasma HDL levels.

Many patients who fail to respond to single agent treatment will respond adequately to the combination of a bile acid sequestrant (such as colestipol) and niacin[74,81]. These two agents exhibit a synergism seemingly similar to that seen in dogs[138] and in man[144] following the addition of lovastatin to a bile acid sequestrant or ileal bypass surgery. In the latter studies, addition of lovastatin

resulted in decreased LDL synthesis and an increase in the FCR of LDL apoB. An increase in hepatic membrane LDL receptors was seen in the dog studies. There have been no similar studies to date on the effects of colestipol and niacin on LDL metabolism and whole body sterol synthesis in man.

In the studies presented here, niacin combined with colestipol produced a 27.1% drop in total cholesterol and a fall of 41.2% in LDL cholesterol; triglycerides decreased by 42.7% (Table 1). The clinical response demonstrated by the FH patients reported in this thesis are consistent with the results of previous longitudinal studies of patients with FH[74,81], except that the decreases in total and LDL cholesterol seen in the patients treated with the single agent niacin are greater than those usually reported. The response to niacin may be greater in that these patients represent a group selected to remain on niacin alone because of the degree of therapeutic response, or intolerance to the side effects of other therapeutic agents. Alternatively, the patients studied here may have been more compliant because of closer follow-up.

While neither plasma nor LDL cholesterol levels were normalized in these patients, data from both the Pooling Project[1] and the Lipid Research Clinics[74] would argue that these changes were physiologically significant and would if

maintained for a period of seven to ten years, decrease the risk of myocardial infarction by about 50%.

In view of the strict definition of FH used in this study, and the comparability of both the initial plasma lipid and lipoprotein values and the changes in plasma lipids and lipoproteins reported here to the responses reported by others, it is likely that the changes in mononuclear cell cholesterol homeostasis, mononuclear cell LDL receptor activity, plasma LDL composition and binding studies are valid and that the magnitude of the reported responses are appropriate.

Several previous studies have shown that freshly isolated mononuclear leukocytes respond in vitro to many of the same factors which influence hepatic cholesterol homeostasis in vivo. Young and Rodwell[37] demonstrated parallel changes in HMG CoA reductase activity in freshly isolated leukocytes and hepatic tissues from rats fed cholesterol (decreased activity), or cholestyramine (increased activity). In other studies, there were parallel decreases in HMG CoA reductase and LDL receptor levels in mononuclear cells freshly isolated from the blood of normal volunteers following cholesterol feeding[38].

McNamara, et al[42] have shown similar effects in human cells with cells isolated from patients with FH treated with cholestyramine, but not with probucol[145], another lipid lowering drug. Rates of cholesterol synthesis were doubled

in patients with FH receiving cholestyramine, but not those receiving clofibrate or probucol. Previous studies by Sundberg and Illingworth[44] showed that the rate of cholesterol synthesis from acetate and the rate of high affinity degradation of LDL were increased about two-fold in freshly isolated mononuclear leukocytes from patients with FH treated with colestipol as a single agent when compared to untreated patients. This effect was not seen in patients treated with niacin or the combination of colestipol and niacin. This study was mainly cross sectional, with longitudinal data being obtained on only 3 patients.

These studies provided the impetus for developing the hypotheses which have been evaluated in the research reported in this thesis. In these earlier studies, no stimulation of cholesterol synthesis or LDL receptor activity was seen in patients treated with niacin or the combination of colestipol and niacin. These results are consistent with changes seen in the livers of FH patients treated with cholestyramine[137]. Overall, the results of these previous studies suggest that relative changes in LDL receptor activity and cholesterol synthesis which occur in mononuclear cells from patients on differing dietary or drug regimens parallel the changes seen in other body tissues, particularly the liver.

In the current study, similar decreases in plasma cholesterol were produced by each of the drug regimens used.

Comparable lowering of total- and LDL- cholesterol was achieved with regimens using niacin in addition to a bile acid sequestrant, or niacin as a single agent. These treatments showed no increase in rates of cholesterol synthesis or LDL degradation. Colestipol as a single agent resulted in a 1.64-fold increase in 2-^{14}C-acetate incorporation into cholesterol in-vitro, as well as a 1.8-fold increase of the high affinity degradation of ^{125}I-LDL by freshly isolated mononuclear leukocytes (Table 2).

Given these results, the mechanism of the increased LDL receptor activity and rates of cholesterol synthesis cannot be a simple homeostatic response to decreased plasma cholesterol levels. This response also could not be a direct effect of colestipol on mononuclear cells since bile acid sequestrants are not absorbed by the body. The involvement of serum bile acid concentration in regulation is unlikely in view of the study of Nilausen and Meinertz[146] which reported that distal ileal bypass surgery produced only a transient increase in LDL receptor activity and rates of cholesterol synthesis in freshly isolated mononuclear cells, despite a permanent decrease in plasma total- and LDL- cholesterol. Ileal bypass surgery physically interrupts the enterohepatic recirculation of bile acids, thus presumably has the same effect as bile acid sequestrant therapy without drug use.

Because bile acid sequestrants are not absorbed by the body, the mechanism(s) of their actions on circulating mononuclear blood cells are not obvious. In the current study, as well as previous work by McNamara, et al[42,145] and Sundberg and Illingworth[44], no correlation was observed between initial plasma cholesterol levels and the rates of cholesterol synthesis or of high affinity degradation of ^{125}I-LDL in freshly isolated mononuclear cells from patients with FH. There was, however, an association between bile acid sequestrant use in the treatment of FH and a doubling of the rates of cholesterol synthesis and LDL receptor activity which is not related to the serum bile acid level[146].

These changes have not been seen with other agents including niacin, clofibrate and probucol[42,44,145], but have been noted in patients with FH who were treated by ileal bypass surgery[146]. This would imply that the mechanisms responsible for the increases in cholesterol synthesis and LDL receptor activity in mononuclear leukocytes isolated from FH patients following colestipol treatment described in the studies reported in this thesis or previously reported as occurring following ileal bypass[146] are attributable to factors other than a decrease in LDL cholesterol or changes in plasma bile acid concentrations. The hypothesis was therefore proposed that the most likely mechanism(s) for these effects in peripheral cells involved changes in the composition of LDL.

In mononuclear cells the rates of sterol synthesis may respond to many things including the magnitude of change in the concentration of LDL induced by drug therapy, variations in LDL composition, changes in the FCR for LDL, decreases in levels of serum bile acids, decreases in the intracellular pool of cholesterol, or fluctuations in the levels of one or more of the hormones known to affect lipid metabolism. The previously cited studies[42,44,145], have indicated that a simple response to plasma LDL cholesterol levels does not explain the responses of mononuclear leukocytes from FH patients to colestipol treatment.

Studies of bile acid regulatory effects upon hepatic cholesterol homeostasis in the hamster[147] have shown several different effects. In the hamster, whose low basal hepatic rates of cholesterol synthesis approximate the situation in man, cholic acid and chenodeoxycholic acid feeding also decreased hepatic cholesterol synthesis. In addition, since the absolute reduction in hepatic cholesterol synthesis could not make up for the increase in sterol absorption caused by the increased dietary bile acids, hepatic levels of cholesterol ester increased, receptor-mediated hepatic uptake of LDL was halved, and plasma LDL cholesterol levels doubled. Acute intravenous infusions of the bile acids (as taurine conjugates) had none of these effects.

The lack of direct effect of colestipol or serum bile acid concentration on rates of cholesterol synthesis or

receptor mediated degradation of LDL supports the idea that the mechanism is related to an increased flux of cholesterol through the liver and not via a direct effect on plasma bile acid levels. The study of Nilausen and Meinertz[146] suggests that serum bile acids are not involved, however, there are no reports on the direct effect of plasma bile acid levels upon cellular cholesterol homeostasis in man. Unlike the results of chronic bile acid sequestrant therapy, the increases in cholesterol synthesis and LDL receptor activity reported following ileal bypass are only temporary and return to normal in a matter of weeks[146]. Since plasma bile acid levels are permanently lowered by ileal bypass surgery, they would be unlikely mediators of the transitory changes in cholesterol synthesis and LDL receptor activity.

Further suggestions regarding possible mechanisms come from studies using other model systems. Reductions in fibroblast intracellular cholesterol pool size have been shown to increase cellular rates of cholesterol synthesis[94,101], as does a decrease in the cell membrane sphingomyelin content[101]. These increases are usually[94,101], but not invariably[148], linked to increases in the number of LDL receptors. Given the recent reports of sterol sensitive regulatory elements being involved in the regulation of HMG CoA reductase[34] and the LDL receptor[29], it is very likely that fluctuations in the intracellular cholesterol pool mediate at least some of these effects. LDL bathes the circulating mononuclear cells and thus may

also influence the net flux of cholesterol across the cell membrane as well as the phospholipid content of the membrane.

In concurrence with the studies of Witztum, et al,[101] and Teng, et al,[102] we found changes in the composition of LDL following colestipol treatment of patients with FH. The initial study to investigate the role of LDL as an intermediary in the responses of mononuclear cells to colestipol treatment was a cross sectional study of the composition of total LDL (Table 3).

Treatment with colestipol but not the combination of colestipol with niacin lowered the content of free cholesterol 10.3%. The content of triglyceride in total LDL was increased 40%. These responses to colestipol treatment yielded values which were indistinguishable from the free cholesterol and triglyceride content of total LDL isolated from normal volunteers. The content of protein and phospholipid components were not changed in their relative amounts present in total LDL.

Colestipol treatment decreased the ratio of free cholesterol-phospholipid, an index of surface composition, from .242 to .211 ($P<0.02$). The ratios of free cholesterol-esterified cholesterol decreased from 0.39 to 0.36 and the ratio of total cholesterol-protein increased from 1.472 to 1.567. While these changes are not statistically

significant, they suggest further investigations as part of a larger longitudinal study.

Eight patients with well characterized FH participated in the longitudinal study of the effects of colestipol treatment on total LDL composition. When measured in absolute terms, LDL protein was lowered by 24%, along with a 20.7% decrease in LDL free cholesterol and a 14.6% drop in LDL cholesterol ester with no change in LDL phospholipid content and an increase in triglyceride (Table 4a).

The changes in absolute composition are difficult to interpret since three components decrease, one increases and one is unchanged. The differences could be explained on the basis of a decreased number of LDL particles circulating, changes in the composition of the LDL particles present, changes in the size of the circulating particle, or a combination of all three. Since there is only one molecule of apoprotein-B present on each LDL particle, the decrease in LDL protein content indicates a decrease in the number of circulating LDL particles. The changes in free cholesterol and cholesterol content could be due to a decrease in the number of LDL particles or to a change in the composition of the particles present. The lack of change in the phospholipid content and the 43% increase in triglyceride content in the presence of a decreased number of particles would argue for a change in composition and/or size of the remaining particles.

After correcting for the changes in protein content, the difference in absolute content of free cholesterol disappeared (Table 4a). The amount of total cholesterol per unit protein increased to the same degree as the cholesterol ester content. The difference in cholesterol ester was not statistically significant, possibly due to the small number of patients studied. The content of triglyceride per unit protein increased 43%. There was also a tendency towards an increase in the phospholipid content per unit protein. These latter changes were not significant because of the great individual variation in response. These results, which correct for changes in the number of particles still do not differentiate between the other issues of change in particle size or change in composition.

Expression of the data in terms of weight percent of the components of the lipoprotein particle has been done in two other studies of human lipoprotein composition[97,102] in order to address particle composition. When addressed in terms of the contribution that each component made to the total particle, free cholesterol decreased, while triglyceride increased (Table 4b). The relative percent contributions of cholesterol ester, protein, and phospholipid were not changed significantly. The ratio of FC/PL decreased as did the ratio of free to esterified cholesterol. A ratio of the sum of the surface component weights (free cholesterol, protein, and phospholipid) to core component weights (cholesterol ester and triglyceride)

was derived to address the issue of size changes versus composition changes. This surface/core ratio decreased from 1.667 to 1.505 but the change was not statistically significant.

Despite the relative frequency of niacin as an additional agent to lower plasma cholesterol in patients with FH, it was infrequently used as a single agent during the majority of these studies. The reasons for this were 1) the frequency of poorly tolerated side effects, and 2) the availability of lovastatin (mevinolin), an equally effective drug with far fewer side effects. By the time the longitudinal LDL composition and functional studies began, most of the patients with FH who had been on niacin alone were switched to lovastatin therapy. The results from the patients who were available for study during the cross sectional portion of the study suggest that the actions of niacin may not produce the composition changes seen with colestipol, and that responses to the combination of colestipol with niacin may be intermediate to the actions of either agent used alone.

Studies by Krauss, et al[97],Teng,et al,[102] and Witztum[101] have shown that particles isolated in the LDL density range are both physically and metabolically heterogeneous. In the studies reported in this thesis, the composition of two LDL subfractions were measured to determine if they were influenced selectively: because LDL

is a heterogeneous series of particles, only a portion of the LDL might be affected.

The decrease in the amount of LDL4 protein present in FH patients following colestipol treatment was half that of the decrease in free cholesterol (Table 5a). The amounts of cholesterol ester and phospholipid present did not change significantly. Triglyceride increased, on average, by 29.6%, but this change was not statistically significant. After correcting to constant protein content to normalize for changes in the number of particles of LDL, the results were similar. The ratio of free cholesterol-protein decreased 11.2% while the ratio of triglyceride-protein increased 30.5%, without changes in the ratios of cholesterol ester-protein, total cholesterol-protein, or phospholipid-protein.

The disproportionate differences between changes in protein content of total LDL with colestipol therapy and the changes in free cholesterol and triglyceride content would support a change in composition within the LDL4 fraction and would argue against the observed changes being due solely to a decrease in particle number. Fewer particles are definitely present following drug treatment, but they also represent a different spectrum of particle compositions.

The relative content of LDL4 free cholesterol decreased with no change in the remainder of the components (Table 5b). There was a tendency towards an increase in

triglyceride, but it was not significant. There were significant decreases in the ratios of free to esterified cholesterol, free cholesterol-phospholipid, and total cholesterol-phospholipid. There was no change in the ratio of surface to core components. Overall, these changes indicate that colestipol therapy leads to a depletion of cholesterol in the LDL particles, even though the particle may be of similar size (inferred from the lack of change in surface to core ratio).

The absolute level of LDL6 free cholesterol decreased substantially, as did protein, cholesterol ester and phospholipid (Table 6a). The decreases in the content of each of the components present was about the same, except free cholesterol, in which the decrease was about half again that of the other components. There was no difference in the amount of LDL6 triglyceride present. When corrected for changes in the content of protein, there were no differences in the content of the other components. There was a significant decrease in the ratio of free cholesterol to protein. This would suggest that the changes seen in absolute levels of LDL6 constituents were related to a decreased number of LDL6 particles rather than a major change in composition. The change in free cholesterol is most likely due to increased turnover or decreased residence time in the plasma. This has been suggested by previous studies[100,101].

The relative content of free cholesterol decreased significantly; however, there were no significant changes in the content of cholesterol ester, protein, or phospholipid (Table 6b). There was a tendency towards an increase in triglyceride content but it did not achieve statistical significance. There were decreases in the ratio of free cholesterol to phospholipid (17.4%) and to cholesterol ester (13.0%) (Table 6b). There were no significant changes in the ratio of surface to core components or in total cholesterol to phospholipid. Again, this would support minimal changes in particle composition with a major change in the number of particles.

There was a three-fold difference in the decrease in LDL6 protein compared to the decrease in LDL4 protein. Since the absolute amount of apoprotein B in each LDL particle is fixed, the changes in protein reflect the numeric distribution of particles in each LDL density range. The observed changes are consistent with the majority of the decrease in the total number of LDL particles being attributable to decreases within the heavier LDL6 fraction with a lesser change in the number of particles being observed in the LDL4 subfraction.

Overall, the composition changes are consistent with a differential metabolism of the LDL subfractions, i.e., drug therapy with colestipol inducing the catabolism of large, triglyceride poor, abnormally cholesterol rich LDL particles

which accumulate in the untreated patients with FH to a less dense, cholesterol depleted particles which more closely resemble those seen in normal individuals. Support for a change in particle size is provided by the tendency for total LDL to have a decreased surface to core ratio without there being any similar decrease in the surface to core ratios of the LDL subfractions. If the shift was only to a smaller particle, decreases in the content of cholesterol ester and triglyceride (core constituents) would be expected; in fact the cholesterol ester content remained constant and triglyceride increased following treatment with colestipol. This provides direct evidence that there are a prevalence of LDL particles of a different composition following colestipol treatment than were present before treatment.

There are several possible ways in which the alterations in LDL composition may cause the increases in mononuclear cell cholesterol synthesis and LDL receptor activity. The first could be directly related to the decreased content of free and esterified cholesterol in LDL. With less cholesterol per LDL particle available to the cell, if the rate of LDL uptake remained constant, there could be a relative decrease in the intracellular cholesterol pool, which in turn, might cause activation of cholesterol synthesis and LDL receptor synthesis via the sterol sensitive elements present in the regulatory regions of those two genes[29,34]. This would account for both the

observed increases in rates of cholesterol synthesis and in LDL receptor activity and provide the linkage between cholesterol synthesis and LDL receptor number. It does not account for the fact that plasma concentrations of LDL even after treatment of FH are still roughly 10-fold higher than levels needed to saturate the LDL receptor.

The effect(s) of clofibrate and probucol on LDL composition have not been studied in detail. It may be inferred from the two patients studied on niacin and the 9 patients studied on the combination of niacin with colestipol, however, that the changes produced by these treatments may not be the same as those produced by colestipol alone. If the free cholesterol content of LDL were not altered by treatment with lipid lowering agents other than colestipol, this might provide a mechanism to explain the differences seen in LDL receptor activity and rates of cholesterol synthesis in freshly isolated mononuclear leukocytes.

The content of cholesterol within the lipoprotein particle may not be the total explanation for changes in cholesterol homeostasis. The relative content of phospholipids and free cholesterol present in the light fraction of LDL may also influence cellular cholesterol pools and cholesterol homeostasis. Studies using free cholesterol in liposomes with phosphatidylcholine (containing little or no sphingomyelin) incubated with

fibroblasts[149] showed that when the free cholesterol-phosphatidylcholine ratio (FC/PC) was 1:1 or greater there was net uptake of surface free cholesterol from the liposomes and accumulation of the cholesterol as intracellular cholesterol ester. In contrast, when the FC/PC ratio was 0.5:1 there was a slight decrease in cellular cholesterol ester implying a net efflux of cellular cholesterol.

The free cholesterol-total phospholipid ratio found in LDL4 isolated from patients with FH was 25% lower following treatment with colestipol than at baseline (Table 5b). On the basis of the liposome studies it is likely that such changes could result in alterations of cholesterol flux with an increased cholesterol efflux from the cell's plasma membrane to LDL following treatment with colestipol. The interactions of other lipoprotein particle constituents, especially protein, cholesterol ester and sphingomyelin makes direct comparisons with liposomes difficult. The change in LDL4 free cholesterol would argue for at least a decreased rate of cellular cholesterol accumulation, or potentially a slow efflux of cholesterol from cells in contact with the LDL4.

Sphingomyelin may also mediate cellular responses. The previous report of Jadhav, et al[100] showed decreases in the content of sphingomyelin in total LDL isolated from treated FH patients. This was believed to be due to the decreased

residence times of LDL in plasma following treatment of FH. The greatest decrease in sphingomyelin content of LDL is in the lighter LDL4 (1.020-1.040 gm/cc) particles. This could potentially decrease the cell membrane content of sphingomyelin as the lipoprotein phospholipids can equilibrate with cell membrane phospholipids by direct exchange as well as following cellular uptake of the LDL particle. Increased LDL sphingomyelin levels are associated with the prolonged catabolism of LDL seen in FH, and have been associated with decreased levels of cholesterol synthesis and with suppression of LDL uptake in cultured fibroblasts[94]. The mechanism responsible for this is unknown.

Total LDL isolated from FH patients on colestipol showed a 14% decrease in the percentage of the total phospholipid present as sphingomyelin when compared to total LDL isolated from untreated FH patients (Table 7a). In a follow-up study involving a different group of FH patients, a 7.7% decrease in sphingomyelin in the LDL4 subfraction (Table 7b) was demonstrated with no significant change in the content of sphingomyelin in LDL6 (Table 7c). There were no significant changes in the content of any other phospholipids (phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, or lysophosphatidylcholine) in the LDL subclasses tested (Tables 7b,7c).

Compared to normal subjects, the patients with FH had lower levels of phosphatidylinositol and higher levels of lysophosphatidylcholine. This may have been related to oxidation of phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine to their lyso- forms either in vivo or in vitro prior to analysis. These potential differences need further characterization.

If the changes in LDL composition described in samples from patients with FH following colestipol treatment are of metabolic significance, the particles must have a measurable difference in their interaction with cells compared to native LDL isolated from untreated FH patients. This was directly evaluated in a series of experiments in which the interactions of LDL particles with cultured human fibroblasts were studied.

High affinity receptor-mediated binding of ^{125}I-LDL occurred up to a protein concentration of 25 ug/ml for samples of LDL4 isolated during dietary treatment, or 50 ug/ml for samples of LDL4 isolated during colestipol treatment (Figure 8a). At these concentrations saturation of the LDL4 binding sites occurred. Binding site saturation did not occur at the tested concentrations of LDL4 isolated from normal subjects. There was insufficient material available from normal controls to carry the binding curves to higher concentrations.

There was a decrease in the slope of the binding curve towards that of the normal subjects for ^{125}I-LDL4 isolated from FH patients following treatment with colestipol. This, it must be remembered, is concurrent with normalization of LDL4 free cholesterol, cholesterol ester, triglyceride and sphingomyelin concentrations.

The LDL6 isolated from FH patients during dietary therapy alone exhibited only slightly higher binding to normal fibroblasts than LDL6 isolated during colestipol treatment. The binding increased rapidly until 15 ug/ml LDL6. After this point binding continued to increase but at a much lower rate, implying saturation of the high affinity binding site had occurred (Figure 9a).

There were no changes in ^{125}I-LDL subfraction internalization. The internalization of ^{125}I-LDL4 and LDL6 were concentration dependent (Figure 8b,9b). There were changes in the slopes of the internalization curves at the same concentrations of LDL subfractions at which saturation of binding occurred. Saturation was not seen with LDL4 isolated from normal subjects, presumably because of the inability to provide sufficient concentrations of normal LDL4 to saturate the LDL receptor. There were no differences in slope which might relate to a difference in the ability of the bound LDL subfraction to be internalized by normal fibroblasts.

High affinity receptor-mediated degradation of ^{125}I-LDL4 isolated from patients decreased following colestipol treatment (Figure 8c). This was expected. Degradation should be proportional to binding and LDL4 isolated from FH patients prior to treatment had higher rates of receptor-dependent binding and receptor-mediated degradation than LDL4 isolated after colestipol treatment. A similar relationship between decreased LDL4 binding and degradation is seen with LDL4 isolated from normal subjects. Thus the differences in high affinity LDL4 degradation reflected the changes in binding affinity of the lipoproteins. There were no differences between the curves for high affinity receptor mediated degradation of LDL6 isolated from patients with FH before or after colestipol treatment, or from normal subjects (Figure 9c). This is consistent with the lack of difference seen in the binding and internalization curves.

The composition changes discussed in the preceding sections were reflected a 3.67-fold increase in the LDL4 Kd from 0.58×10^{-5} ng/ml to 2.18×10^{-5} ng/ml ($p<0.01$ by F statistic) as shown in Table 8. The post-treatment figure is virtually identical to the 2.42×10^{-5} ng/ml value for normal subjects. This represents a decrease in the LDL receptor affinity for the LDL4 particles present in the circulation of FH patients following colestipol treatment. The decrease in the Kd of LDL6 following colestipol treatment was not significant.

The binding affinity of LDL4 isolated following colestipol treatment is the same as the affinity of LDL4 isolated from normal subjects. When the data were subjected to Scatchard analysis, the differences between the mean lines for LDL4 from normal subjects and LDL4 from FH patients before and after treatment with colestipol are consistent with the differences predicted by the computer analysis (Figure 11a, 11c). The data for LDL6 show no significant differences (Figure 11b, 11d).

Preliminary data show that LDL4 isolated from two patients treated with colestipol was about 57% as effective as their baseline LDL4 in competing with LDL4 isolated before treatment for LDL binding sites (IC50 12ug/ml vs 7.1 ug/ml). LDL4 from normal subjects was about 55% as effective in competing for LDL binding sites (IC50 13 ug/ml) as seen in Figure 10a. These changes are of the same magnitude and direction as the increases in Kd described. There was insufficient data generated in these studies to allow statistical analysis of the differences. Different IC50 values were also noted for LDL6 samples isolated from patients with FH before and after treatment with colestipol, and for that isolated from normal subjects (65, 51, 29 ug/ml respectively). The relationship (if any) of these changes to the calculated dissociation constants need further investigation.

The pattern of changes in Kd are consistent with differences shown in the binding and competition curves. This supports the concept that the LDL4 isolated from untreated FH patients has a higher affinity for the LDL receptor than LDL4 isolated following colestipol treatment or LDL4 from normal controls. The values for Kd and LDL concentration at receptor saturation are consistent with published values[150].

These results are expected given that the normal LDL receptor in vivo will preferentially select those LDL particles with the highest affinity leaving the lower affinity particles in the plasma and thus available for analysis. The FH patient, having a defective receptor, will accumulate many particles with a higher binding affinity which would be cleared by a normal receptor. Following treatment with colestipol, the number of functional LDL receptors increases with a resultant increase in LDL clearance from plasma. As the residence time for LDL4 in plasma decreases towards normal, the composition of LDL4 from the patient treated with colestipol becomes more like the LDL4 isolated from normals, and the FH LDL4 acquire a binding affinity closer to normal. Since normal levels of LDL receptor activity and normal rates of LDL catabolism are not always achieved, the LDL4 isolated from any given patient with FH being treated with colestipol may continue to have a higher binding affinity for the LDL receptor than LDL4 from normal controls.

The basis for this difference is probably related to the differences in composition. Differences in the content of sphingomyelin on the lipoprotein surface could affect either the charge density (by displacing phosphatidylserine, phosphatidylethanolamine, or phosphatidylinositol) or membrane fluidity because of steric hindrances imposed by the N-linked fatty acid attached to the second carbon. Either of these could affect the ability of the apoB binding site to be recognized by the LDL receptor or increase the chances of the particle detaching from the receptor before the complex is internalized. Increased sphingomyelin content has been shown by Gatt, et al[94] to decrease LDL uptake via the LDL receptor and to decrease cellular cholesterol synthesis.

Cholesterol ester content has also been shown to have an impact on lipoprotein interaction with the receptor. Increases in cholesterol ester content decrease the binding affinity of LDL particles for the LDL receptor[151]. The mechanism for this is not known. As cholesterol ester is primarily located in the core of the particle, the most obvious mechanism would involve changes in the particle radius affecting the presentation of the LDL binding site to the receptor and thus the affinity of the interaction. The data for FH patients presented here associates a decreased cholesterol ester content with increased binding affinity of the lipoprotein, however, the change in cholesterol ester

Other parameters of cellular cholesterol homeostasis which were measured were the ability of the internalized LDL cholesterol to suppress cholesterol synthesis (Figures 12a-d) and to stimulate cholesterol esterification (Figures 13a-d). The ability of the LDL fractions to suppress rates of cholesterol synthesis was maximal at LDL cholesterol concentrations under 25 ug/ml (representing receptor saturating concentrations of LDL protein). There were no significant differences between the curves for LDL4 (Figures 12a,12c) and LDL6 (Figures 12b,12d) when the curves were plotted as a function of cholesterol content. There was no difference between LDL subfractions isolated from untreated FH patients, from the same patients after colestipol treatment or LDL subfractions isolated from normal volunteers in this respect.

The stimulation of cholesterol esterification showed its maximal rate of increase over the range of 0 to 34 ug/ml LDL4 cholesterol (Figures 13a,13c) and 0 to 40 ug/ml LDL6 cholesterol (Figures 13b,13d). These amounts of cholesterol are provided by of the amount of LDL protein needed to saturate the LDL receptor. This again supports LDL receptor binding as the limiting step in the regulation of cellular cholesterol homeostasis.

The LDL subfractions showed a great deal of intra-individual variation in their abilities to stimulate oleic acid incorporation into cholesterol ester. When LDL4 and

LDL6 stimulation of oleic acid incorporation into cholesterol ester were plotted against the total amount of LDL subfraction cholesterol added to the medium, differences between individuals were greater than the differences noted between the LDL4 and LDL6 subfractions (Figures 13a,13b). There were no consistent differences between the amounts of ^{3}H-oleate incorporated into cholesterol ester by cells incubated with LDL subfractions from FH patients isolated before or after treatment with colestipol.

Most of the difference seen in the ability of the LDL preparations to stimulate cholesterol ester formation in cultured normal human fibroblasts was related to the relative amount of esterified cholesterol present in the particle. Those LDL particles with the highest content of esterified cholesterol showed the most ability to stimulate the incorporation of oleic acid into cholesterol ester. The two apparent outlying lipoproteins (pretreatment NC and DG) both had a very high cholesterol ester content compared to other FH patients both in the LDL4 (40.11% and 38.07% versus 33.68%) and in the LDL6 (46.1% and 41.6% versus 36.16%). NC also had an abnormally low binding affinity before treatment (Table 8) compared to other FH patients. No LDL receptor binding data were obtained on DG before treatment with colestipol. The curves for LDL4 samples isolated from these two patients following treatment with colestipol were similar to those of the other patients studied.

The lipoprotein subfractions functioned to suppress cellular cholesterol synthesis as would be expected from the cholesterol content of the lipoprotein. In the same manner, the incorporation of oleic acid into cholesterol ester was stimulated by incubation with the LDL subfractions. This is consistent with the lack of changes in internalization and degradation of LDL subfractions by normal fibroblasts and implies that once the lipoprotein binds to the receptor, the cholesterol is delivered to the cell and regulates the subsequent events of cholesterol homeostasis in a normal manner.

CONCLUSIONS

Treatment of patients with FH using colestipol is effective in lowering plasma concentrations of LDL cholesterol. This reduction of circulating LDL cholesterol levels provides a decreased cholesterol supply to peripheral cells as reflected by increased rates of cholesterol synthesis and LDL receptor activity in freshly isolated mononuclear leukocytes. The results of these leukocyte studies extend the results of previous reports and confirm that treatment of heterozygous FH patients with a bile acid sequestrant as a single agent causes an increase in the rates of cellular cholesterol synthesis and LDL uptake which are presumably related to depletion of the hepatic cholesterol pool. The increases in mononuclear leukocyte rates of cholesterol synthesis and receptor-mediated degradation of LDL do not represent a simple response to decreased LDL cholesterol levels in the plasma. As colestipol is not absorbed by the body, an indirect mechanism is proposed.

There is both a decrease in the total number of LDL particles present in plasma and a change in the density distribution and composition of those particles which remain. There is a smaller decrease in the number of particles present in the LDL4 density range (1.020-1.040 g/cc) as compared to the larger falls measured in the LDL6 density range (1.040-1.055). Consistent with the changes

seen in the composition of total LDL isolated from patients with FH before and after treatment with colestipol, both LDL4 and LDL6 reflected decreases in free cholesterol and ratio of free to esterified cholesterol. However, there were no decreases in the relative content of cholesterol ester of either subfraction. Triglyceride content of the LDL4 subfraction increased but despite its magnitude, the change was not significant.

This would suggest both a decline in the total number of LDL particles and a shift in the relative distribution and composition of LDL4 and LDL6 towards lighter particles as shown by the decreased cholesterol to protein and free to esterified cholesterol ratios. The particles also contain relatively more triglyceride and less free cholesterol as evidenced by the increase in the triglyceride to protein ratio and the decrease in both the ratios of free cholesterol to phospholipid and free to esterified cholesterol. The changes in LDL6 are more likely to reflect free cholesterol depletion of the particle than a change in size.

The size changes are supported by decreases in the cholesterol to phospholipid ratio reflecting a decrease in core size. Composition change following colestipol therapy is documented by a shift in the ratio of two surface components of the particle, free cholesterol and phospholipid. The surface phospholipids also contained

relatively less sphingomyelin following colestipol treatment.

These composition changes are reflected in a decreased binding ability of the altered lipoprotein with LDL receptors on cultured normal human fibroblasts when compared to the same lipoprotein fraction isolated before colestipol treatment. The published data indicate that LDL4 may be more actively catabolized than LDL6 and is the fraction whose FCR is affected most by colestipol treatment[104]. This increase in FCR reflects a greater receptor mediated rate of clearance from plasma, and was assumed to reflect a greater affinity of LDL4 for the receptor. The particles which were isolated and studied directly, however, had a measured decrease in the binding affinity.

The best explanation for this seeming contradiction is that the increased turnover rates for LDL seen in-vivo following colestipol treatment reflect an induction in hepatic LDL receptors as a relatively direct effect. This increase in LDL receptor number may provide the largest contribution to increased LDL clearance as more receptors gives more sites for uptake. The LDL particles are cleared by the receptors in relative order of decreasing affinity i.e. the particles with the highest binding affinity are taken from plasma first. This would leave the particles with lesser binding affinities available in the plasma for analysis.

The LDL isolated from patients with FH before treatment with colestipol reflects an abnormal preponderance of higher affinity particles which are cleared rapidly when the hepatic LDL receptor number is increased by drug treatment. Following colestipol treatment the peripheral cells are exposed to this low affinity LDL and upregulate their cellular LDL receptors and rates of cholesterol synthesis. Thus a drug like niacin, which is presumed to decrease LDL levels by decreasing net LDL synthesis would not be expected to have the same effects as colestipol on LDL composition or peripheral LDL receptor activity or rates of cholesterol synthesis even with the same observed net decrease in plasma LDL cholesterol. The preliminary studies of LDL composition and mononuclear leukocyte cholesterol homeostasis reported here support this view but further study is indicated to confirm these speculations.

The LDL4 and LDL6 particles isolated before or after treatment with colestipol were not different in their ability to regulate intracellular synthesis of cholesterol or storage as cholesterol ester under the steady state conditions studied. Thus, the changes seen in freshly isolated mononuclear leukocytes isolated from FH patients following treatment with colestipol may be mediated by changes in LDL composition, predominantly in the LDL4 fraction by the mechanisms outlined above, rather than by changes in post-receptor cholesterol homeostasis.

FUTURE DIRECTIONS

There are a number of different directions suggested by the studies reported in this thesis. The differences in receptor binding in-vitro should be expanded to human LDL turnover studies in order to simultaneously measure the effects of colestipol treatment on in-vivo rates of light and heavy LDL catabolism. It would also be of value to know whether these receptor binding affinity changes would affect the relative amounts of light and heavy LDL cleared by the receptor and non-receptor mediated pathway. This could be addressed through the use of chemically modified LDL using agents such as cyclohexanedione which abolish the receptor mediated uptake of LDL. These studies would allow greater understanding of normal lipoprotein regulation in man and the mechanisms of the drugs which are used for a lifetime to treat elevated plasma cholesterol.

Further studies on the effects of other classes of lipid lowering drugs upon LDL composition and on LDL receptor binding need to be done. The metabolic effects of lovastatin and its congeners are only beginning to be understood. Those of the fibric acid derivatives and probucol are not well characterized, and those of niacin are known only on a phenomenonological level. The wide usage of these pharmaceuticals in patients with hypercholesterolemia deserves further attention at the whole animal or human level as well as at the cellular level.

More questions directed at the mechanism(s) of the clinically observed effects include: Is the altered binding affinity related to free cholesterol depletion causing an increased fluidity in the particle surface? There are reports of cholesterol loading of HDL increasing microviscosity[154,155], but none regarding LDL or lipoprotein binding. This could be tested in two ways. The first would be to load the isolated lipoprotein particles with free cholesterol by exchange,then measure the binding of the lipoprotein to the LDL receptor and to measure the fluidity of the particle. The second would be to take the LDL (cholesterol loaded or native) and incubate it with whole serum. This would allow LCAT to esterify the surface free cholesterol and cholesterol ester transfer protein to transfer the new cholesterol ester to HDL. The fluidity of this free cholesterol depleted particle could then be measured with fluorescence polarization using probes such as perylene and diphenyl hexatriene, and the receptor binding measured.

Further questions include whether the changes in surface phospholipids cause a fluidity or charge density change thus affecting binding. This could be addressed by altering the phospholipid composition of the lipoprotein particles and increasing the total phospholipid content by exchange with synthetic liposomes. A related issue is whether there is an effect on the fibroblast receptor from the phospholipid composition of the cell membrane. There

are several studies which give some evidence for an effect of phospholipid substitution on receptor binding and internalization independent of fluidity changes in the cell membrane[156,157,158].

Additionally, the receptor binding site could be mapped using the range of monoclonal antibodies which have been developed to different epitopes of the region near the binding site. Could these antibodies detect a conformational change which causes an altered affinity for the receptor?

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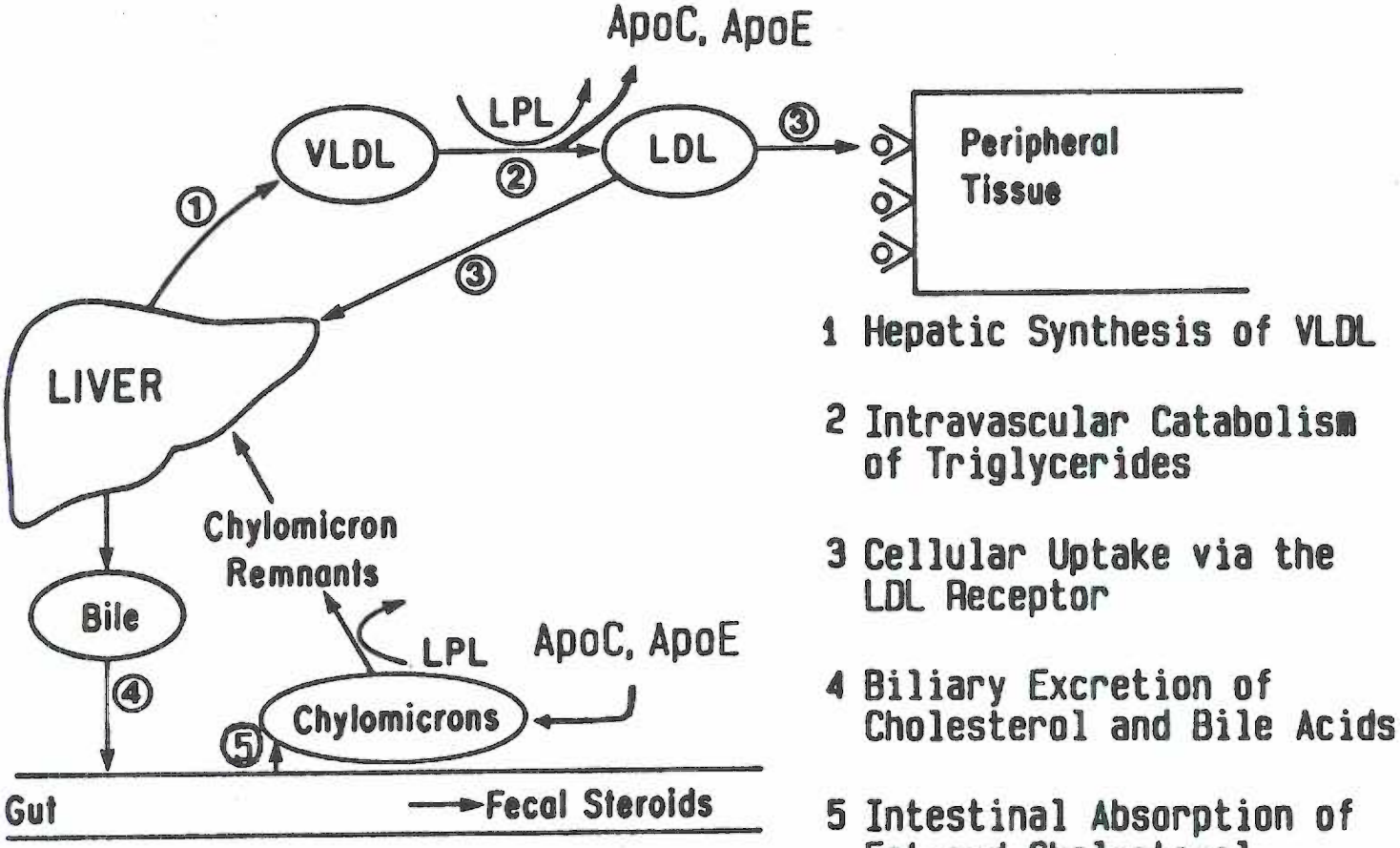
Figure 1. CHOLESTEROL AND LIPOPROTEIN METABOLISM

Dietary fats and cholesterol are taken up from the gut lumen into enterocytes and packaged into large apoB containing lipoproteins called chylomicrons. The chylomicrons enter lacteals in the gut wall and make their way through the lymphatic system to enter the circulation at the superior vena cava. In the circulation these particles acquire apoC and apoE by exchange from HDL. One of the C apoproteins (CII) serves as an activator for lipoprotein lipase (LPL). The apoB and apoE act as recognition signals for uptake by the chylomicron remnant receptor. Intravascular catabolism of chylomicrons with loss of triglyceride to peripheral cells and to adipocytes yield chylomicron remnants which are taken up by the liver.

The liver repackages dietary cholesterol and fat along with endogenously synthesized cholesterol into VLDL. VLDL contains apoB as an intrinsic structural protein, as well as associated apoC and apoE. Intravascular catabolism of VLDL with loss of some of the triglyceride and some of the apoC leads to IDL and with further loss of triglyceride and apoC and apoE to LDL. LDL is the metabolic endproduct of this cascade. LDL then circulates in the plasma until it is bound by the receptor and taken up by cells. The first step in the cellular metabolism of LDL is the binding of the lipoprotein particle to a specific high affinity receptor on the surface of the cell membrane. After LDL binds to the receptor, the complex is internalized via endocytosis. Dissociation of the receptor-LDL complex and release of the receptor from the endosome occurs before fusion of the endocytic vesicle with the lysosome. The internalized LDL maintains its integrity until the endocytic vesicle fuses with a lysosome. The LDL receptor is recycled to the cell surface. The pathways are not yet clear, but are thought to involve at least two distinct types of vesicles.

In the lysosome, enzymes hydrolyze the LDL apoB to amino acids and oligopeptides and release the cholesterol ester in the core. Cholesterol ester is broken down by lysosomal acid hydrolase allowing free cholesterol to enter the cytosol and become available for metabolism. Free cholesterol both suppresses HMG CoA reductase which decreases endogenous cholesterol synthesis and it activates acyl CoA:cholesterol acyl transferase, thereby facilitating storage of cholesterol as the ester. When a cell has accumulated sufficient cholesterol for its needs, synthesis of the receptor is suppressed, and the number of cell surface receptors decreases. This decreases the cellular uptake of cholesterol and prevents the accumulation of excessive amounts of cholesterol in either the free or esterified form.



LIPOPROTEIN METABOLISM

Figure 2. Time curve for the incorporation of 2-^{14}C-acetate into sterols by freshly isolated normal human mononuclear leukocytes. Cells were incubated for up to 6 hours with 1.35 umoles/ml 2-^{14}C-acetate. Sterols were separated by thin layer chromatography and the incorporation of label into each fraction was measured. Results present the mean and SEM for 3 experiments.

FIGURE 2. TIME COURSE FOR THE INCORPORATION OF 2-14C ACETATE INTO STEROLS

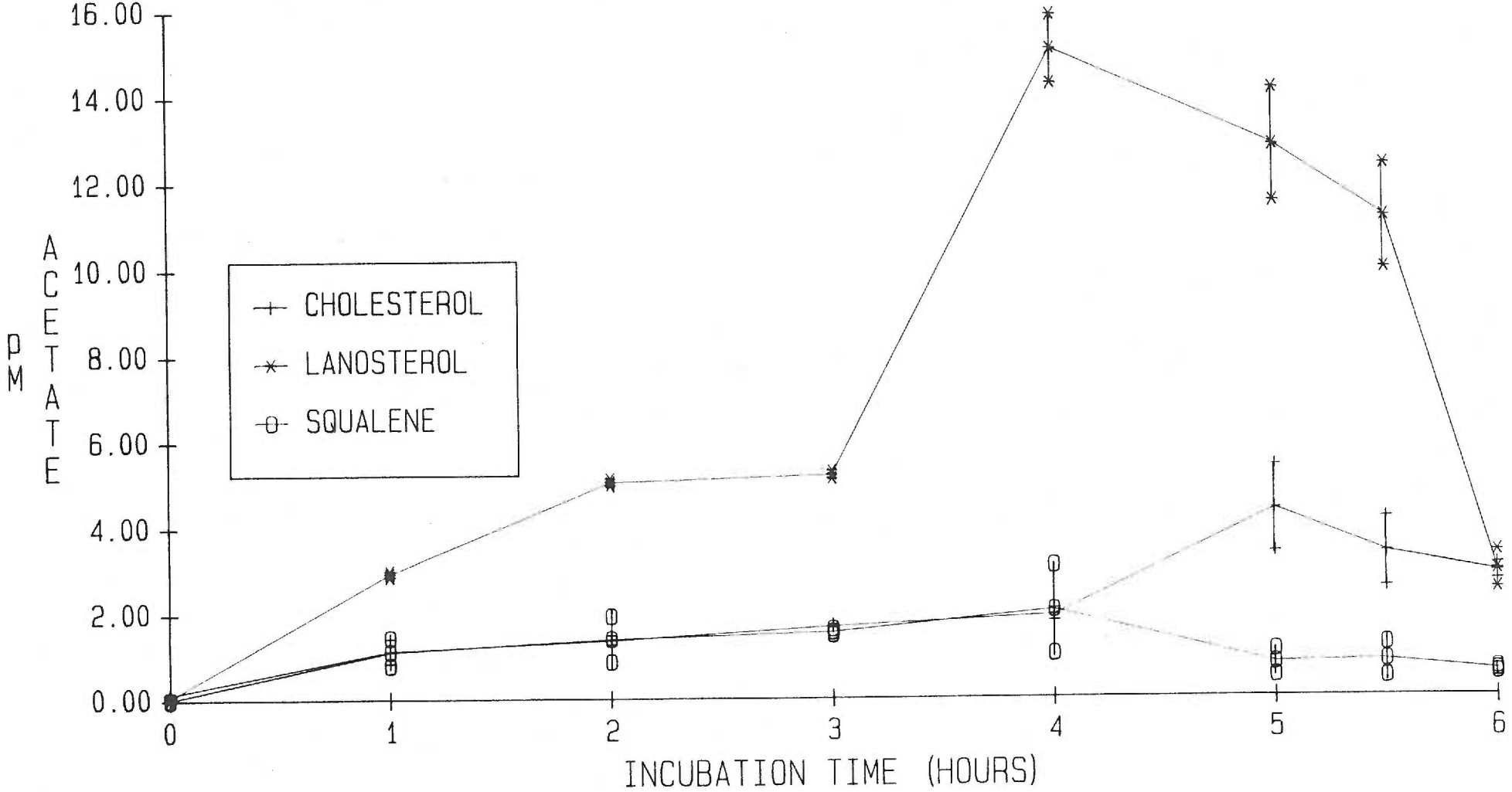


Figure 3. Incorporation of 2-^{14}C-acetate by freshly isolated normal human mononuclear leukocytes into sterols as a function of acetate concentration. Ten million freshly isolated mononuclear cells were incubated for 5 hours at 37 C with acetate concentrations from 0.45 umoles/ml to 3.35 umoles/ml. Results shown are the mean and SEM of 3 duplicate incubations.

FIGURE 3. INCORPORATION OF 2-14C ACETATE INTO STEROLS
EFFECT OF ACETATE CONCENTRATION

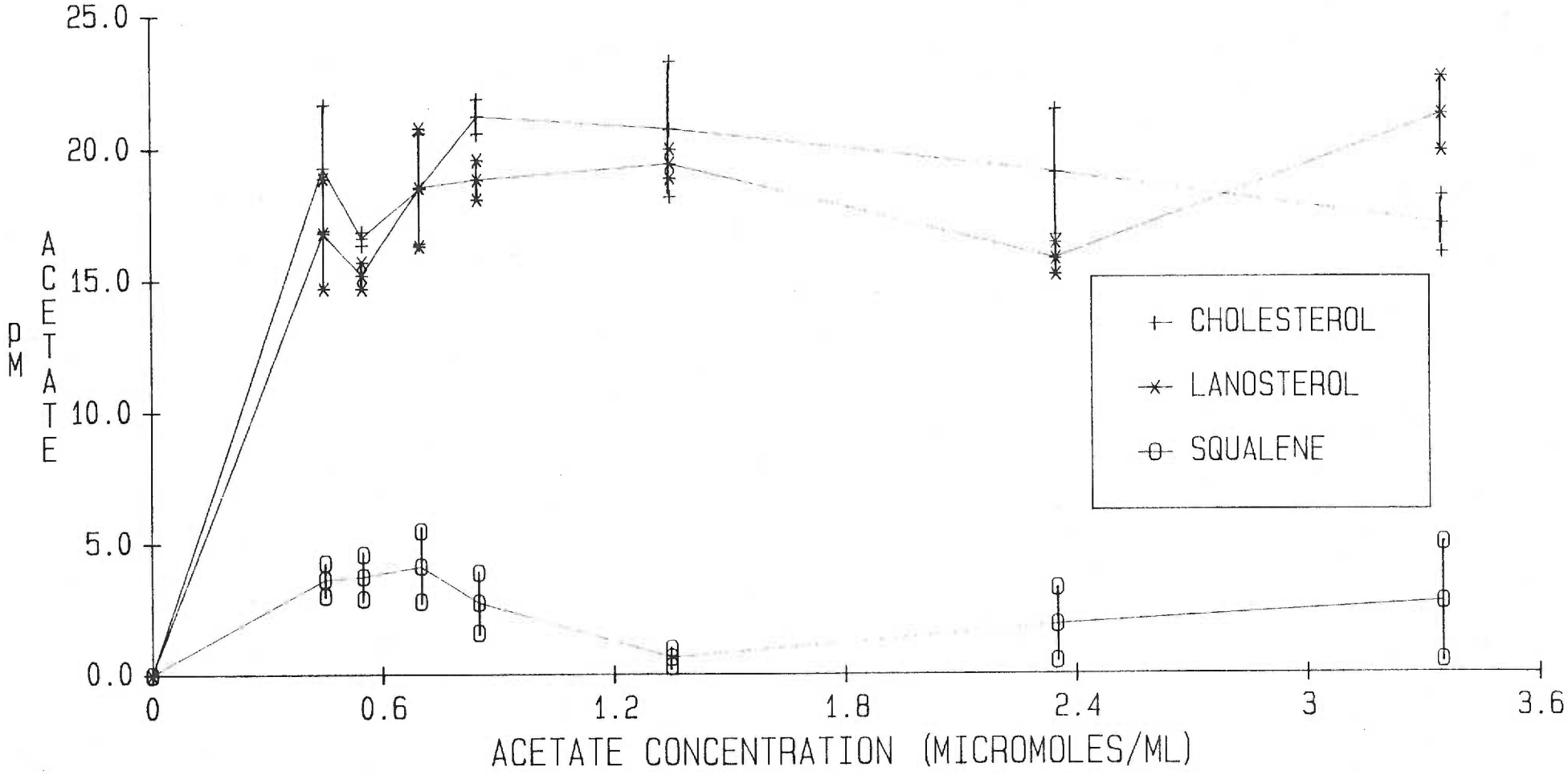


Figure 4. The effect of cell number on the degradation of ^{125}I-LDL by freshly isolated normal human mononuclear leukocytes. Total degradation of ^{125}I-LDL was measured by incubating the indicated number of freshly isolated mononuclear cells in 1 ml of medium containing 25 ug/ml ^{125}I-LDL. Nonspecific degradation was measured in replicate wells which contained an additional 500 ug/ml cold LDL. Specific high affinity degradation was measured by difference between total and nonspecific degradation. Results shown are the mean and SEM of two incubations each done in duplicate for a total of four observations at each point.

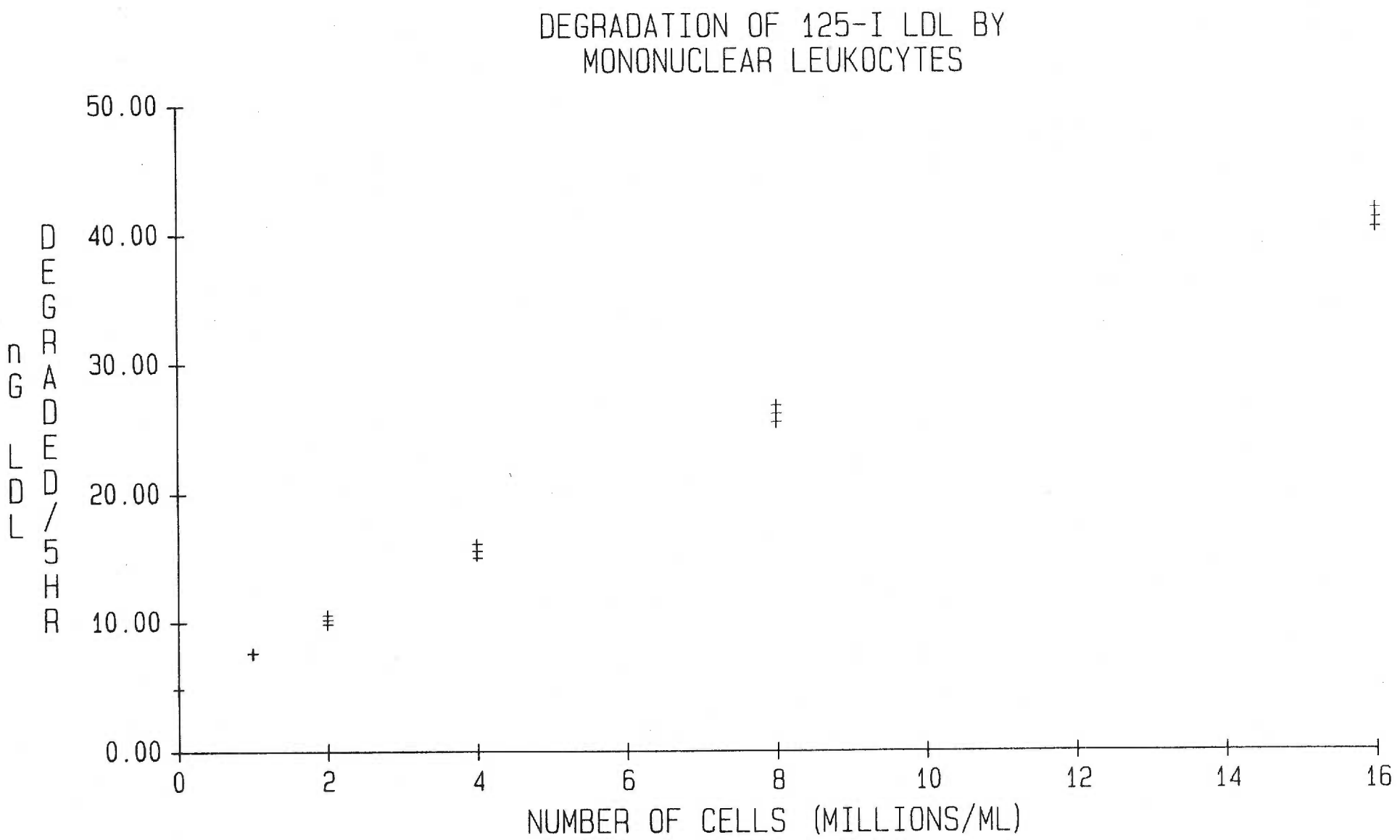

DEGRADATION OF 125-I LDL BY
MONONUCLEAR LEUKOCYTES
ng LDL DEGRADED/5HR
50.00
40.00
30.00
20.00
10.00
0.00
0
2
4
6
8
10
12
14
16
NUMBER OF CELLS (MILLIONS/ML)

Figure 5. The effect of duration of incubation on the degradation of ^{125}I-LDL by freshly isolated normal human mononuclear leukocytes. Specific high affinity degradation was defined as the difference between the total amount of ^{125}I-LDL degraded (^{125}I-tyrosine produced) and the amount of ^{125}I-LDL degraded in the presence of a 20 fold excess of unlabeled LDL. The data from a representative experiment are shown. In these experiments 3.51 million cells were incubated in each well for the time period shown. The 1 hour and 2 hour points are at the lower limit of sensitivity of the assay.

FIGURE 5. DEGRADATION OF 125-I LDL
BY MONONUCLEAR LEUKOCYTES

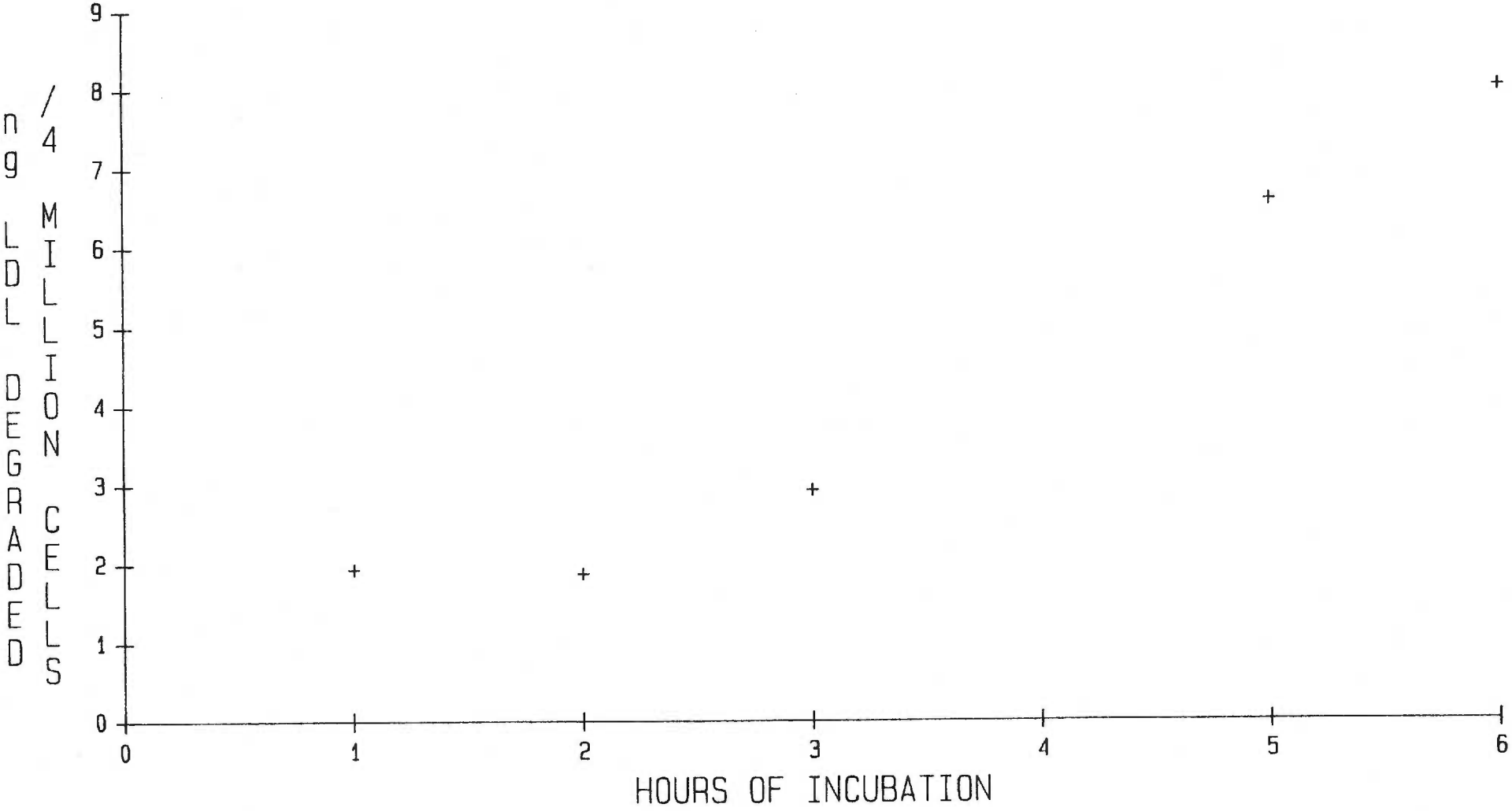


Figure 6. The effect of calcium concentration on specific high affinity degradation of ^{125}I-LDL by freshly isolated normal human mononuclear leukocytes. Increasing amounts of calcium (as the gluconate salt) were added to the incubation medium containing 4 x 10^6 cells per ml. Calcium concentrations greater than 1.8 mM produced a precipitate and thus could not be used. Concentrations less than 0.3 mM were not tested because this was the basal concentration of calcium in the RPMI-1640. The data shown are the mean and SEM of four experiments.

FIGURE 6. EFFECT OF CALCIUM ON DEGRADATION OF 125-I LDL BY MONONUCLEAR LEUKOCYTES

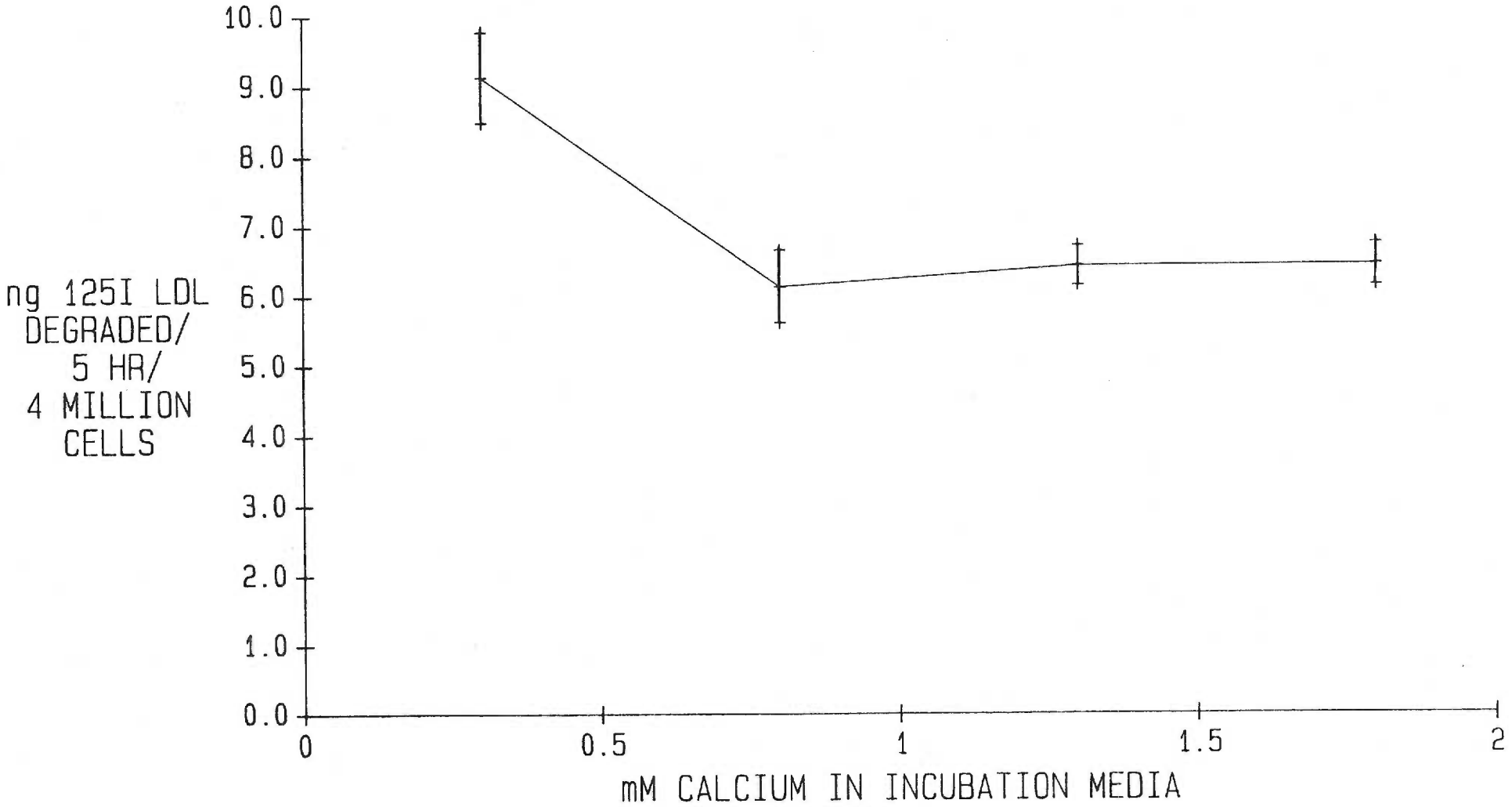


Figure 7. The effect of Low Density Lipoprotein concentration on the degradation of ^{125}I-LDL in freshly isolated human mononuclear leukocytes. 4 x 10^6 cells were incubated in one ml of RPMI-1640 for 5 hours at 37 C in the presence of 0.75 to 200 ug/ml ^{125}I-LDL. The data presented are the mean and SEM of three duplicate experiments.

Total degradation is the amount of degraded LDL present in the medium following incubation of labeled LDL with normal human skin fibroblasts.

Non-specific degradation is the amount of LDL degraded by normal skin fibroblasts when incubated with labeled LDL and a 20-fold excess of unlabeled LDL.

Specific degradation is the difference between the two measurements.

Total degradation represents the summation of all LDL uptake pathways (receptor mediated, scavenger, and bulk uptake). Nonspecific degradation is via those pathways not involving the receptor, and specific degradation is the LDL receptor mediated uptake of ligand.

FIGURE 7. DEGRADATION OF 125-I LDL
BY MONONUCLEAR LEUKOCYTES

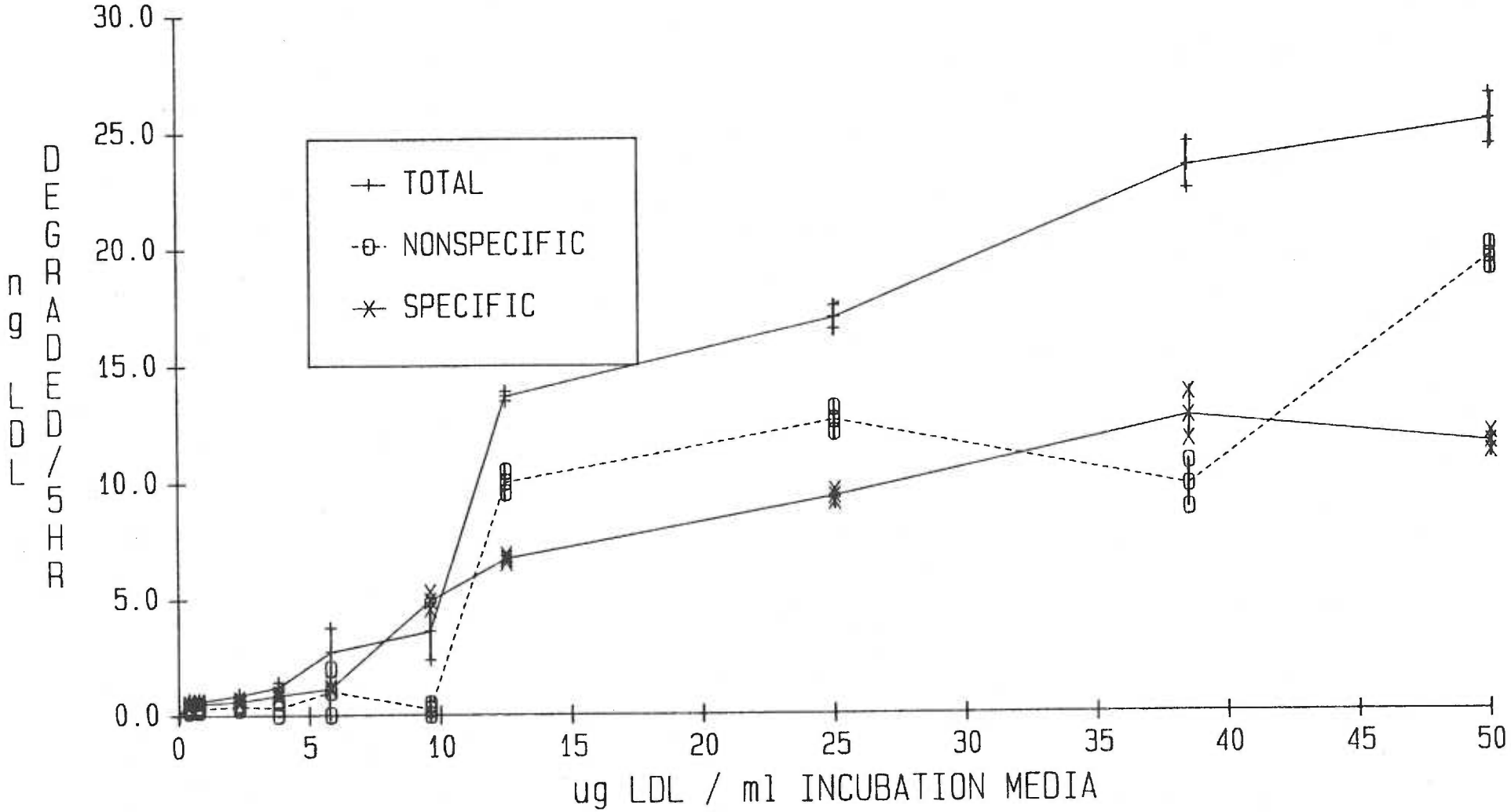


Figure 8a. LDL4 Binding to Normal Skin Fibroblasts- LDL4 isolated from 4 normal subjects and 8 patients with FH off drug and again after stabilizing on 10-15g colestipol twice daily. Binding studies were done in normal human fibroblasts which had been derepressed by a 48 hour preincubation in 10% LPDS to optimize the expression of LDL receptors on the cell surface. The cells were incubated with graded amounts of ^{125}I-LDL4 (density 1.020-1.040 g/cc) in the presence and absence of a 20-fold excess total LDL. The data presented represent the mean and SEM of 8 assays for each of the FH patient series and the mean and SEM of 4 determinations for the normal controls

FIGURE 8a. LDL4 BINDING BY
CULTURED NORMAL FIBROBLASTS

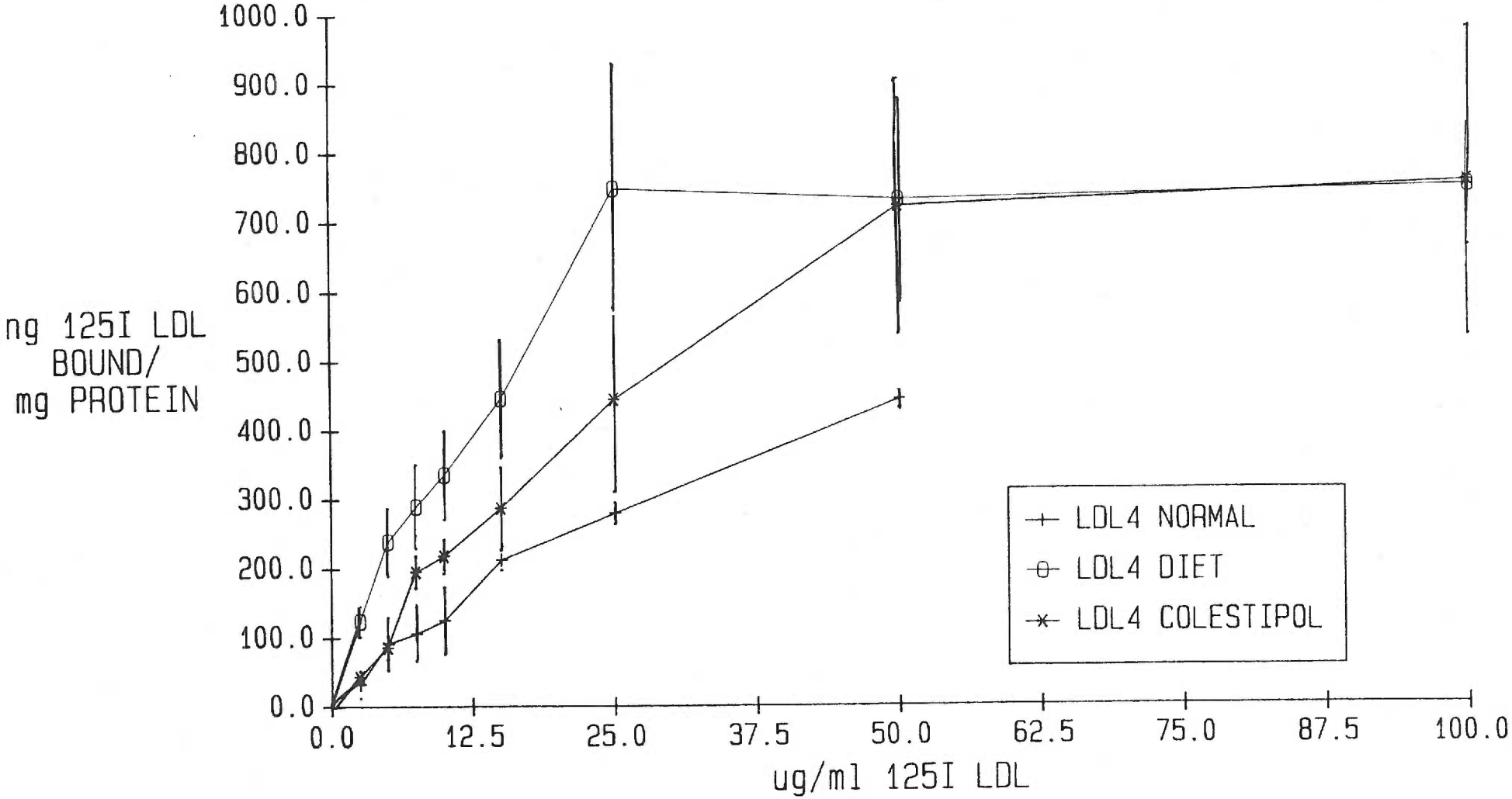


Figure 8b. LDL4 Internalization by Normal Skin Fibroblasts- LDL4 (1.020-1.040 gm/cc) was isolated from 4 normal subjects and from 8 patients with FH before and after treatment with colestipol. These studies were done in normal human fibroblasts which had been derepressed by a 48 hour preincubation in 10% LPDS to optimize the expression of LDL receptors on the cell surface. The cells were incubated with graded amounts of ^{125}I-LDL4 (density 1.020-1.040 g/cc) in the presence and absence of a 20-fold excess total LDL. The data presented represent the mean and SEM of 8 assays for each FH patient series and the mean and SEM of 4 determinations for the normal controls

FIGURE 8b. LDL4 INTERNALIZATION BY CULTURED NORMAL FIBROBLASTS

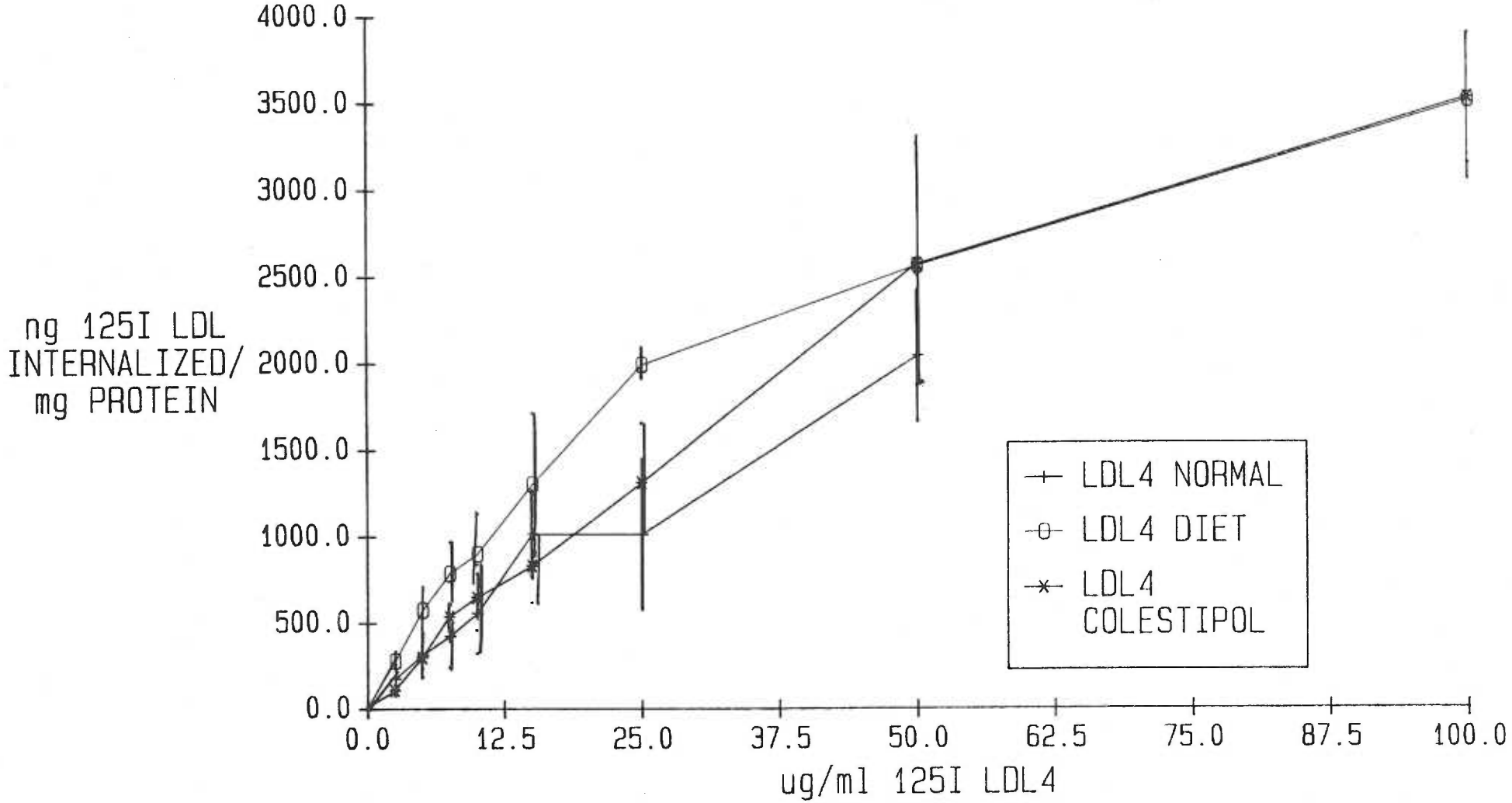


Figure 8c. LDL4 Degradation by normal skin fibroblasts- High affinity degradation was measured in normal human skin fibroblasts using lipoprotein isolated from 4 normal subjects and from 8 patients with FH before and after colestipol therapy. Degradation studies were done in normal human fibroblasts which had been derepressed by a 48 hour preincubation in 10% LPDS to optimize the expression of LDL receptors on the cell surface. The cells were incubated with graded amounts of ^{125}I-LDL4 (density 1.020-1.040 g/cc) in the presence and absence of a 20-fold excess total LDL. High affinity degradation is defined as the difference between the total amount degraded and that degraded in the presence of a 20-fold excess of unlabeled LDL. The data presented represent the mean and SEM of 8 assays for each FH patient series and the mean and SEM of 4 determinations for the normal controls.

FIGURE 8c. LDL4 DEGRADATION BY CULTURED NORMAL FIBROBLASTS

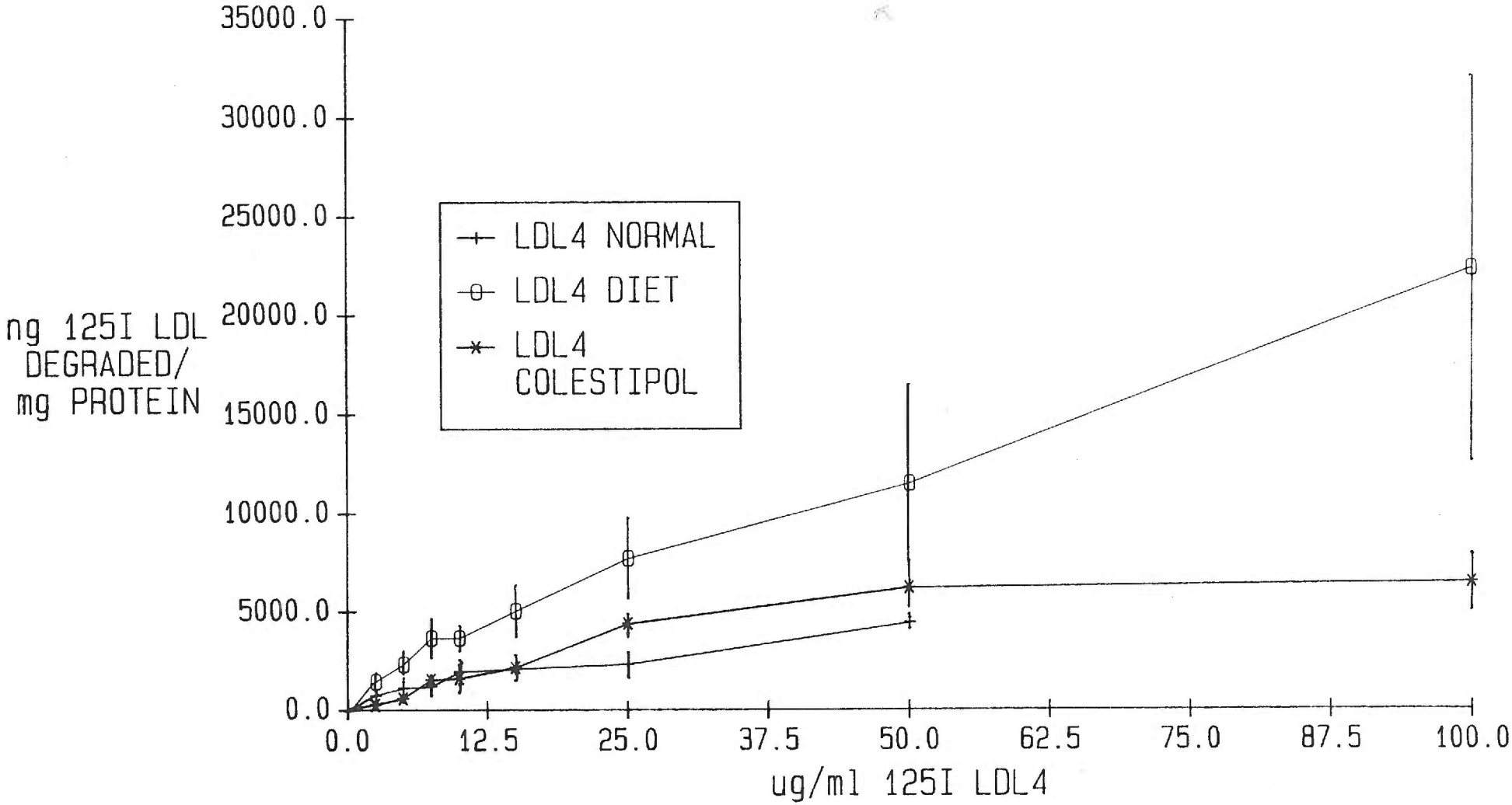


Figure 9a. LDL6 binding to normal human skin fibroblasts- To assess whether colestipol treatment affected LDL6 in such a way that its binding to normal human fibroblast LDL receptors was altered, samples were obtained from 8 patients with FH off drug and after stabilizing on 10-15g colestipol twice daily. Binding studies were done using LDL6 subfractions isolated in the density range 1.040-1.055 g/cc. The fibroblasts were derepressed by incubation in 10% LPDS for 48 hours before adding the ^{125}I-LDL6 to enhance the expression of cell surface LDL receptors. The receptor mediated component was measured by incubating graded amounts of ^{125}I-LDL6 subfraction with normal skin fibroblasts in the absence and presence of a 20-fold excess unlabeled total LDL. The data presented represent the mean and SEM of 8 assays for each FH patient series and the mean and SEM of 4 determinations for the normal controls.

FIGURE 9a. LDL6 BINDING BY CULTURED NORMAL FIBROBLASTS

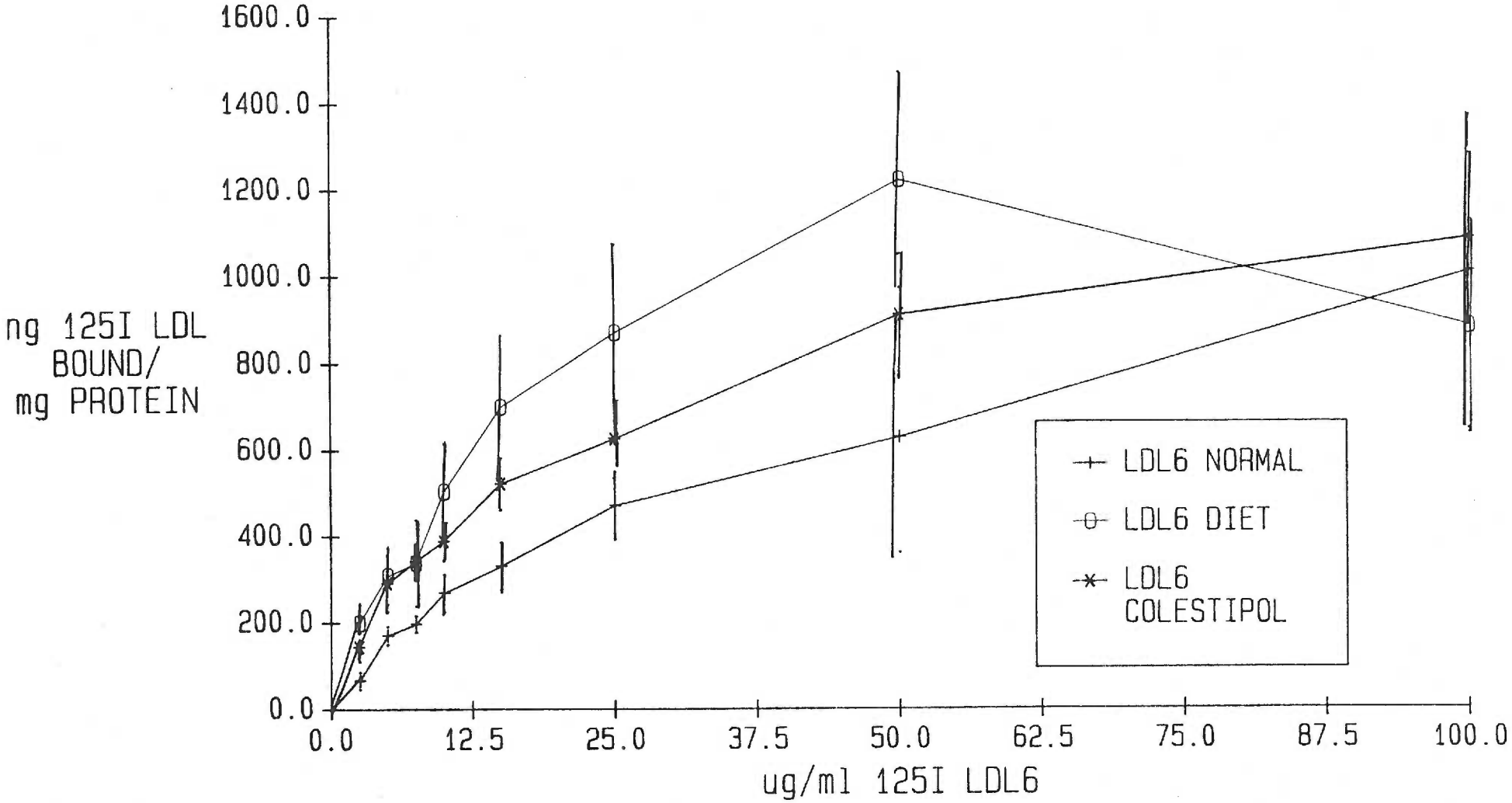


Figure 9b. LDL6 internalization by normal human skin fibroblasts- The amount of ^{125}I-LDL6 internalized was measured following incubation of normal human fibroblasts, which had been derepressed for 48 hours in 10% LPDS, with graded amounts of ^{125}I-LDL6 in the presence and absence of a 20-fold excess unlabeled total LDL. The data presented represent the mean and SEM of 8 assays for each FH patient series and the mean and SEM of 4 determinations for the normal controls

FIGURE 9b. LDL6 INTERNALIZATION BY CULTURED NORMAL FIBROBLASTS

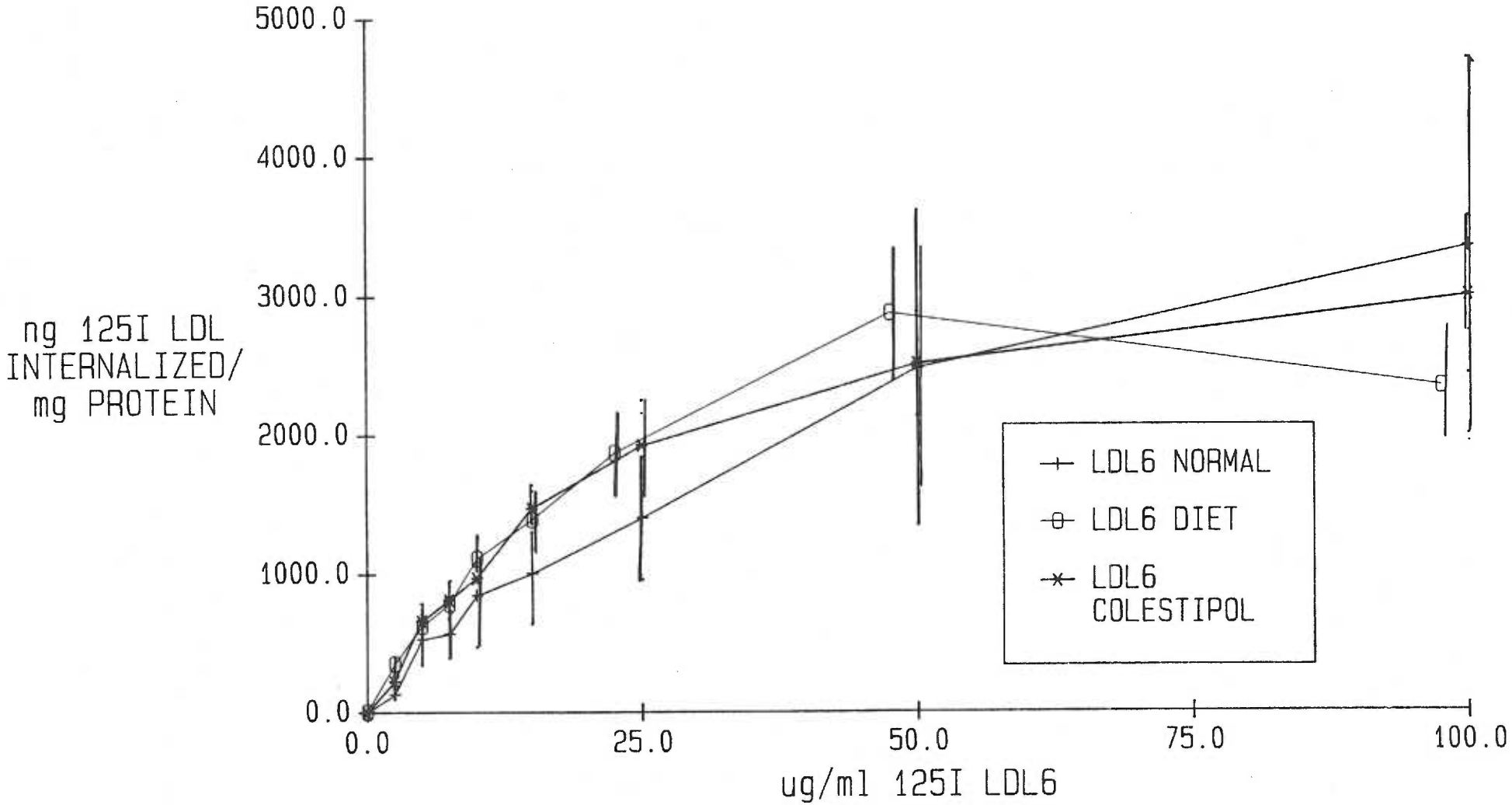


Figure 9c. LDL6 Degradation by normal skin fibroblasts- Cultured normal human fibroblasts which had been grown for 48 hours in 10% LPDS to enhance the expression of cell surface LDL receptors were incubated with graded amounts of ^{125}I-LDL6 in the presence and absence of 20-fold excess unlabeled total LDL. High affinity degradation is defined as the difference between the total amount degraded and that degraded in the presence of a 20-fold excess of unlabeled LDL. The data presented represent the mean and SEM of 8 assays for each FH patient series and the mean and SEM of 4 determinations for the normal controls

FIGURE 9c. LDL6 DEGRADATION BY CULTURED NORMAL FIBROBLASTS

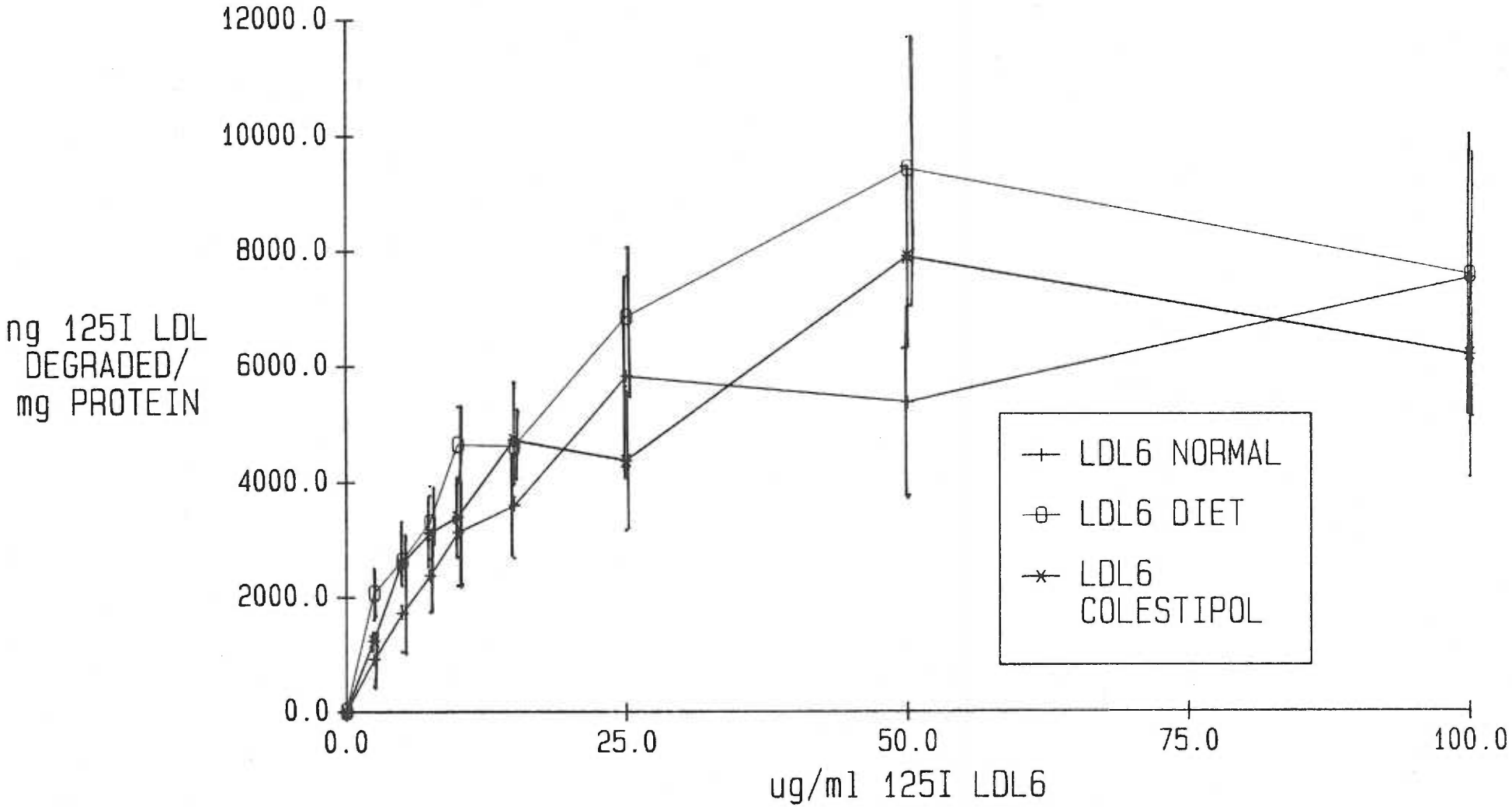


Figure 10. Competition of LDL subfractions for binding to normal skin fibroblasts- The IC50 is that concentration of competitor which will inhibit the binding of half of the ligand which would normally bind to a site. The IC50 was measured by incubating normal human skin fibroblasts which had been derepressed for 48 hours in 10% LPDS to maximize the expression of receptors with graded amounts of unlabeled LDL4 (Figure 10a) or LDL6 (Figure 10b) isolated from patients with FH treated with diet alone or with the combination of diet and colestipol, and normal subjects with 7.5 ug/ml of the comparable ^{125}I-LDL subfraction isolated from two patients with untreated FH. Data are the mean and SEM of triplicate incubations.

FIGURE 10a. LDL4 BINDING COMPETITION
NORMAL SKIN FIBROBLASTS

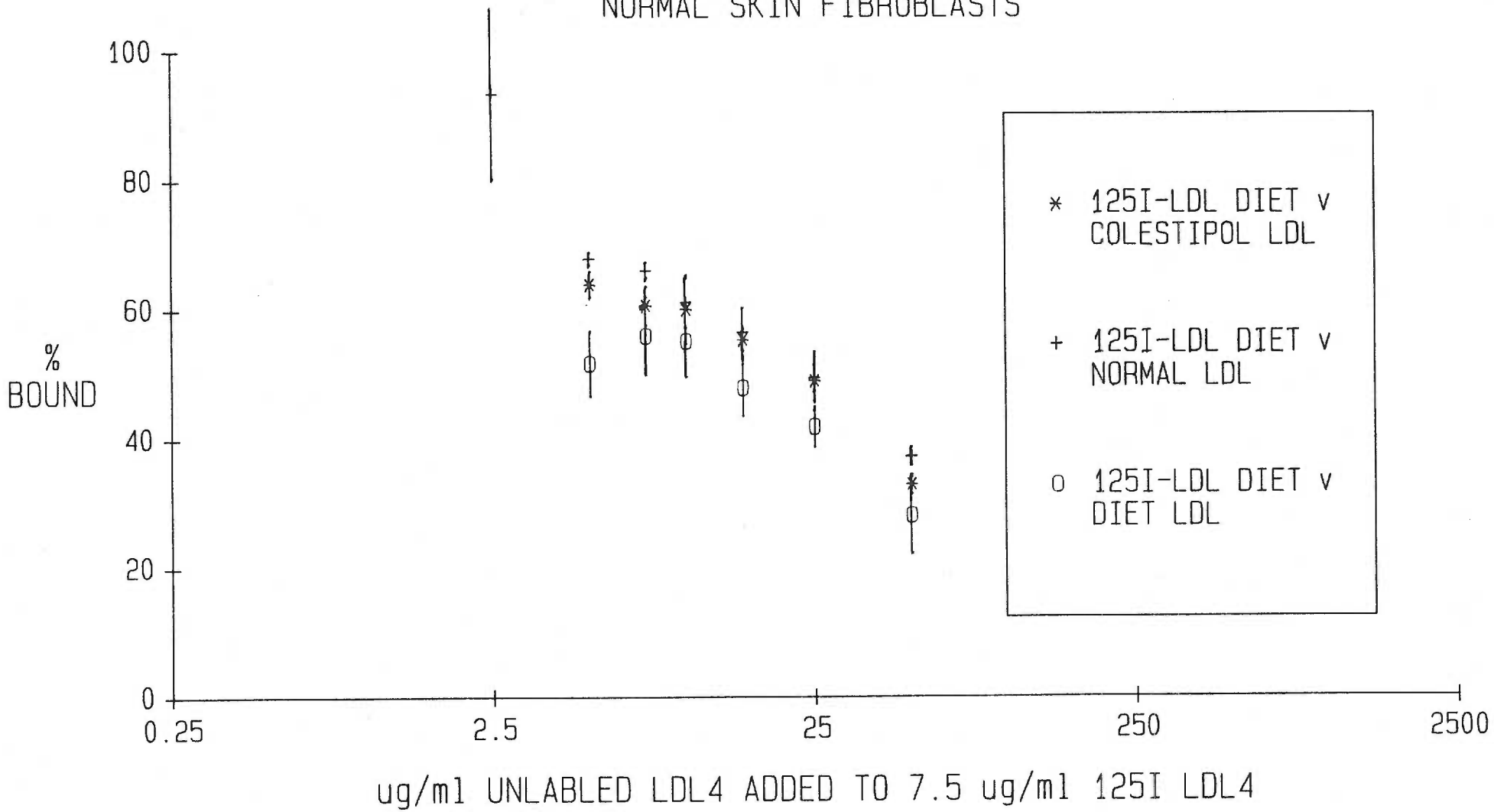


FIGURE 10b. LDL6 BINDING COMPETITION
NORMAL SKIN FIBROBLASTS

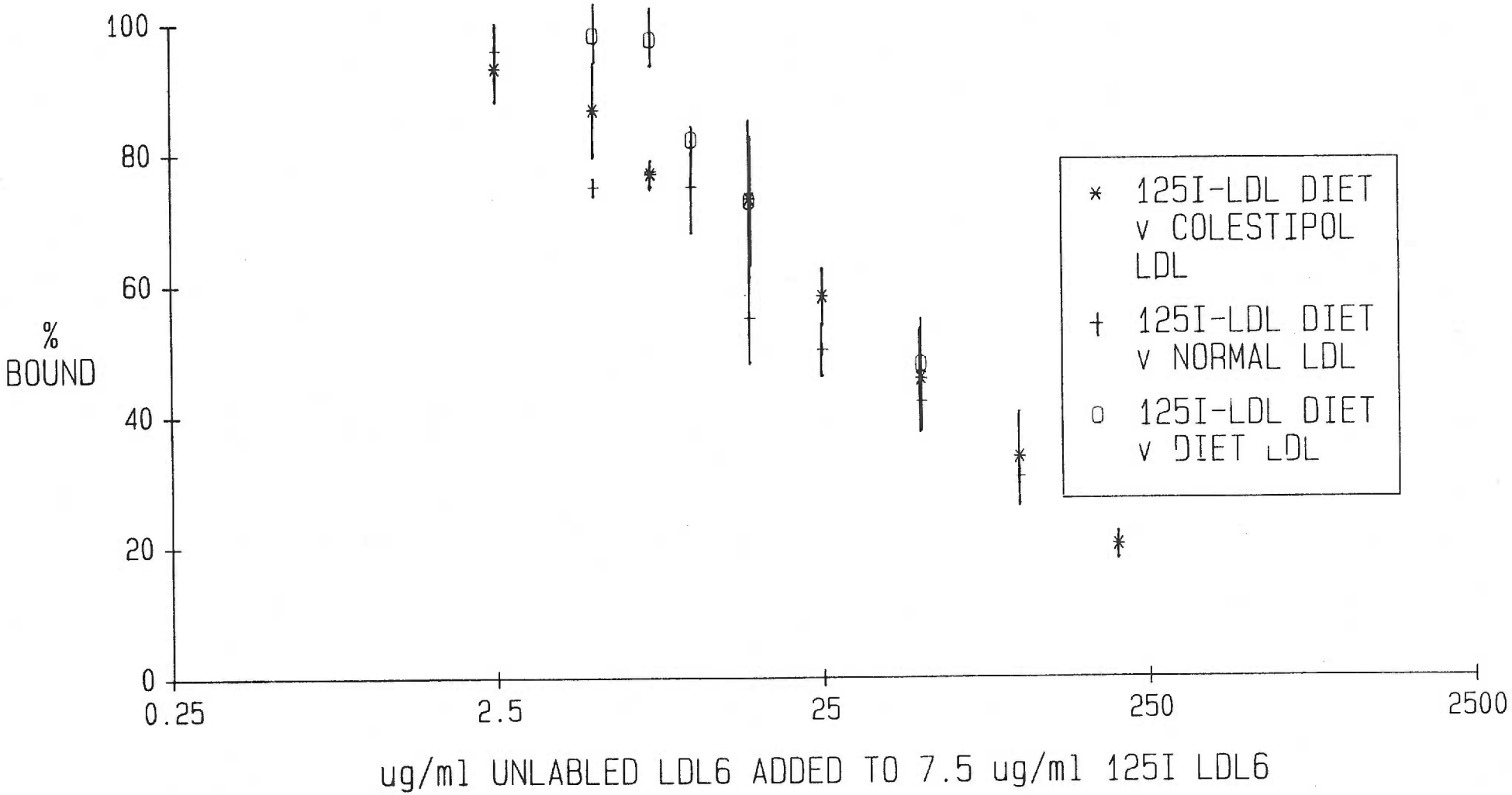


Figure 11. Scatchard plots of LDL subfraction binding data.

Figure 11a LDL4 subfractions. The pooled LDL4 scatchard plots as derived by LIGAND analysis is shown for LDL4 from FH patients before treatment (LDL4 Diet), after treatment with colestipol (LDL4 colestipol). Data for 4 pooled normal controls is also shown (LDL4 normal. The data for the 6 individual patients is shown in table 8.

Linear regression of the data against the logarithm of substrate concentration gave IC50 values as follows:

Normal	13.0 ug/ml
Colestipol	12.5 ug/ml
Diet	7.1 ug/ml

Figure 11b. LDL6 subfractions. The pooled LDL6 scatchard plots as derived by LIGAND analysis is shown for LDL6 from FH patients before treatment (LDL6 Diet), after treatment with colestipol (LDL6 colestipol). Data for 4 pooled normal controls is also shown (LDL6 normal. The data for the 6 individual patients is shown in table 8.

Linear regression of the data against the logarithm of substrate concentration gave IC50 values as follows:

Normal	29 ug/ml
Colestipol	51 ug/ml
Diet	65 ug/ml

FIGURE 11a. SCATCHARD ANALYSIS OF LDL4
BINDING TO NORMAL SKIN FIBROBLASTS

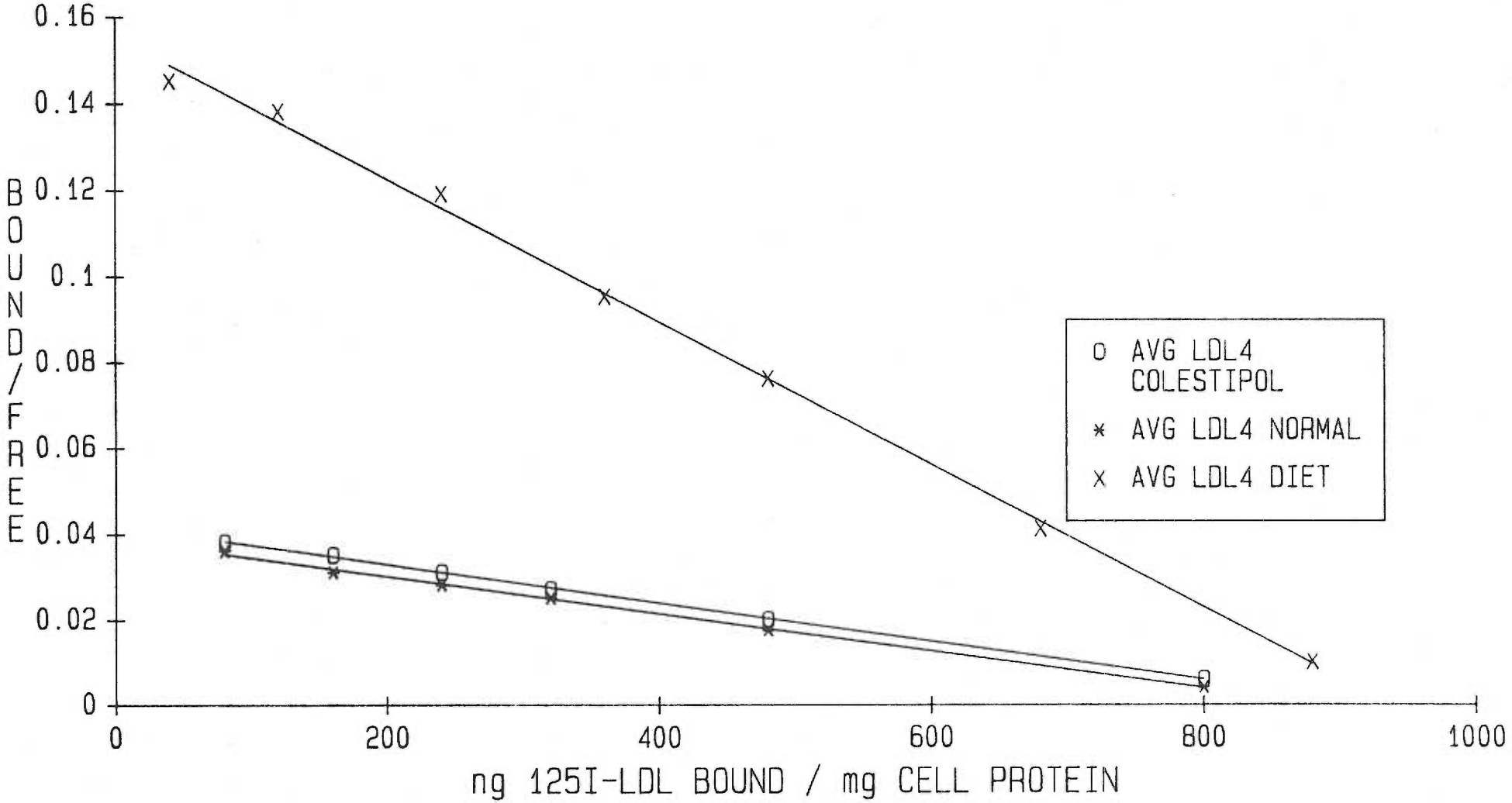


FIGURE 11b. SCATCHARD ANALYSIS OF LDL6 BINDING TO NORMAL SKIN FIBROBLASTS

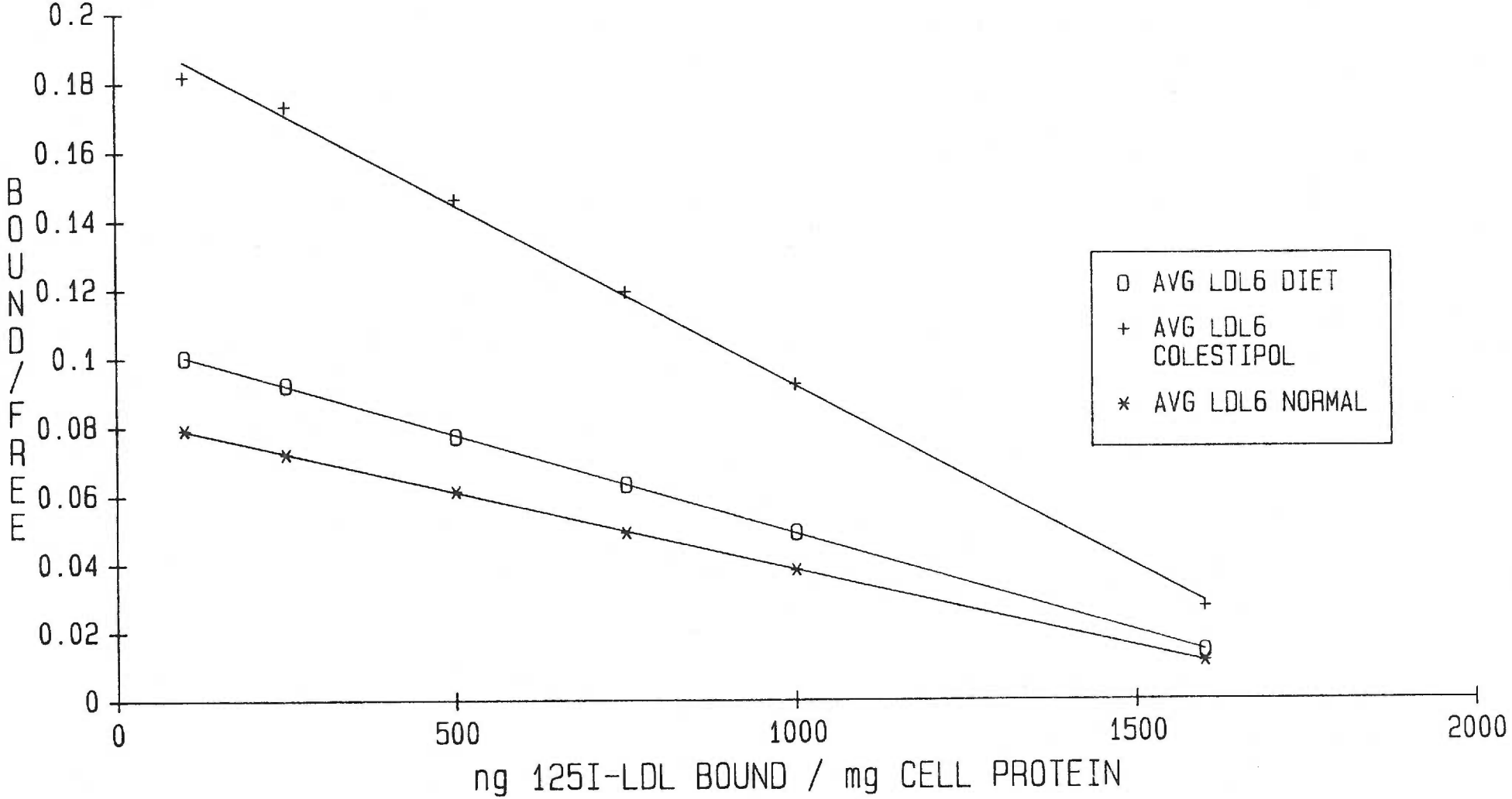


Figure 11. (cont.) Scatchard plots of LDL subfraction binding data.

Figure 11c. LDL4 isolated from a representative patient before and after colestipol treatment. The LDL4 scatchard plot as derived by LIGAND analysis is shown for LDL4 from a single patient with heterozygous FH before treatment (LDL4 Diet) and after treatment with colestipol (LDL4 colestipol). Units for Kd are ng/ml.

DIET		COLESTIPOL	
BOUND	BOUND/FREE	BOUND	BOUND/FREE
249.1	.296	27.7	.030
447.0	.186	122.9	.032
599.0	.131	207.7	.016
762.6	.095	227.3	.013
1126.2	.071	289.0	.014
1764.3	.049		
Binding sites (ug)	1953		588
Regression coefficient (r)	0.797		0.876
Kd	0.26×10^{-5}		3.28×10^{-5}

Figure 11d. Representative patient LDL6 before and after colestipol treatment. The LDL6 scatchard plot as derived by LIGAND analysis is shown for LDL6 from a single patient with heterozygous FH before treatment (LDL6 Diet) and after treatment with colestipol (LDL6 colestipol). Units for Kd are ng/ml.

DIET		COLESTIPOL	
BOUND	BOUND/FREE	BOUND	BOUND/FREE
296.8	.210	111.7	.094
315.5	.186	167.5	.061
380.3	.121	195.8	.038
533.1	.056	266.0	.040
717.8	.036	310.6	.031
		351.4	.015
		546.5	.012
		592.2	.006
Binding sites (ug)	750		574
Regression coefficient (r)	0.930		0.862
Kd	1.31×10^{-5}		2.00×10^{-5}

FIGURE 11c. SCATCHARD ANALYSIS OF LDL4
BINDING TO NORMAL SKIN FIBROBLASTS

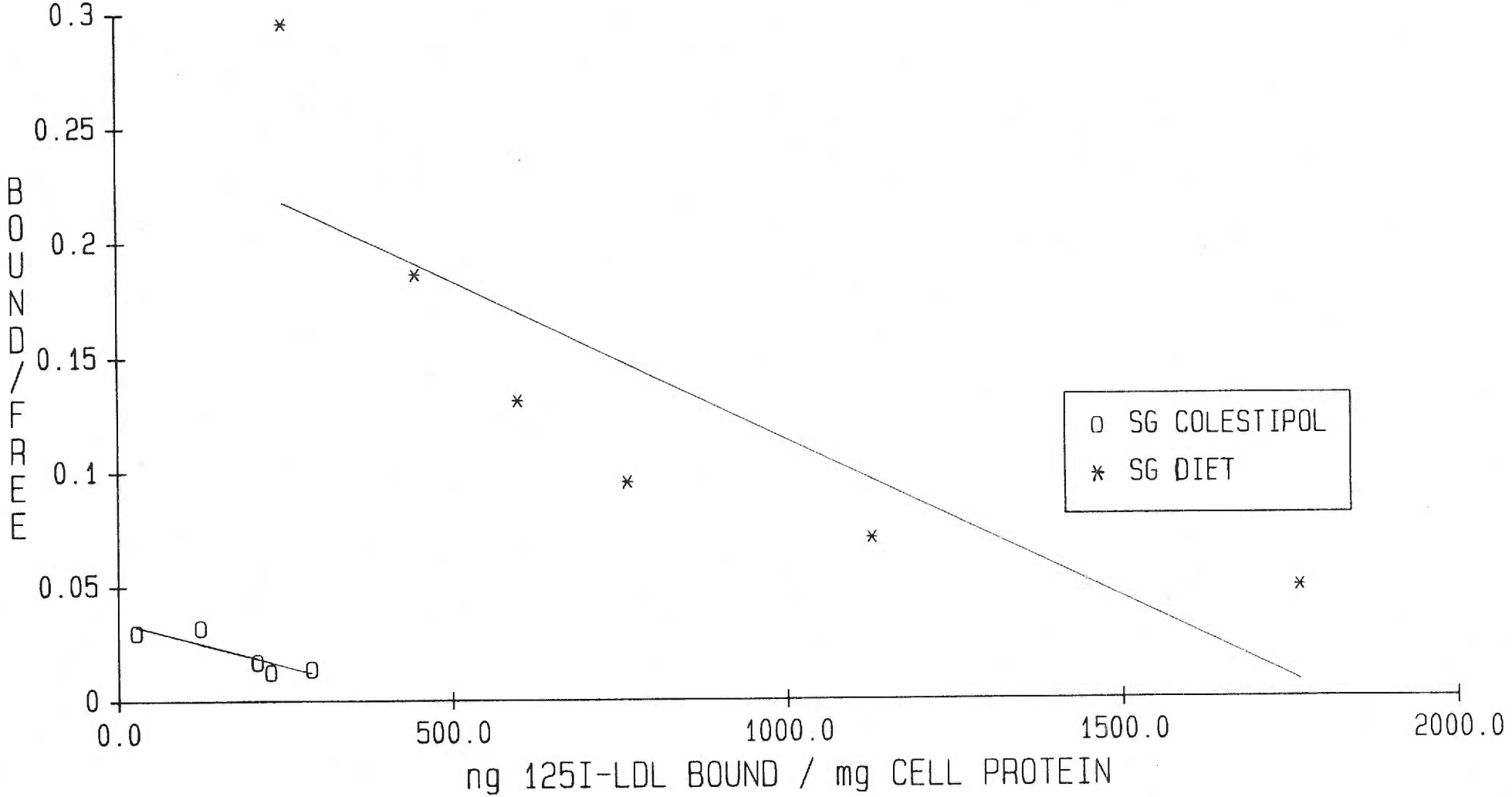


FIGURE 11d. SCATCHARD ANALYSIS OF LDL6
BINDING TO NORMAL SKIN FIBROBLASTS

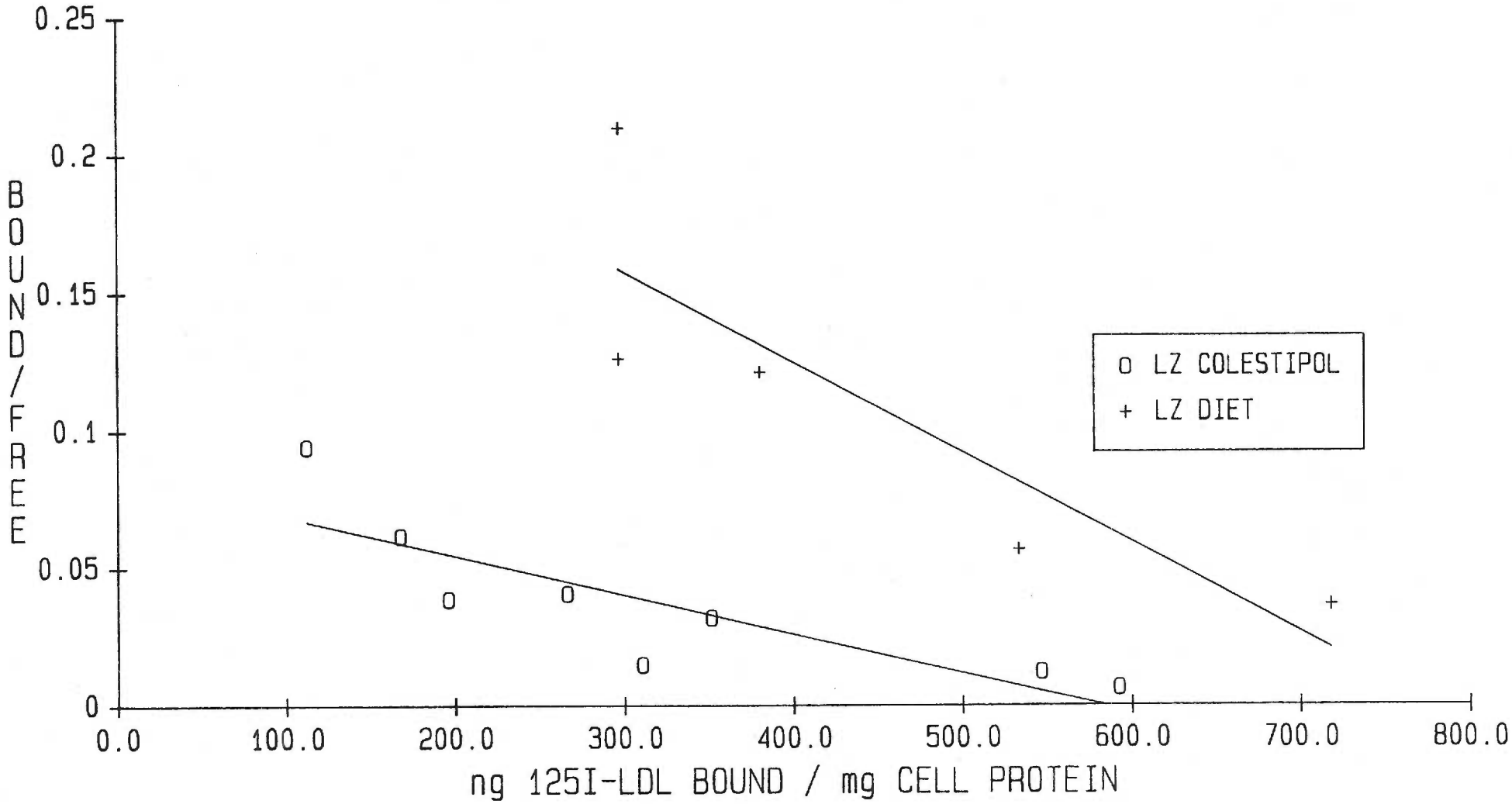


Figure 12. The effect of preincubation with LDL subfractions on the incorporation of 2-^{14}C-acetate into cholesterol by cultured human fibroblasts. Cultured normal human skin fibroblasts were derepressed for 24 hours in 10% LPDS, incubated 16 hours in 10% LPDS containing graded amounts of LDL4 or LDL6, then incubated an additional 3 hours following the addition of 1.5 millimoles 2-^{14}C-acetate. The incorporation dose response curves are shown in figures 12a (LDL4) and 12b (LDL6).

Symbols representing individual patients are listed by initials in the side box. Solid lines represent data obtained during the diet treatment period. Dashed lines indicate data collected after treatment with colestipol. Means and SEM for the groups are shown in figures 12c (LDL4) and 12d (LDL6).

FIGURE 12a. 2-14C ACETATE INCORPORATION INTO CHOLESTEROL

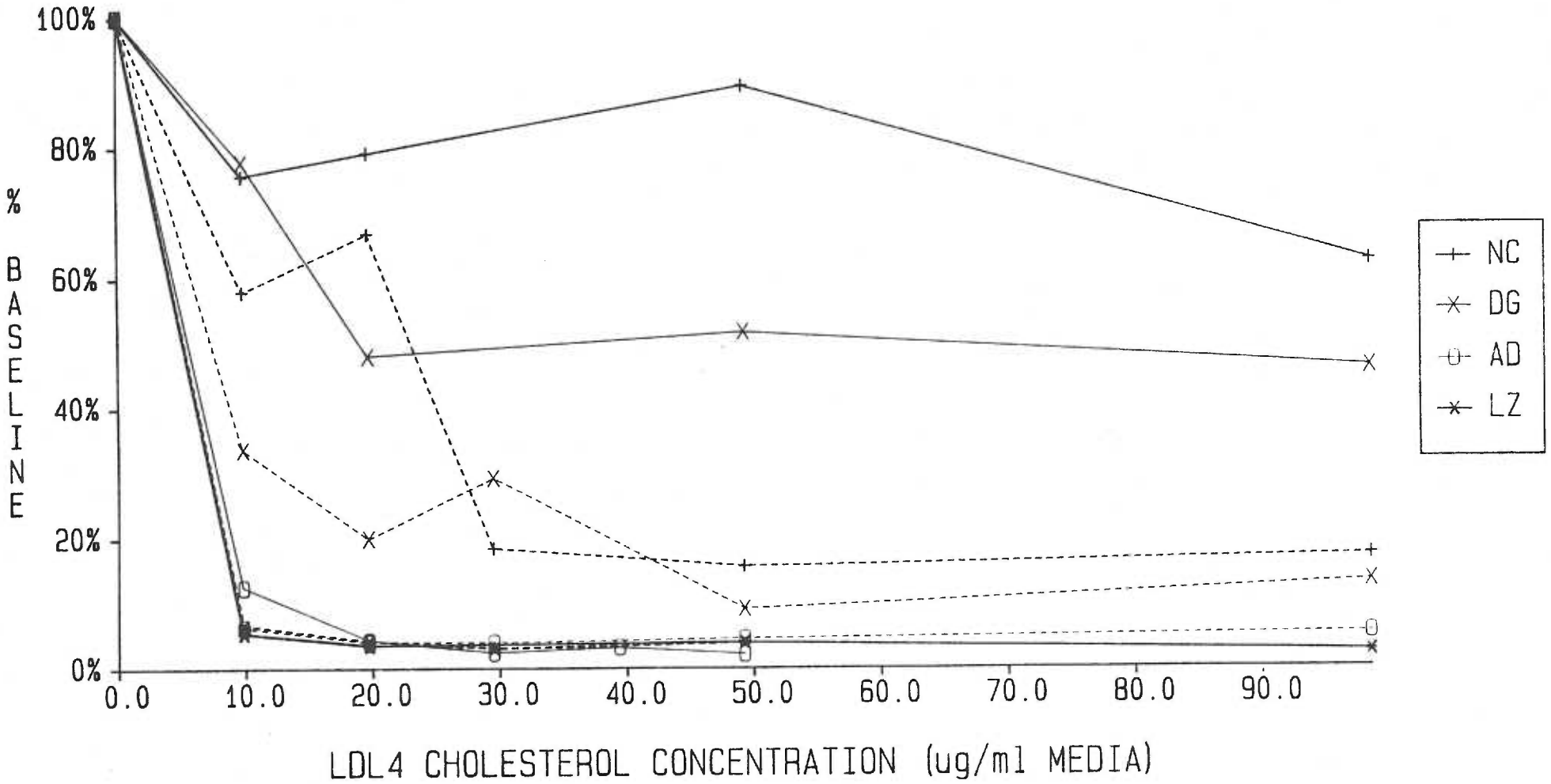


FIGURE 12b. 2-14C ACETATE INCORPORATION INTO CHOLESTEROL

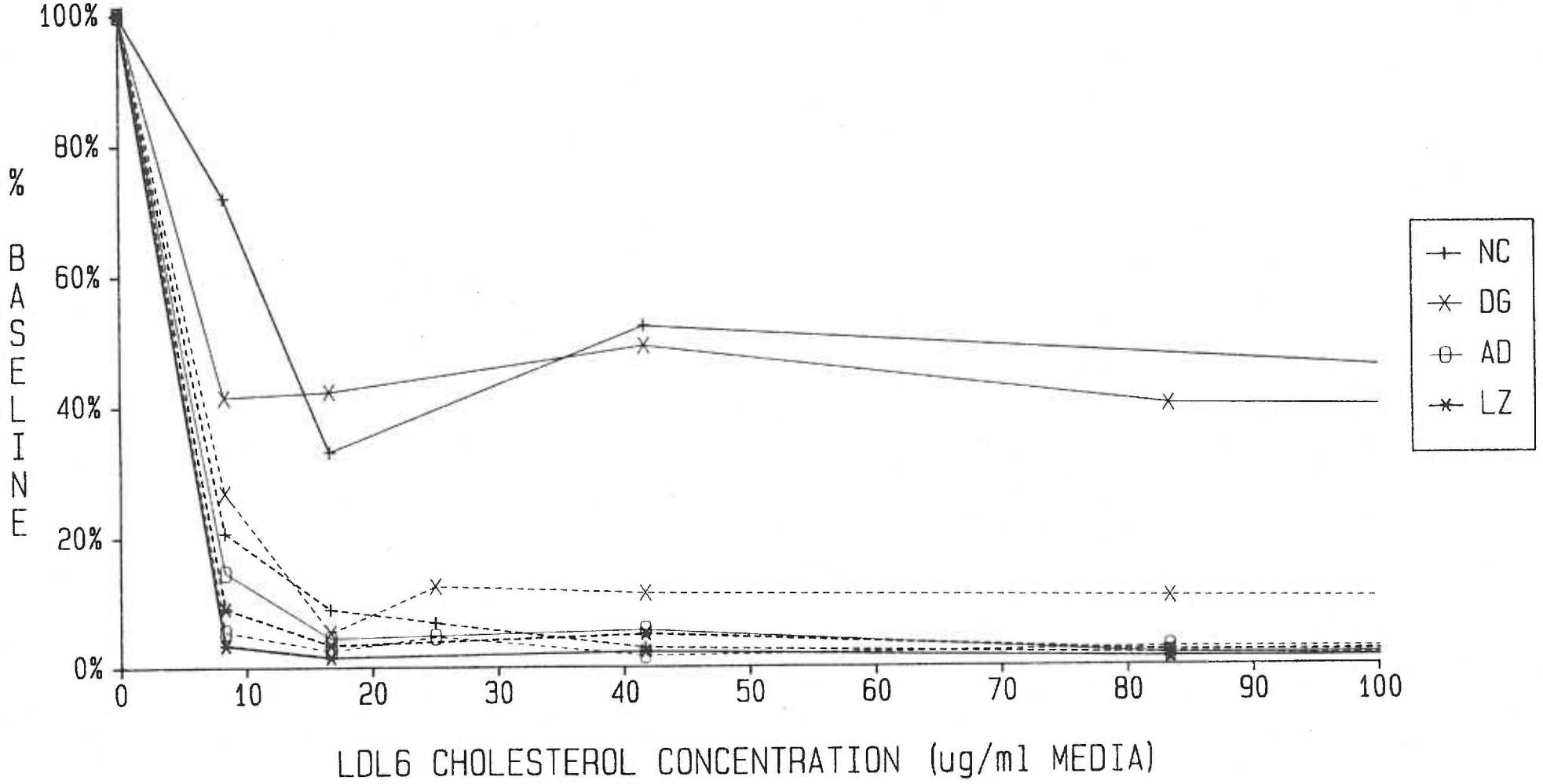


FIGURE 12c. 2-14C ACETATE INCORPORATION INTO CHOLESTEROL

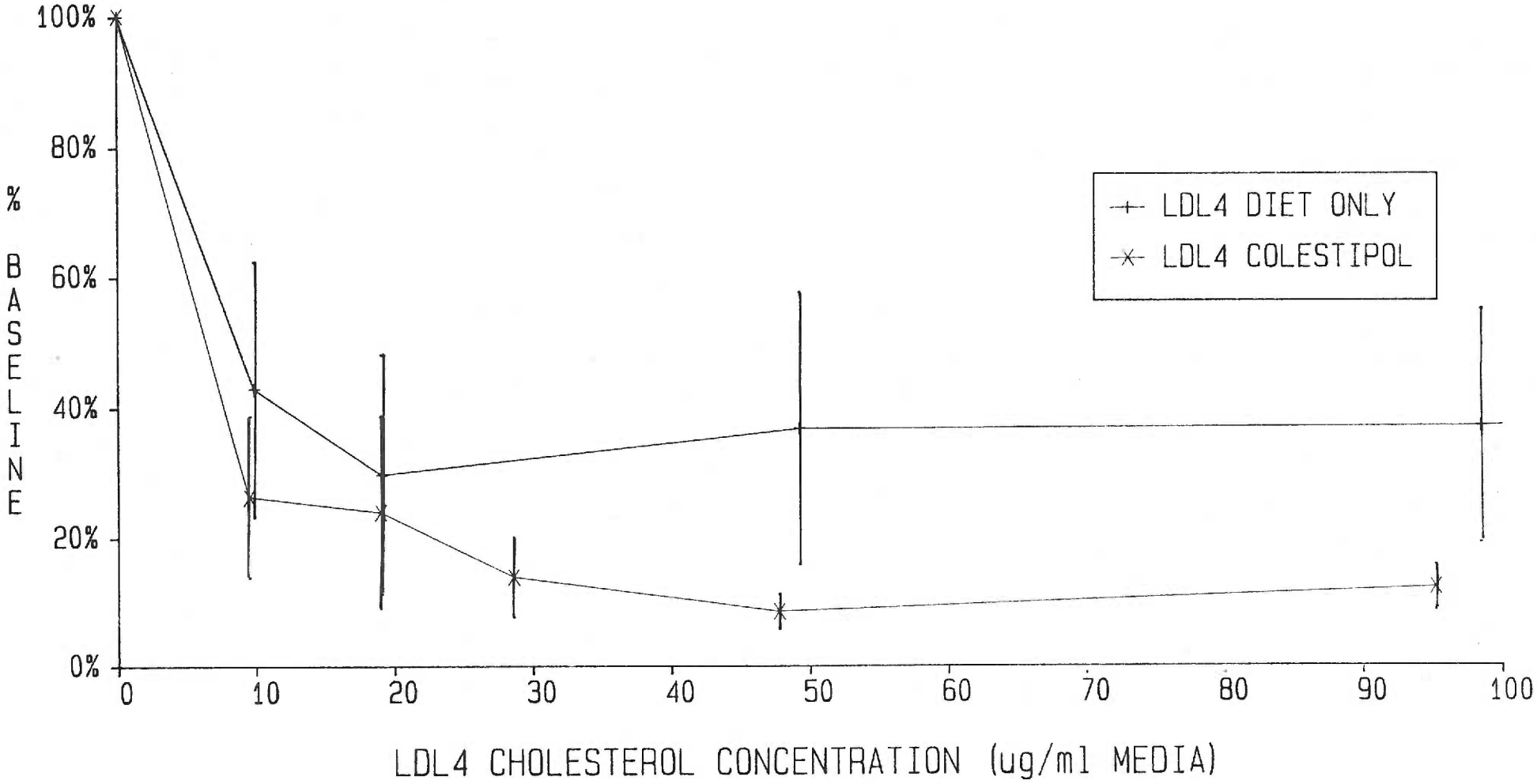


FIGURE 12d. 2-14C ACETATE INCORPORATION INTO CHOLESTEROL

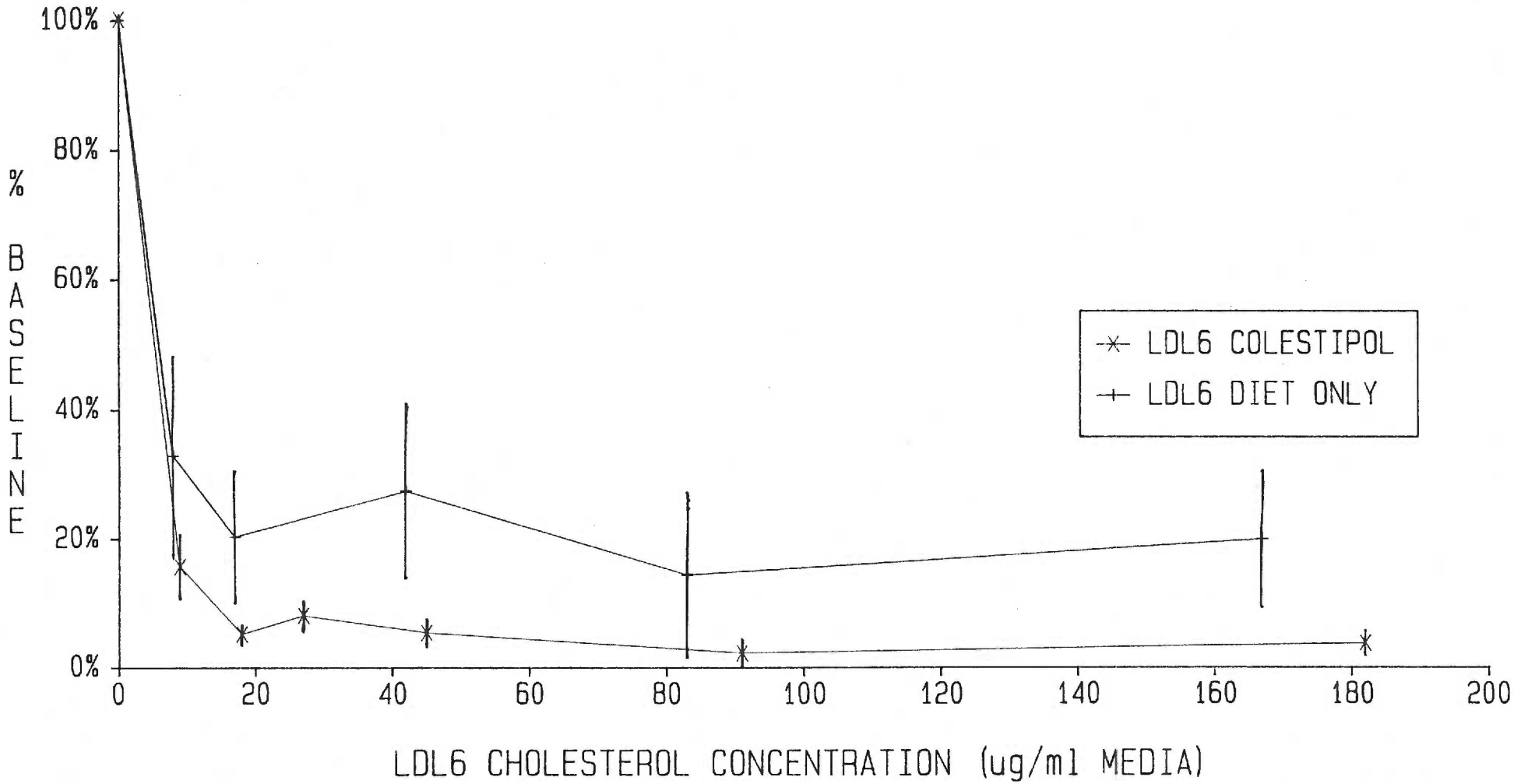


Figure 13. Effect of preincubation with LDL subfraction on incorporation of ^{3}H-oleic acid into cholesterol esters. Normal skin fibroblasts were incubated with 10% LPDS for 24 hours then incubated with graded amounts of LDL4 or LDL6 for 16 hours. The medium was then changed to one containing 10% LPDS, LDL4 (13a) or LDL6 (13b) and 9.9mM ^{3}H-oleic acid complexed with albumin. The cells were incubated an additional 3 hours to allow incorporation of the label into cholesterol ester.

Symbols representing individual patients are listed by initials in the side box. Solid lines represent data obtained during the diet treatment period. Dashed lines indicate data collected after treatment with colestipol. Mean data and SEM for the treatment groups are shown in figures 13c (LDL4) and 13d (LDL6)

FIGURE 13a. 3H OLEATE INCORPORATION INTO CHOLESTEROL ESTER

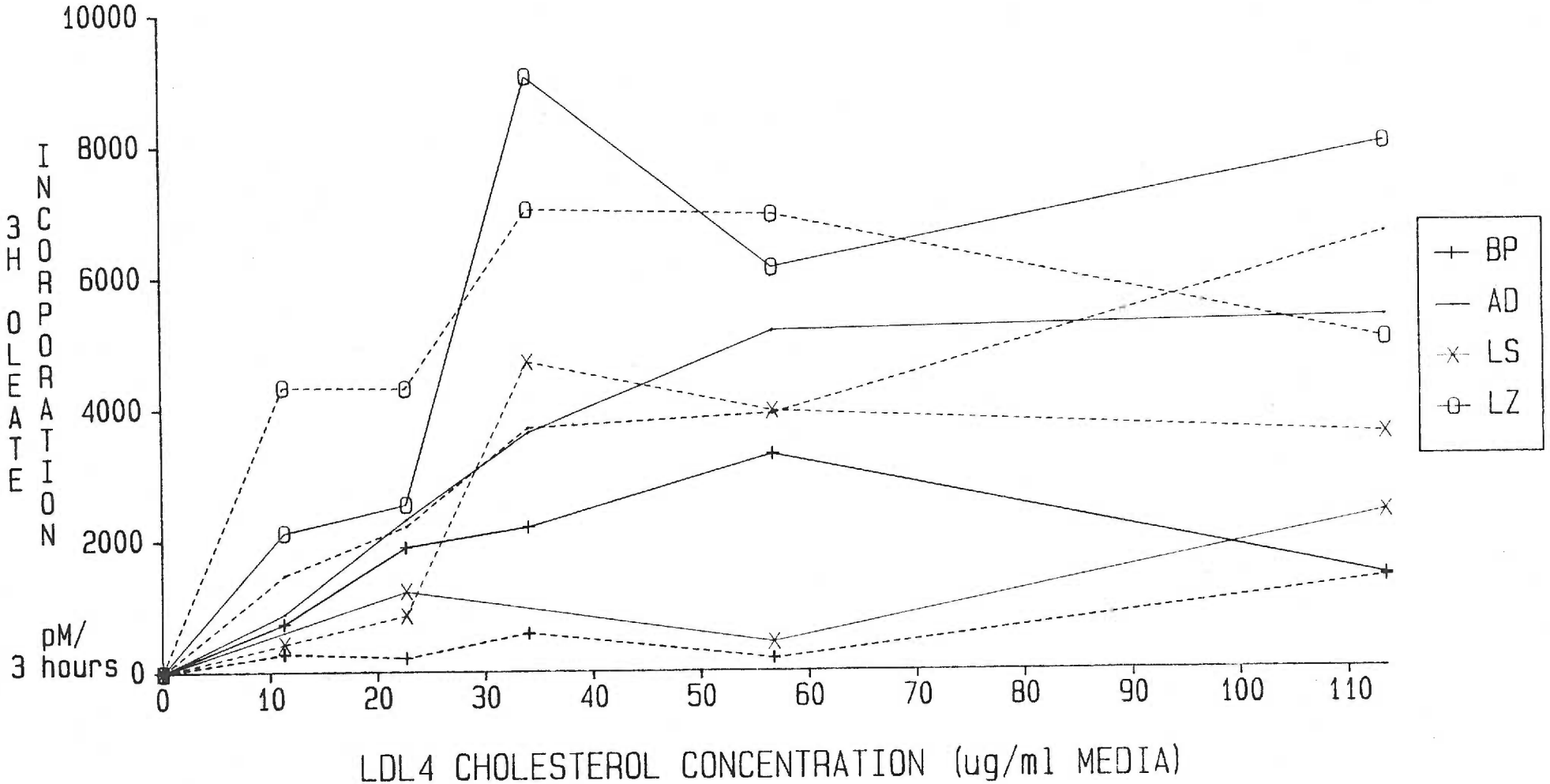


FIGURE 13b. 3H OLEATE INCORPORATION INTO CHOLESTEROL ESTER

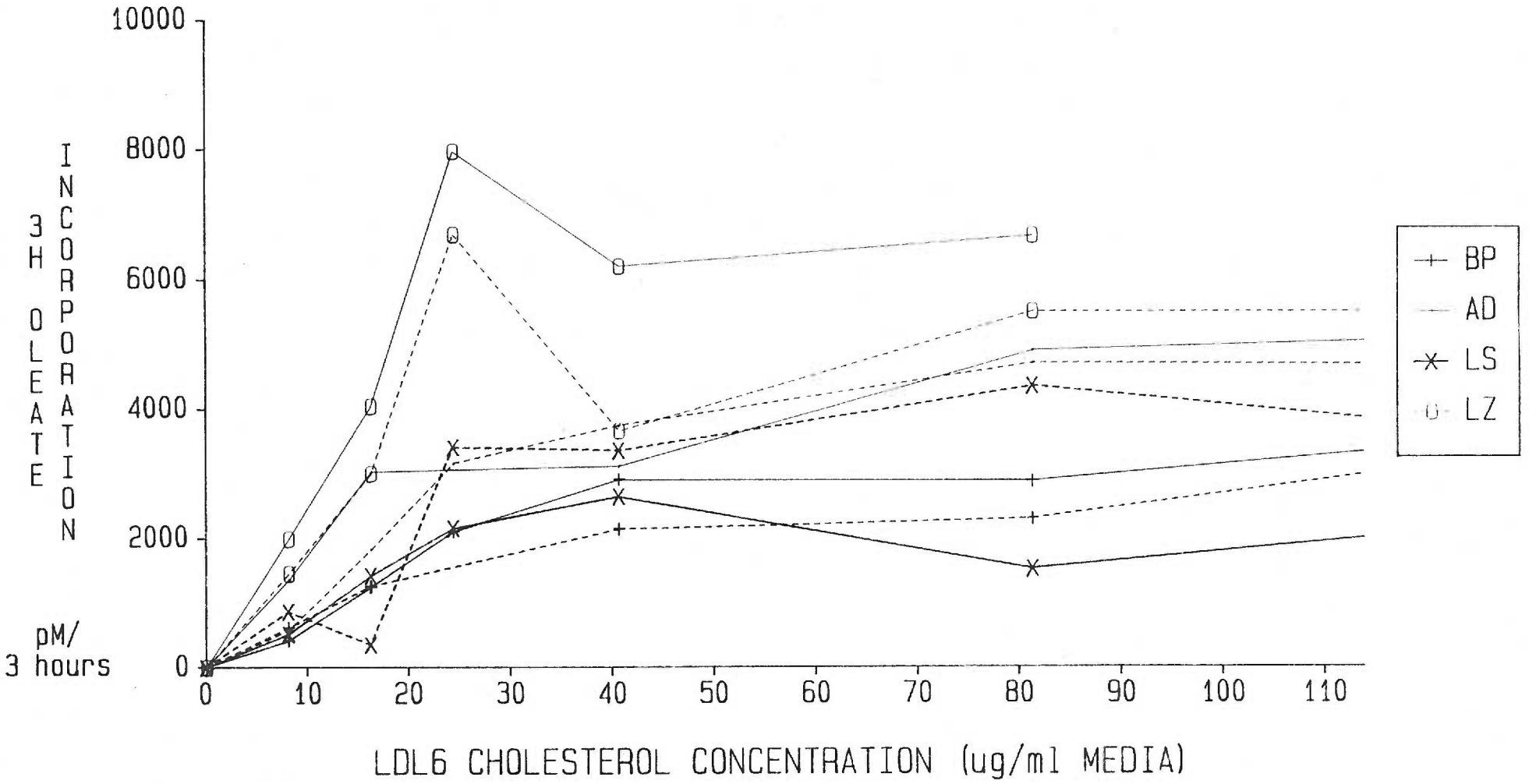


FIGURE 13c. 3H OLEATE INCORPORATION INTO CHOLESTEROL ESTER

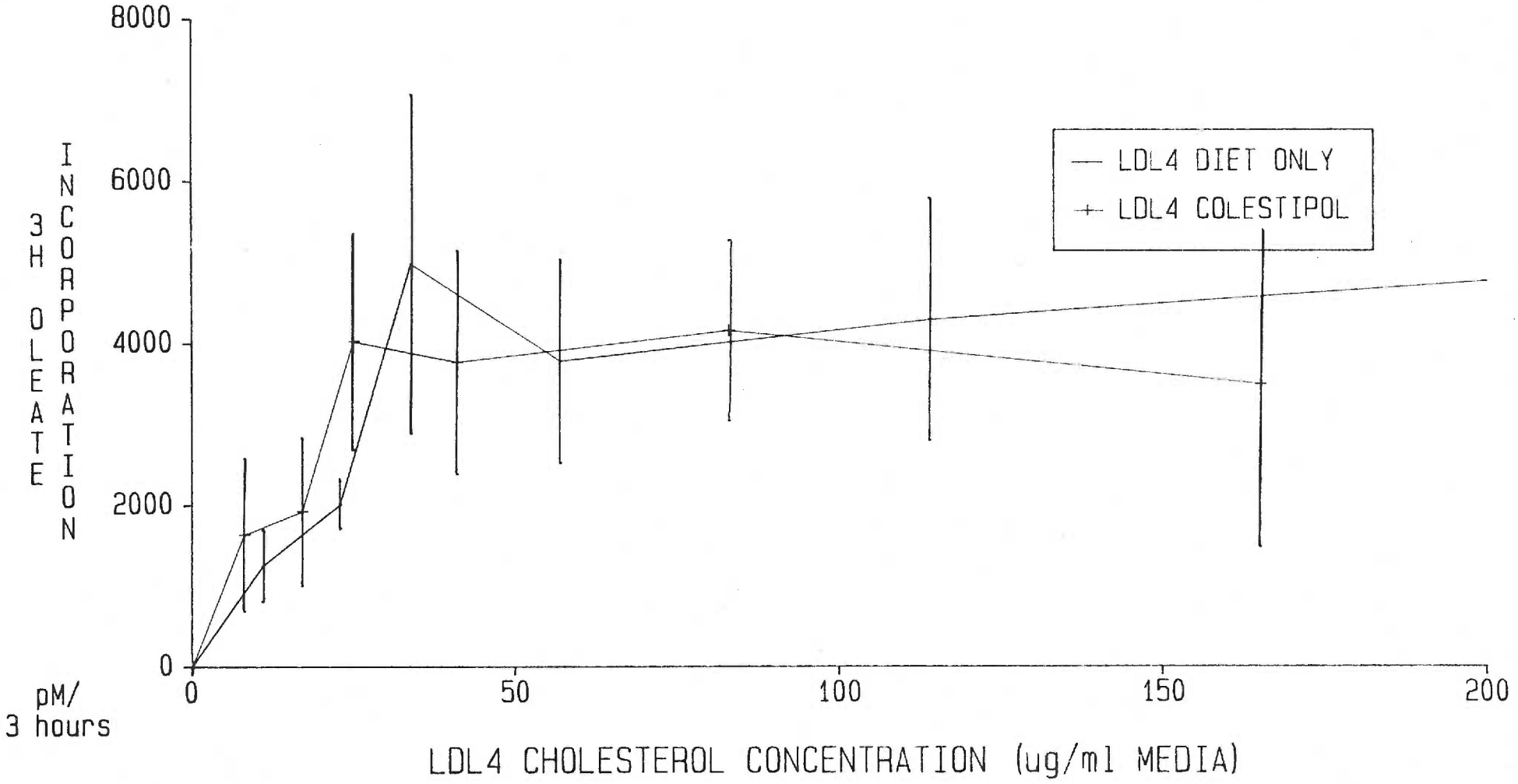


FIGURE 13d. 3H OLEATE INCORPORATION
INTO CHOLESTEROL ESTER

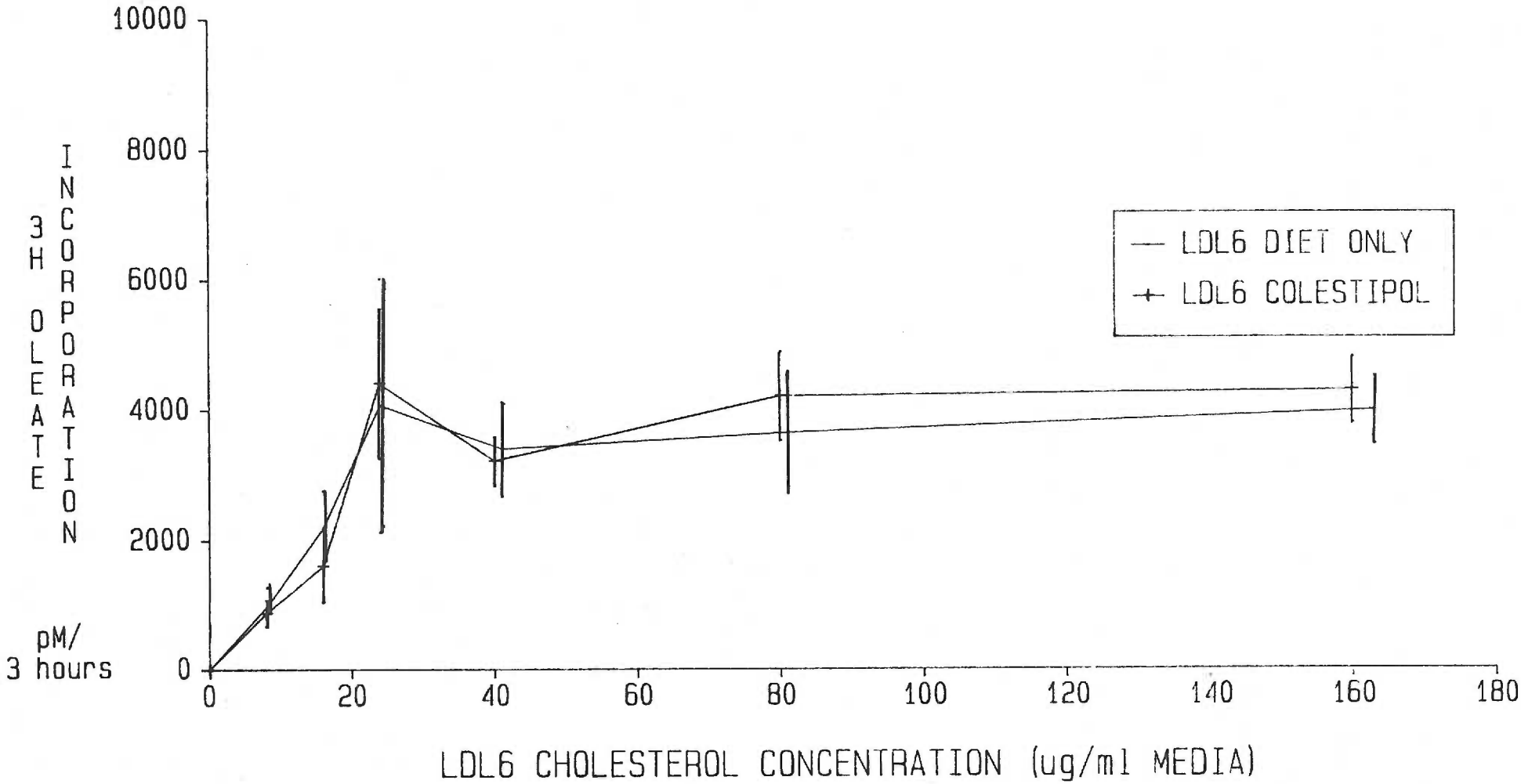


Table 1
The Effect of Lipid Lowering Drugs on the Concentrations of Plasma Lipids and Lipoproteins in Patients with Heterozygous Familial Hypercholesterolemia

	Plasma Chol	LDL Chol	HDL Chol	LDL/ HDL	Plasma Triglyceride	n
Diet	401 ± 17	330 ± 17	48 ± 3	7.4 ± 0.7	130 ± 14	23
Colestipol	352 ± 16*	267 ± 15**	47 ± 3	5.9 ± 0.4**	176 ± 37+	23
% Change	(-12.2)	(-19.1)			(+35.4)	
Diet	414 ± 22	331 ± 19	55 ± 6	6.0 ± 0.6	143 ± 30	9
Niacin	313 ± 26*	226 ± 20**	65 ± 3	3.8 ± 0.4**	105 ± 30	9
% Change	(-24.0)	(-31.5)			(-26.6)	
Diet	418 ± 18	335 ± 18	45 ± 5	7.4 ± 0.7	178 ± 31	11
Colestipol/ Niacin	302 ± 21*	197 ± 22**	66 ± 7	3.8 ± 0.7**	102 ± 14+	11
% Change	(-27.7)	(-41.2)			(-42.7)	
Normal	184 ± 4	113 ± 7	57 ± 2	2.0 ± 0.3	69 ± 8	18

All patients were studied prospectively and the mean baseline values for each group are shown. Normal values are shown for comparison purposes.
Values are mean ± SEM and percent change from diet treatment for the indicated number of patients in each group. All measurements are in mg/dl. Chol - cholesterol.
Differences between mean values were established by paired t test of the data.

+p<0.05 vs Diet *P<0.01 vs Diet **P<0.001 vs Diet

Table 2
Parameters of Cholesterol Homeostasis in Freshly Isolated Mononuclear Cells From Patients With Heterozygous FH Treated With Lipid Lowering Drugs

	Total Cholesterol	LDL Cholesterol	Acetate to cholesterol pM/10^7 cells/5hrs	LDL degradation ng/4x10^6cells/4 hours	n
Diet	425.5 ± 34.8	352.8 ± 33.5	67.3 ± 14.8	7.53 ± 2.96	11
Colestipol	378.5 ± 30.6+	289.3 + 28.6+	110.5 ± 10.4*	13.59 ± 7.16**	11
% Change	(-12.4)	(-21.9)	(+64.2)	(+80.5)	
Diet	416.1 ± 20.7	331.4 ± 17.8	65.2 ± 17.3	3.74 ± 0.88	7
Niacin	282.2 ± 12.9+	200.3 ± 12.5+	58.3 ± 13.8	3.92 ± 0.42	7
% Change	(-32.2)	(-39.6)	(-10.6)	(+4.8)	
Diet	433.2 ± 20.7	351.9 ± 20.2	71.6 ± 19.2	3.95 ± 0.82	6
Colestipol/ Niacin	291.0 ± 25.9+	203.5 ± 25.2+	56.6 ± 12.4	4.23 ± 1.22	6
% Change	(-32.8)	(-42.2)	(-20.9)	(+7.1)	

Values are mean ± SEM. All patients were studied prospectively and the diet treatment values shown. Differences in mean plasma cholesterol concentration and rates of acetate incorporation were established using paired t test. Wilcoxon rank sum was used to test the changes in rates of LDL degradation.

* $p<0.025$ vs Diet ** $p<0.005$ vs Diet + $p<0.001$ vs Diet

Table 3
Composition of Total Low Density Lipoprotein (Density 1.020-1.060 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs

	TREATMENT GROUP				
COMPONENT	DIET ONLY	COLESTIPOL	COLESTIPOL/NIACIN	NIACIN	NORMAL
FC (WT %)	8.09 ± 0.20	7.20 ± 0.35*	7.52 ± 0.35	7.37 ± 0.45	7.46 ± 0.23*
CE (WT %)	35.44 ± 0.55	35.03 ± 0.59	36.52 ± 0.72	36.39 ± 0.65	34.63 ± 0.71
TG (WT %)	3.70 ± 0.36	5.20 ± 0.65*	4.53 ± 0.56	4.02 ± 1.16	5.22 ± 0.83
PRO (WT %)	19.11 ± 0.43	18.13 ± 0.97	19.84 ± 0.84	17.20 ± 0.27**	20.25 ± 0.66
PL (WT %)	33.65 ± 0.62	34.38 ± 0.55	31.87 ± 0.85	35.02 ± 0.21*	32.44 ± 0.78
FC/PL	0.242 ± .007	0.211 ± .010+	0.237 ± .009	0.211 ± .014	0.233 ± .012
TC/PL	0.856 ± .018	0.830 ± .025	0.897 ± .030	0.828 ± .038	0.831 ± .020
TC/PRO	1.472 ± .037	1.567 ± .042	1.490 ± .075	1.541 ± .119	1.365 ± .062
FC/CE	0.394 ± .013	0.360 ± .020	0.358 ± .020	0.350 ± .015*	0.375 ± .014
N	34	21	8	2	18

Values are mean ± SEM. The data presented is cross sectional. Composition data for 18 normal subjects is shown for comparison purposes. Comparisons of the differences between means were performed with an unpaired t test. FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein, PL- phospholipid, TC- total cholesterol

* P<0.05 vs Diet
+ P<0.02 vs Diet
**P<0.01 vs Diet

Table 4a
Composition of Total Low Density Lipoprotein (Density 1.020-1.060 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study

	TREATMENT GROUP		
COMPONENT	DIET ONLY	COLESTIPOL	% CHANGE
FC (ug/ml)	821 ± 43	651 ± 61+	-20.67
CE (ug/ml)	2090 ± 103	1786 ± 141	-14.55
TG (ug/ml)	460 ± 77	581 ± 122	26.21
PRO (ug/ml)	2152 ± 67	1632 ± 90*	-24.21
PL (ug/ml)	3256 ± 165	3417 ± 341	3.09
FC/PRO	0.401 ± .016	0.407 ± .035	1.47
CE/PRO	1.673 ± .080	1.907 ± .101	12.27
TC/PRO	1.376 ± .053	1.564 ± .063+	12.02
TG/PRO	0.202 ± .027	0.356 ± .061*	43.26
PL/PRO	1.668 ± .100	1.951 ± .128	14.51
N	8	8	

Values are mean ± SEM. Differences between means were assessed with the Wilcoxon rank sum.
FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein,
PL- phospholipid, TC- total cholesterol

* $P<0.008$ vs Diet + $P<0.039$ vs Diet

Table 4b
Composition of Total Low Density Lipoprotein (Density 1.020-1.060 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study

	TREATMENT GROUP		
COMPONENT	DIET ONLY	COLESTIPOL	NORMAL
FC (WT %)	8.12 ± 0.29	7.18 ± 0.41+	7.46 ± 0.23
CE (WT %)	33.96 ± 0.55	33.84 ± 1.24	34.63 ± 0.71
TG (WT %)	4.08 ± 0.53	6.11 ± 0.88+	5.22 ± 0.83
PRO (WT %)	20.37 ± 0.54	18.28 ± 1.07	20.25 ± 0.66
PL (WT %)	33.59 ± 1.31	34.46 ± 0.77	32.44 ± 0.78
FC/PL	0.244 ± .011	0.208 ± .001+	0.233 ± .012
SURF/CORE	1.667 ± .100	1.505 ± .051	1.509 ± .055
TC/PL	0.837 ± .035	0.822 ± .038	0.831 ± .020
FC/CE	0.422 ± .026	0.366 ± .022**	0.375 ± .014
N	8	8	18

Values are mean ± SEM. Composition data for 18 normal subjects is shown for comparison purposes. Differences between means were assessed with the Wilcoxon rank sum.FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein, PL- phospholipid, TC- total cholesterol, SURF/CORE- the ratio of the sum of the weights of the surface components (FC, PRO, PL) to the sum of the weights of the core components (CE, TG).

+ $P<0.039$ vs Diet only

Table 5a
Composition of Light Low Density Lipoprotein (Density 1.020-1.040 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study

	TREATMENT GROUP		
COMPONENT	DIET ONLY	COLESTIPOL	% CHANGE
FC (ug/ml)	276 ± 15	226 ± 15**	-18.3
CE (ug/ml)	758 ± 45	688 ± 45	- 9.4
TG (ug/ml)	137 ± 23	177 ± 27	29.6
PRO (ug/ml)	687 ± 29	625 ± 16+	- 9.9
PL (ug/ml)	1231 ± 82	1184 ± 79	- 3.8
FC/PRO	0.416 ± .024	0.374 ± .022*	-11.2
CE/PRO	1.971 ± .121	1.942 ± .128	1.5
TC/PRO	1.508 ± .076	1.499 ± .080	- 0.6
TG/PRO	0.206 ± .032	0.269 ± .034*	30.5
PL/PRO	1.834 ± .098	1.906 ± .113	3.8
N	12	12	

Values are mean ± SEM. Differences between means were assessed with the Wilcoxon rank sum.
FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein,
PL- phospholipid, TC- total cholesterol

* P<0.046 vs Diet + P<0.021 vs Diet **P<0.005 vs Diet

Table 5b
Composition of Light Low Density Lipoprotein (Density 1.020-1.040 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study

	TREATMENT GROUP		
COMPONENT	DIET ONLY	COLESTIPOL	NORMAL
FC (WT %)	7.66 ± 0.32	6.86 ± 0.33*	7.62 ± 0.25
CE (WT %)	35.99 ± 0.89	34.94 ± 0.90	30.68 ± 1.14
TG (WT %)	3.89 ± 0.61	4.95 ± 0.71	5.63 ± 1.39
PRO (WT %)	18.78 ± 0.74	18.81 ± 0.93	22.32 ± 1.66
PL (WT %)	33.68 ± 0.82	34.44 ± 0.65	33.75 ± 1.35
FC/PL	0.230 ± .013	0.201 ± .001**	0.228 ± .016
SURF/CORE	1.520 ± .052	1.517 ± .043	1.754 ± .055**
TC/PL	0.817 ± .027	0.795 ± .019*	0.834 ± .018
FC/CE	0.369 ± .016	0.342 ± .018+	0.431 ± .015+
N	12	12	8

Values are mean ± SEM. Composition data for 8 normal subjects is shown for comparison purposes. Statistical comparisons were performed with the Wilcoxon rank sum for the colestipol group, and with an unpaired t test for the normal subjects. FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein, PL- phospholipid, TC- total cholesterol, SURF/CORE- the ratio of the sum of the weights of the surface components (FC, PRO, PL) to the sum of the weights of the core components (CE, TG)

* P<0.046 vs Diet + P<0.026 vs Diet **P<0.011 vs Diet

Table 6a
Composition of Heavy Low Density Lipoprotein (Density 1.040-1.060 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study

	TREATMENT GROUP		
COMPONENT	DIET ONLY	COLESTIPOL	% CHANGE
FC (ug/ml)	553 ± 29	339 ± 23*	-38.6
CE (ug/ml)	1506 ± 78	1183 ± 93+	-27.3
TG (ug/ml)	232 ± 35	240 ± 42	3.6
PRO (ug/ml)	1416 ± 30	1047 ± 28*	-26.1
PL (ug/ml)	2352 ± 93	1810 ± 115*	-23.2
FC/PRO	0.391 ± .023	0.344 ± .022+	-14.7
CE/PRO	1.851 ± .101	1.912 ± .123	3.1
TC/PRO	1.395 ± .074	1.441 ± .060	1.4
TG/PRO	0.166 ± .032	0.219 ± .044	27.3
PL/PRO	1.674 ± .068	1.716 ± .073	2.3
N	12	12	

Values are mean ± SEM. Differences between means were assessed with the Wilcoxon rank sum.
FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein,
PL- phospholipid, TC- total cholesterol

* $P<0.0046$ vs Diet + $P<0.006$ vs Diet

Table 6b
Composition of Heavy Low Density Lipoprotein (Density 1.040-1.060 g/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study

	TREATMENT GROUP		
COMPONENT	DIET ONLY	COLESTIPOL	NORMAL
FC (WT %)	7.73 ± 0.30	6.55 ± 0.37**	7.34 ± 0.18
CE (WT %)	36.16 ± 0.86	36.55 ± 0.90	35.28 ± 1.56
TG (WT %)	3.27 ± 0.53	4.06 ± 0.55	3.48 ± 0.74
PRO (WT %)	20.00 ± 0.69	19.72 ± 0.72	19.25 ± 0.90
PL (WT %)	32.84 ± 0.65	33.13 ± 0.57	34.66 ± 2.17
FC/PL	0.282 ± .012	0.233 ± .014**	0.233 ± .012+
SURF/CORE	1.540 ± .060	1.481 ± .055	1.580 ± .055
TC/PL	0.858 ± .020	0.840 ± .016	0.831 ± .020
FC/CE	0.369 ± .018	0.321 ± .016**	0.375 ± .014
N	12	12	8

Values are mean ± SEM. Composition data for 8 normal subjects is shown for comparison purposes. Statistical comparisons for the colestipol group were done with the Wilcoxon rank sum and for the normal subjects with an unpaired t test. FC- free cholesterol, CE- cholesterol ester, TG- triglyceride, PRO- protein, PL- phospholipid, TC- total cholesterol, SURF/CORE- the ratio of the sum of the weights of the surface components (FC, PRO, PL) to the sum of the weights of the core components (CE, TG)

* P<0.05 vs Diet + P<0.01 vs Diet **P<0.005 vs Diet

Table 7a
Composition of Total Low Density Lipoprotein (Density 1.020-1.060 gm/cc)
Isolated From Patients with FH on Lipid Lowering Drugs During Longitudinal Study
Contribution of Individual Phospholipids (Weight %)

	TREATMENT GROUP		
PHOSPHOLIPID	DIET ONLY		PHOSPHOLIPID
NORMAL			DIET ONLY
SPHINGOMYELIN	19.29 ± 1.62	16.58 ± 2.05*	17.47 ± 2.31
LYSOPHOS-PHATIDYLCHOLINE	4.58 ± 1.07	1.91 ± 0.31	1.76 ± 0.07
PHOSPHATIDYL CHOLINE	66.11 ± 2.95	72.80 ± 0.43	63.59 ± 1.89
PHOSPHATIDYL INOSITOL	3.95 ± 1.00	4.86 ± 1.70	8.36 ± 0.79*
PHOSPHATIDYL ETHANOLAMINE	2.75 ± 0.67	2.11 ± 0.63	4.10 ± 1.18
PHOSPHATIDYL SERINE	3.32 ± 0.79	1.71 ± 0.41	4.52 ± 1.21
N	9	9	8

Values are mean ± SEM. The data for 8 normal subjects is shown for comparison. Statistical analysis of the colestipol group used the Wilcoxon rank sum. Comparisons with the normal subjects was using an unpaired t test.

* $P<0.05$ vs Diet

Table 7b
Composition of Light Low Density Lipoprotein (Density 1.020-1.040 gm/cc)
Isolated From Patients with FH on Lipid Lowering Drugs

Contribution of Individual Phospholipids (Weight %)

PHOSPHOLIPID	DIET ONLY	COLESTIPOL	NORMAL
SPHINGOMYELIN	20.62 ± 2.10	19.03 ± 2.88*	16.71 ± 2.76
LYSOPHOS-PHATIDYLCHOLINE	4.92 ± 0.91	5.41 ± 1.10	1.78 ± 0.51*
PHOSPHATIDYL CHOLINE	61.61 ± 2.97	61.51 ± 3.18	63.10 ± 5.01
PHOSPHATIDYL INOSITOL	5.49 ± 1.36	7.03 ± 1.76	8.54 ± 1.45
PHOSPHATIDYL ETHANOLAMINE	4.15 ± 1.49	3.11 ± 0.68	3.71 ± 1.25
PHOSPHATIDYL SERINE	3.04 ± 0.87	3.46 ± 0.66	6.30 ± 1.91
N	11	11	14

Values are mean ± SEM. The data for diet and colestipol represent the same patients studied prospectively. Statistical analysis of the colestipol group used the Wilcoxon rank sum, that for the normals used an unpaired t test.

* $P<.05$ vs Diet

Table 7c
Composition of Heavy Low Density Lipoprotein (Density 1.040-1.060 gm/cc)
Isolated From Patients with FH on Lipid Lowering Drugs

Contribution of Individual Phospholipids

PHOSPHOLIPID	DIET ONLY	COLESTIPOL	NORMAL
SPHINGOMYELIN	16.53 ± 2.26	17.71 ± 2.25	17.85 ± 1.68
LYSOPHOS-PHATIDYLCHOLINE	4.17 ± 0.90	4.04 ± 1.00	1.96 ± 0.55
PHOSPHATIDYL CHOLINE	66.83 ± 3.44	62.28 ± 3.95	63.59 ± 3.58
PHOSPHATIDYL INOSITOL	5.56 ± 1.26	5.25 ± 1.32	8.28 ± 1.43
PHOSPHATIDYL ETHANOLAMINE	3.28 ± 1.16	5.67 ± 1.93	4.29 ± 1.02
PHOSPHATIDYL SERINE	3.64 ± 0.93	5.04 ± 1.62	4.13 ± 1.50
N	12	12	16

Values are mean ± SEM The data for diet and colestipol represent the same patients studied prospectively. Statistical analysis used the Wilcoxon rank sum for the colestipol group and an unpaired t test for the normal subjects.

* $P<0.05$ vs Diet

Table 8. Dissociation Constants for LDL4 and LDL6 Isolated from Six Patients with Familial Hypercholesterolemia Before and After Treatment with Colestipol

Patient	LDL4 DIET	LDL4 COLESTIPOL	LDL6 DIET	LDL6 COLESTIPOL
DC	1.59×10^{-5}	4.51×10^{-5}	0.82×10^{-5}	2.04×10^{-5}
SG	0.26×10^{-5}	3.28×10^{-5}	0.27×10^{-5}	0.88×10^{-5}
VH	0.58×10^{-5}	6.76×10^{-5}	1.08×10^{-5}	1.31×10^{-5}
LZ	2.47×10^{-5}	1.48×10^{-5}	1.31×10^{-5}	2.00×10^{-5}
NC	2.17×10^{-5}	0.09×10^{-5}	3.42×10^{-5}	0.91×10^{-5}
RW	0.53×10^{-5}	14.6×10^{-5}	2.35×10^{-5}	1.28×10^{-5}
ARITHMETIC MEAN	1.17×10^{-5}	5.37×10^{-5}	1.54×10^{-5}	1.40×10^{-5}
($\pm$1 SEM)	0.34×10^{-5}	1.81×10^{-5}	0.40×10^{-5}	0.18×10^{-5}
	$P<0.05$ by paired t analysis		NS	
LIGAND POOLED MEAN	0.58×10^{-5}	2.18×10^{-5}	1.75×10^{-5}	0.92×10^{-5}
RANGE ($\pm$ 1 SEM)	0.48 - 0.71	1.77 - 2.67	1.58 - 1.93	0.78 - 1.19
	$P<0.01$ by F statistic		NS	

Range given because values calculated by LIGAND are log-normally distributed.

NORMAL (N=4)	2.42×10^{-5}	2.24×10^{-5}
RANGE ($\pm$ 1 SEM)	2.10 - 2.79	1.88 - 2.66
	$P<0.01$ vs Diet by F statistic	NS
	P NS vs Colestipol by F statistic	NS

NS- not significantly different The units for Kd are in ng/ml rather than in M/l because of the variation in "molecular weight" of the LDL species being tested.