INFLUENCE OF TREATMENT OF FAMILIAL HYPERCHOLESTEROLEMIA ON HUMAN MONONUCLEAR CELL LOW DENSITY LIPOPROTEIN RECEPTOR ACTIVITY: A MODEL FOR WHOLE BODY RESPONSES

by

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LDL receptor activity and cholesterol synthesis rates in mononuclear cells, as well as plasma and lipoprotein cholesterol levels were measured in patients with heterozygous familial hypercholesterolemia. Changes in these parameters were used to examine the metabolic effects of hypolipidemic drug therapy. Treatment with bile acid sequestrants increased LDL receptor activity and cholesterol synthesis rates in freshly isolated mononuclear cells. Nicotinic acid as a single agent had no effect on LDL receptor activity in mononuclear cells. When nicotinic acid was combined with a bile acid sequestrant, the increase in cholesterol synthesis was less than with a bile acid sequestrant alone.

LIST OF ABBREVIATIONS

VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
HDL	High density lipoprotein
HDL_C	An abnormal HDL present after cholesterol feeding
FH	Familial Hypercholesterolemia
LPDP	Lipoprotein depleted plasma

Specific degradation - The difference between the total amount of ^{125}I-LDL degraded and the non-specific degradation of $^{125}ILDL$ in the presence of excess unlabeled LDL.

FCR fractional catabolic rate Type IIa hypercholesterolemia - A 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 HDC remaining at normal levels.

HMG-CoA reductase - 2-hydroxy-3-methyl glutaryl Coenzyme A reductase. This is the rate-limiting enzyme in cholesterol synthesis. The direct product of the enzyme is mevalonic acid.

apo apolipoprotein

Introduction

Familial hypercholesterolemia (FH) is an autosomal, dominantly inherited disorder of metabolism which results in increased concentrations of low density lipoprotein (LDL) in plasma. Since LDL is the major cholesterol carrying lipoprotein, this increase is paralled by increases in plasma cholesterol levels. This lifelong hypercholesterolemia results in lipid deposition in the skin (xanthoma) and arterial wall (atherosclerosis). While xanthoma are of mainly cosmetic importance, cholesterol deposition in the arterial wall results in premature coronary disease. The reported gene frequency of heterozygous FH in the U.S.A. is 1/200-1/500 (1,2), and thus a common cause of hypercholesterolemia. The incidence of premature coronary artery disease and myocardial infarction in FH is 8 to 25 times greater than that of normolipidemic subjects (3,4,5). Average life expectancy is reduced 20 to 25 years.

The biochemical defect in FH involves a deficiency in the number of cell membrane high affinity LDL receptors. These receptors are responsible for specific recognition and transport of LDL into the cytosol. After LDL internalization and lysosomal degradation of the protein moiety, the liberated cholesterol decreases cholesterol synthesis and down regulates the number of LDL receptors. Studies of monozygotic and dizygotic adult twins have shown that the maximally stimulated activity of LDL receptors in peripheral mononuclear cells (6) as well as fibroblasts (7) is under genetic control although environmental changes may have some effects. FH heterozygotes possess approximately half the normal number of functioning receptors, thus

requiring circulating LDL levels twice that found in normolipidemic subjects for normal regulation of cholesterol metabolism. In the rare subject with homozygous FH cellular LDL receptors are either absent or present in markedly reduced numbers.

The rationale for hypolipidemic therapy in FH is based on the premise that long term lowering of LDL cholesterol will decrease or prevent the lethal complication of the disease: atherosclerosis. Several questions remain unanswered. Although it is known that FH is fully expressed (e.g. hypercholesterolemia) at birth (8), the question of how soon therapy must be instituted to prevent atherosclerosis and when the atherosclerotic lesion becomes irreversible remains to be answered.

It has been proposed that because of the decreased receptor number patients with heterozygous FH require an increased plasma LDL level to deliver cholesterol to peripheral tissues and suppress cholesterol synthesis (9). However, it is not known if the 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 lipid lowering therapy. More understanding about this question may be gained by studying lipid synthesis in other altered states of cholesterol metabolism where the receptors are normal, but the plasma low density lipoprotein concentration is increased (as in hypothyroidism and anorexia nervosa) or in hypobetalipoproteinemia or abetalipoproteinemia (in which LDL concentrations are either reduced or absent).

Treatment of patients with FH, using a combination of nicotinic acid and colestipol will reduce plasma total and LDL

cholesterol levels by 30% to 50% (10). The hypolipidemic mechanisms differ. Nicotinic acid depresses LDL levels by reducing free fatty acid mobilization from adipocytes, with a resultant secondary suppression of hepatic VLDL and LDL synthesis. Colestipol and cholestyramine act more directly, and increase bile acid excretion (by binding bile acids in the intestinal lumen) thus producing a drain on the hepatic cholesterol pool and a secondary increase in LDL catabolism. The decrease in serum cholesterol produced by these drugs may yield two effects. In the steady-state there should be an increase in LDL receptor activity towards normal levels and a rise in cellular cholesterol synthesis rates.

By examining changes in serum cholesterol and lipoprotein levels in comparison to alterations of LDL receptor activity and cholesterol synthesis rates in mononuclear cells, some insights into mechanisms and metabolic consequences of hypolipidemic therapy should be gained.

Cholesterol Metabolism

Cholesterol is an essential component of animal cell membranes and can be derived from either de novo synthesis or from the diet (11). In animals fed a cholesterol-free diet hepatic synthesis of cholesterol increases, and together with the intestine, may be able to synthesize enough cholesterol for the whole body. While almost all tissues can synthesize cholesterol they do not increase production under conditions of dietary cholesterol deprivation. In contrast, dietary cholesterol can almost completely suppress hepatic and, to a lesser extent, intestinal synthesis, whereas other tissues continue low rates

of synthesis (12,13).

Regardless of source, cholesterol, is transported from the liver to peripheral tissues as a component of lipoproteins. Tissue culture studies have documented cell surface receptors which recognize apolipoprotein B (apo-B) present in both VLDL and LDL, and thereby mediate cellular cholesterol uptake (14). Since VLDL is too large to enter the interstitial space from plasma (15), it is thought to be LDL that delivers cholesterol to peripheral cells. The two major organs of cholesterol synthesis, the liver and the intestine, are the sole sources of apo-B (16,17). In man, apo-B is secreted as a component of chylomicrons and VLDL. Intravascular catabolism of VLDL, with loss of triglyceride and apolipoprotein-C (18) leads to the production of LDL.

The first step in cellular metabolism of LDL involves the binding of the lipoprotein particle to a specific LDL receptor on the surface of the cell membrane. This receptor fulfills the classic requirements, it is highly specific, exhibits high affinity, and is saturable. The receptor is able to bind LDL (which contains only apoprotein B (19)), but can also bind some lipoprotein particles which contain apolipoprotein E. One of these particles (HDLc) is synthesized in response to cholesterol feeding in man and several other species. (20,21).

Recent studies have characterized the receptor. The receptor is an acidic glycoprotein with a molecular weight of 164,000. It has an isoelectric point of pH 4.6 (22). Each receptor can bind four particles of LDL (Kd~3 nM) or a single particle of HDLc (Kd .13nM) illustrating the binding abilities of the respective apoproteins. There is no evidence of cooperativity (23). Only one class of binding site is

implied by these studies because the response to radiation damage is linear (24). Whether this functional unit is a single polypeptide or several tightly linked subunits remains to be established. In addition to the binding site for lipoproteins, there seems to be a separate subunit responsible for internalization of the loaded receptor. This has been implied by the identification of a patient whose cells can bind LDL normally, but in whom the bound LDL is not internalized (25,26).

The receptor binding of both apoprotein B and apoprotein E is dependent upon lysyl and arginyl residues. Modification of these amino acids, whether the charge is preserved or eliminated, decreases or eliminates receptor-dependant uptake of lipoproteins bearing these apoproteins (27,28).

Cholesterol taken up via the receptor pathway can suppress cholesterol biosynthesis (20,29,30). The LDL concentration at which fibroblasts exhibit one-half maximal binding is about 20 μg/ml (31), well below the amounts circulating in plasma, but only about half the level present in human lymph (15). The LDL concentration in lymph probably more closely approximates that which most human cells see in vivo.

After LDL binds to the receptor active site, the complex is internalized via endocytosis but the lipoprotein maintains its structure until the endocytotic vesicle fuses with a lysosome (32,33). The lysosomal enzymes hydrolyze the apoproteins to constitutent amino acids and oligopeptides (31), releasing the cholesterol ester in the core. The cholesterol ester is hydrolyzed by a lysosomal hydrolase allowing the free cholesterol to enter the cytosol and become available for metabolism (14,32,33). This liberated cholesterol regulates two

microsomal enzymes. It suppresses HMG CoA reductase, decreasing endogenous cholesterol synthesis (33,34), and it activates acyl CoA: cholesterol acyl transferase (ACAT), thereby facilitating cholesterol storage in the ester form (35,36), chiefly as monounsaturated palmitoleate and oleate. When a cell accumulates sufficient cholesterol for membrane production and ester storage, synthesis of the receptor is suppressed (37), and the number of surface LDL receptors decreases. This, in turn, reduces the cell's ability to take up cholesterol and prevents the accumulation of excess cholesterol in either the free or esterified form. (37).

In cultured human fibroblasts receptor mediated uptake of LDL produces a net increase in cellular cholesterol (14) and shifts the ester composition from one rich in polyunsaturated fatty acids to a monounsaturated fatty acid enriched form (33). If the mechanisms of cholesterol regulation in the whole body are similar to this cell culture model, then the LDL receptor plays a major role in modulating cholesterol homeostasis by mediating the uptake and degradation of cholesterol from plasma LDL, thereby aiding the regulation of extracellular LDL concentration. The same process also maintains extrahepatic cholesterol synthesis at a low level by providing sufficient cholesterol for cellular needs (9,38,39).

Hepatic cholesterol synthesis is suppresed by dietary cholesterol through feedback inhibition of 3-hydroxy-3-methyl glutaryl Co-enzyme A reductase (HMG CoA reductase) an NADPH dependent microsomal enzyme which irreversably converts HMG CoA to mevalonic acid. There appear to be two mechanisms for inactivation of hepatic HMG CoA reductase. In the rat, within 12 hours after cholesterol feeding, there

is decreased enzyme activity without loss of immune reactive enzyme (40,41). After 24-hour cholesterol feeding, there is lowered immunochemically detectable enzyme, apparently because of decreased enzyme synthesis. (42,43)

In contrast to the inhibitory effects of cholesterol feeding, factors which increase cholesterol removal from the liver result in an increase in the rate of de novo cholesterol synthesis.The best understood example of this involves synthesis and losses of bile acids. Removal of bile acid through an external fistula, ileal bypass or bile acid sequestrant treatment increases bile acid synthesis (1,15) apparently through increased microsomal 7-α hydroxylase activity (44). This, in turn, causes a secondary increase in hepatic cholesterol synthesis (1,15,44).

The concept that changes in cholesterol synthesis in the hepatocyte may be paralleled by similar changes in non-hepatic cells has been addressed by Young and Rodwell (45). They showed that factors which altered HMG CoA reductase in rat hepatocytes caused parallel changes in enzyme activity in leukocytes. Thus leukocytes would appear to be a potentially useful and accessible cell in which factors affecting cholesterol homeostasis and LDL receptor activity in man can be evaluated.

McNamara (46) showed that cholestyramine treatment increases mononuclear cell cholesterol synthesis rates, while clofibrate had no effect. These results are consistant with the data on the effects of these same drugs on whole body cholesterol synthesis. Unfortunately these data were obtained using a very heterogeneous group of patients with many etiologies for their hypercholesterolemia, unlike the group of

patients involved in the current study.

Anderson and Johansen (47) have also used rates of sterol synthesis by white blood cells as an indirect measurement of LDL receptor activity in 10 kindreds with FH. They showed the expected impairment in LDL suppression of sterol synthesis in most of the FH heterozygotes. They did not confirm receptor defects in any of their subjects other than by these measurements, which were not always consistant. They did not mention what effects hypolipidemic agents, if any, made on sterol synthesis, nor did they discuss the hypertriglyceridemia present in several of their subjects. Despite these minor defects, their study reinforces the view that human mononuclear cells provide an easily accessible cell in which the influences of hyperlipidemic and hypolipidemic states on cholesterol synthesis rates and LDL receptor number may be examined.

Most non-hepatic tissues exhibit low cholesterol synthesis rates (12,13). Human fibroblasts possess the potential to produce large amounts of cholesterol, but the ability is usually suppressed by exposure to whole serum (containing LDL) via the same mechanism as hepatocytes; that is, an inhibition of HMG CoA reductase (34,48). That this pathway operates in mononuclear cells <u>in vivo</u> is supported by studies which show low rates of cholesterol synthesis and low HMG CoA reductase activity in freshly isolated cells. These may be further suppressed by excess LDL (49). Conversely, after 12 hours of incubation in a lipoprotein deficient medium the activity of both these parameters increase greatly but this increase can be prevented by inclusion of lipoproteins in the medium.

Another line of evidence supporting suppressive effects of LDL

in vivo is that fresh skin slices from abetalipoproteinemic patients who totally lack LDL show five-fold higher cholesterol synthesis rates than those of control slices (50). These findings are similar to those which occur in mononuclear cells from abetalipoproteinemic patients in which cholesterol synthesis from acetate was reported to be 2 to 8 times higher than normal cells (51). This is in contrast to the results of Reichl, et al (52) who have shown no differences in cholesterol synthesis or ^{125}I LDL binding or degradation in freshly isolated mononuclear cells. Further studies are needed in this area.

The LDL Receptor and Familial Hypercholesterolemia

Studies on the metabolism of LDL by fibroblasts from patients with homozygous FH have demonstrated at least three mutations affecting LDL receptor function (19,53,54,55). In one group of homozygotes, functional receptors are absent and cells from these patients are designated receptor negative because they show no specific binding of LDL (19,54). This results in reduced catabolism of LDL (19), lack of suppression of HMG CoA reductase activity and cholesterol synthesis, and a lack of stimulation of cholesterol ester synthesis (54,56,57). In contrast to this, fibroblasts from obligate heterozygotes show a 50% reduction in the number of receptors from the levels present in normal subjects (37,58).

A second clinically indistinguishable group of FH homozygotes has been designated receptor defective (54). Unlike the receptor negative cells, fibroblasts from these patients do possess some receptors, but the receptors appear to be abnormal, and are only able to bind approximately 10% of the normal amount of LDL. This allows limited

suppression of HMG CoA reductase (54,59) and detectable cholesterol ester synthesis but only at the expense of very high plasma LDL concentrations. It has been proposed that this type of homozygous FH is a combination between two different mutants at a single locus, analogous to SC hemoglobinopathy. This presumably would occur from possessing one receptor negative allele and one allele from a functional but reduced binding affinity receptor. There are, however, no consistant clinical differences between receptor-defective and receptor-negative homozygotes, and too few families have been studied to show independent segregation.

The third receptor abnormality (identified in two FH homozygotes) involves a lack of LDL internalization after normal binding (25,26). Cells from these patients exhibit a normal number of LDL receptor binding sites with normal binding affinity, but after binding of LDL to the receptor, there is no internalization of the bound LDL. This block in LDL internalization results in a lack of net uptake of lipoprotein bound cholesterol, impaired suppression of HMG CoA reductase and cholesterol synthesis and a lack of the normal increase in cholesterol esterification and storage. The clinical result is identical to that seen in the other known receptor defects. In none of the cases is it known whether the gene defect is in the coding for the receptor protein or in some accessory protein required for proper function.

Recent studies (60,61) have demonstrated allelic variation in LDL receptor activity in normal subjects. Maartmann-Moe, et al (61) have proposed that the variance seen in patients with FH as well as normal subjects is due to the presence of at least four normal alleles

at a single LDL receptor locus. These alleles code for LDL receptors with varying activities, whether measured by binding and internalization or by degradation of labeled LDL. The two- to three-fold differences in activity between the alleles may also explain the slight overlap in receptor activity between FH patients and normolipidemic individuals.

In cultured fibroblasts from receptor negative FH homozygotes the defect in regulation can be overcome if a non-lipoprotein (artificial presentation) form of cholesterol is used allowing cellular uptake without LDL receptor mediation (14,34,35,62). If fibroblasts from patients with homozygous FH are exposed to cholesterol dissolved in ethyl alcohol, HMG CoA reductase is suppressed (35), as in normal cells. Therefore the intracellular mechanisms for cholesterol metabolic regulation are available and functioning <u>if</u> the sterol is delivered intracellularly. Also of interest are data which show that oxygenated cholesterol derivatives such as 7-keto-cholesterol and 25-hydroxy-cholesterol are much more potent suppressors of HMG CoA reductase (62,63,64) or activators of esterification (36) than cholesterol. There is no difference in response of either normal or homozygous fibroblasts to these oxygenated sterols (36,62).

The metabolic defect in FH is also expressed by lymphoid cells in culture. HMG CoA reductase is suppressed and cholesterol esterifying activity is increased by LDL in normal leukocytes as in the fibroblasts discussed before (65). Cells from FH homozygotes, as would be expected, do not respond this way to LDL. Ho and coworkers (51) have demonstrated that these metabolic effects in cultured normal leukocytes are due to LDL binding to high affinity receptors. Observations that the receptors are missing from homozygotes and reduced in heterozygotes have been

reported by Fogelman, et al (66).

Studies on the binding of ^{125}I labeled LDL by cultured fibroblasts have shown that obligate heterozygotes (parents of homozygotes) possess on the average one-half the normal number of LDL receptors. This is similar to results in other subjects who show classic clinical and genetic features of heterozygous FH (38,58). This genetic dosage effect and allelism has been documented by Goldstein, et al (55) and Bilheimer, et al (26). As would be expected at saturating concentrations of LDL, only one-half the normal number of particles bind to the cells, and the rates of degradation of LDL and formation of cholesterol esters are half of that seen in normal cells (54,58). The suppression of HMG CoA reductase activity and cholesterol synthesis is also reduced (58,67). When the concentrations of LDL are below those necessary for LDL receptor saturation, cells from heterozygotes require surrounding LDL concentrations twice as high as normal cells for any of the genotypes to bind and degrade equivalent amounts of lipoprotein (9). The suppression of HMG CoA reductase and activation of cholesterol esterification in fibroblasts parallels the changes seen in receptor activity (19,35).

Current knowledge of LDL and cholesterol metabolism in intact humans and fibroblasts permits speculation about a model for LDL receptor deficiency (68). For heterozygotes the LDL receptor model predicts that the only observable steady-state abnormality would be a two- to threefold elevation in concentration of plasma LDL (9,68). As long as this elevated LDL concentration is maintained, the cells of heterozygotes utilize the same amount of LDL as normal cells, therefore their absolute rate of cholesterol synthesis and LDL degradation would be the same as normals. The fractional catabolic rate for LDL would be

decreased due to the increase in cholesterol pool size.

It is also worth noting that the two to threefold maximal increase of cholesterol synthesis by some homozygotes is less than the fourfold increase over normal rates of production seen in fibroblasts cultured from homozygotes in the presence of high extracellular LDL levels (56). This shows that there has been some suppression of synthesis perhaps from receptor independent endocytosis of LDL.

Sterol balance studies in patients with heterozygous FH have confirmed these predictions and have shown normal whole body cholesterol synthesis rates (69,70,71). LDL turnover studies have shown normal or slightly increased LDL synthetic rate along with a reduction in fractional catabolic rate (55,72).

Treatment of Hypercholesterolemia

Dietary management is the foundation of all current treatment methods for familial hypercholesterolemia. Recommendations include a total cholesterol intake less than 300 mg/day and a decrease in total fat intake with a relative increase in the amount of polyunsaturated fat over the amount of saturated fat and an increase in the complex carbohydrate content (73,74,75). For most heterozygotes such a diet will not completely normalize plasma cholesterol levels although it will yield a modest (10-15%) reduction (76).

There currently is no perfect agent for treating FH. The ideal agent would be a compound which would either allow LDL penetration into cells via the LDL receptor mediated pathway or directly inhibit cholesterol biosynthesis. Although this has not been achieved, several theraputic regimens have been observed to have empiric

hypocholesterolemic effects in heterozygotes (75,77,78).

Cholestyramine and colestipol are anion exchange resins which are not absorbed. They bind bile salts in the intestinal lumen, thereby preventing their absorption 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 (81), which depletes the hepatic cholesterol pool and results in an increased number of hepatic LDL receptors and an enhanced rate of LDL catabolism (79). Clinically, plasma LDL levels fall. Unfortunately, the increased drain on the hepatic cholesterol pool stimulates a marked increase in hepatic cholesterol synthesis, thus decreasing somewhat the effectiveness of this therapy (69,80,81). Even though hepatic cholesterol synthesis is increased, a bile acid sequestrant in conjunction with diet in heterozygotes will usually give a long term reduction in plasma cholesterol levels of about 30%, leaving the plasma cholesterol levels at the upper limits of normal (82,83,84) in many patients. Recent studies suggest that combined used of nicotinic acid and cholestyramine may be more effective than either drug alone (10,85). Nicotinic acid appears to reduce the compensatory increase in hepatic cholesterol synthesis and enhances the overall effectiveness of the drain on the body cholesterol pool brought about by bile acid sequestrant therapy.

Two recently discovered drugs may have considerable therapeutic application (86,87). Compactin [ML-236B] and Mevinolin [Monacolin K] are both fungal metabolites which have been shown to be specific competitive inhibitors of HMG CoA reductase and are active _in vitro_ and _in vivo_. Their structures and mode of action are virtually

identical. Experiments in the dog (88) have shown a decrease in cholesterol synthesis rate, an increase in the LDL fractional catabolic rate and an increase in LDL receptor activity. In recent studies 7 patients with heterozygous FH were treated for 24 weeks with compactin (30-60 mg/day). Plasma cholesterol levels fell by 22% (86) which is less than the response seen in combined therapy with colestid and niacin. Although these agents appear to be a potentially useful new class of hypolipidemic agents, several major cautions should be mentioned. All of the animal and human studies to date have been of relatively short duration (days to weeks), whereas therapeutic intervention is for life. Therefore these studies may not have given sufficient time for any possible long term effects to develop. Cholesterol functions not only in a structural role in membranes and as a digestive adjunct when converted to bile acids, but is also the precursor for vitamin D and steroid hormones. Mevalonic acid is also an obligatory precursor of ubiquinone and dolichol. In one study (86) an unchanged serum ubiquinone level was noted after treatment with compactin.

Indications of the importance of the LDL receptor are given by the data of Brown, et al (89) and Cummings, et al (127) which show that LDL receptor activity is increased in tissues with a high demand for cholesterol. The tissues with the greatest number of LDL receptors per milligram tissue are those involved in steroid hormone production, the adrenal cortex, the gonads and the placenta.

In normal subjects the adrenal response to stress involves increased cortisol secretion which may be derived from both endogenous adrenal cholesterol synthesis and increased uptake of LDL from plasma.

In patients with abetalipoproteinemia, where adrenal hormone response is dependant almost completely upon endogenous synthesis, there is decreased output of steroid hormones and metabolites during stimulation with ACTH (90,91). Whether compactin or mevinolin will have clinically significant effects upon corticosteroid levels in response to ACTH stimulation needs to be examined.

Despite these shortcomings the potential therapeutic benefit from specific inhibitors of HMG CoA reductase is large, and they may be a major addition to medical treatment of FH in the future.

Secondary Causes of Hypercholesterolemia

Several other disorders which cause hypercholesterolemia may alter cholesterol metabolism without primary alterations in the LDL receptor. Hypothyroidism is one of the more common secondary causes of hypercholesterolemia. It is a disorder of several etiologies, but the clinical presentation is the same, a generalized hypometabolic state. In many ways hypothyroidism is a phenocopy of FH, and is associated with an increase in plasma LDL concentrations, an acquired decrease in the number of LDL receptors and a decreased fractional catabolic rate of LDL (27). In a recent study of hypothyroid patients, Abrams and Grundy (92) showed a decrease in total sterol synthesis with no change in bile acid synthesis. This suggests that the increase of plasma LDL levels is related to a decreased excretion of neutral steroids rather than due to reduced conversion of cholesterol to bile acids.

Anorexia Nervosa may also be a secondary cause of hypercholesterolemia (93,94). While this disease is primarily of psychological origin, the physical and endocrine effects may be

profound. Forty to sixty percent of patients with anorexia develop significant hypercholesterolemia. This was originally thought to result from decreased levels of thyroid hormones, but on further examination thyroid status has been found to be normal, and the etiology of hypercholesterolemia remains unknown.

DESIGN OF THE STUDY

Aims

The aims of the study were: 1) to investigate the effects of hypolipidemic drug therapy in patients with familial hypercholesterolemia upon mononuclear cell LDL receptor activity and cholesterol synthesis and 2) to examine whether the freshly isolated mononuclear cell is a suitable model system for future investigations into drug and dietary effects on cholesterol metabolism.

Patient Popluation

Patients were characterized as having familial hypercholesterolemia on the basis of:

a. Persistant plasma cholesterol greater than 300 mg/dl with triglycerides lower than 200 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 consistant with an autosomal dominant trait.

c. The presence of tendon xanthomata 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 270 mg/dl in children within the family.

f. Documentation of decreased ^{125}I LDL degradation by lymphocytes or fibroblasts from the patient as compared with normal controls after initial incubation in lipoprotein deficient medium (95).

Most of the patients were not able to meet all of the

criteria. In those cases a in combination with b, and/or c or e were considered diagnostic. Subjects were drawn from patients currently being followed at the OHSU Lipid Clinic as well as new referrals.

Six patients currently being followed through the Clinical Research Center who have hypobetalipoproteinemia or abetalipoproteinemia were also studied.

Patients on estrogens, corticosteroids, or patients with additional disorders, such as diabetes, which are known to affect lipid metabolism or patients with exessive alcohol intake were excluded from the study.

Outline of Studies

One group of patients with FH had blood drawn for analysis of plasma cholesterol, triglyceride, lipoprotein composition, freshly isolated white blood cell receptor number and white blood cell sterol synthesis measurements when on no medication (diet therapy only). Thyroid function was checked when initially seen to ensure euthyroid status. The maximum inducible number of LDL receptors was also measured in most patients.

Studies in patients with abetalipoproteinemia and hypobetalipoproteinemia, (lipoproteins, LDL receptor activity and sterol synthesis rates in mononuclear cells) were undertaken as patients became available.

Patients with FH were studied under each of four possible treatment regimens. The treatment groups consisted of no drug (diet only), bile acid sequestrant (colestipol or cholestyramine) or nicotinic acid (as single drug therapy). A fourth group of patients used both a

bile acid sequestrant and nicotinic acid. The patients with FH on diet therapy only as well as a group of normal subjects were used as controls. The purpose of the study was explained and informed consent was obtained in all cases. The study was approved by the Human Research Committee of the Oregon Health Sciences University.

MATERIALS AND METHODS

Materials

Penicillin, streptomycin and RPMI-1640 with glutamine were purchased from Gibco. [^{3}H] Choline, 2-[^{14}C]-Acetate and ^{125}I were purchased from Amersham. Culture flasks and dishes were purchased from Falcon. All other chemicals were reagent grade.

Plasma Lipids and Lipoproteins

Blood was drawn by venipuncture into tubes containing 1 mg/ml EDTA as an anticoagulant. Plasma was separated by centrifugation and total cholesterol and triglyceride determined with the Auto-Analyzer II (96). Lipoproteins were separated by the methods described in the LRC Manual of Operations (97). Phospholipid phosphorus was assayed in aliquots of the total lipid extract (98) by the method of Bartlett (99). When indicated, major lipid classes and individual phospholipids were separated by thin layer chromatography on silica gel H which had been prewashed with chloroform methanol 1:1. The solvent system was heptane: diethyl ether: chloroform: acetic acid (80:10:10:1 v/v) for total lipids and chloroform: methanol: acetic acid: water (75:45:12:6 v/v) for phospholipids.

Low density lipoprotein (LDL) was isolated for composition analysis by preparative ultracentrifugation (100). The very low density lipoprotein was first removed by centrifugation at 180,000 xg for 11.5 hours with a Beckman type 50.3 rotor in a L8-70 ultracentrifuge at a density of 1.019 g/ml. The density of the infranate was adjusted to

1.063 g/ml by the addition of solid potassium bromide (101). The LDL was then isolated by flotation as above. The LDL was washed by reflotation at a density of 1.063 g/ml.

The purified LDL was dialyzed exhaustively against phosphate buffered saline (pH 7.4) and analyzed. Protein was measured by the method of Hartree (102), free and esterfied cholesterol by gas-liquid chromatography (103), and triglyceride and phospholipid as above.

Iodination of LDL

Low density lipoproteins were isolated by ultracentrifugation between the densities of 1.019 and 1.062 using a type 60 Ti rotor and a Beckman L8-70 ultracentrifuge. The lipoprotein was respun once at this density, dialysed to remove the potassium bromide and labeled with ^{125}I by the method of McFarland (104). Iodination was done at pH 10 at which incorporation into lipids is minimal. Iodine monochloride was added to give a molar ratio of iodine atoms to protein of less than one; for this calculation a molecular weight of 100,000 for LDL apoprotein was assumed.

The purified ^{125}I-LDL was filtered through a .45 μ Millipore R filter into a sterile vial and aliquots taken for counting and lipid and protein determinations. During isolation and purification of the LDL all glassware, solutions and tubes were autoclaved. With these methods less than 1% of the label was Trichloroacetic acid soluble, less than 4% was not precipitated by heparin-manganese, and 3% to 5% of the ^{125}I was bound to lipids, mostly in the lecithin and triglyceride fractions. The efficiency of protein labeling varied from 10% to 25%.

Lipid Synthesis Studies in Mononuclear Cells

Mononuclear cells were separated from 40cc to 50cc anticoagulated blood by centrifuging the whole blood, and aspirating off the plasma. About one-half of the red blood cells were aspirated from the bottom of the tube, and the remaining cells and plasma were diluted to a total volume of 25cc with RPMI-1640. This diluted blood was then layered over 20cc Ficoll-paque^{R} density gradient medium. After centrifugation at 1000g for 25 minutes the mononuclear cells were harvested from the interface between the diluted plasma and the gradient. The cells were washed 3 times in saline or RPMI-1640 by repeated suspension and centrifugation. These preparations contained 85% to 90% lymphocytes, 10% to 15% monocytes, and less than 2% granulocytes as determined by differential white cell counts of Wright's stained smears. Cells were counted by a Coulter^{R} cell counter and their viability assessed by Trypan Blue exclusion. For measurement of cholesterol and phospholipid synthesis, aliquots of 6 to 9 million cells were incubated at 37°C for 5 hours in homologous serum buffered to pH 7.4 containing 3.0 mM [^{3}H] choline and 1.5 mM 2[-^{14}C] acetate. At the end of the incubation period the lipids were extracted by the addition of chloroform-methanol 1:1 as previously described. The incorporation of labeled acetate into cholesterol, lanosterol, squalene (105), and total fatty acids, as well as incorporation of labeled choline into choline containing phospholipids was determined on aliquots of the lipid extracts by thin layer chromatography.

LDL Receptor Studies

Degradation of ^{125}I-LDL by mononuclear cells was measured as proposed by Bilheimer (95). Mononuclear cells, isolated as above, were incubated in 75 cm^2 flasks (cell concentration 2 x 10^6 cells/ml) at 37°C for 66 hours in RPMI-1640 medium supplemented with 2mM glutamine, 100 u/ml penicillin, 100 µg/ml streptomycin and 10% pooled human lipoprotein depleted plasma (LPDP). This treatment maximized the number of receptors on the cell surface. Two milliliter aliquots of the derepressed cell suspension were placed in 25 cm^2 flasks and 12.5 µg/ml ^{125}I-LDL (specific activity 200-400 cpm/ng) added to all flasks. 500µg/ml unlabeled LDL was added to some of the flasks to suppress degradation via the receptor mediated pathway. The flasks were then incubated an additional 5 hours at 37°C. The incubation was stopped by placing the flasks on ice and adding RPMI-1640 at 4°C. Cells and medium were harvested by pipetting the medium into 15cc conical tubes and centrifuging at 1500 g for 15 minutes at 4°C. After decanting the medium into 13 X 100 mm tubes, the cells were washed twice with saline and assayed for protein using the Lowry method (106). The medium was precipitated by addition of 0.5 ml of 50% trichloroacetic acid after addition of 0.5 cc plasma as carrier protein. After centrifugation, the free iodide in the trichloroacetic acid supernatant was oxidized to iodine by addition of 50 µl of 30% hydrogen peroxide and 20 µl of 40% potassium iodide as a carrier. The free iodine was then removed by repeated washing with chloroform. The final washed supernatant was counted on a Packard model 5160 gamma counter to measure the trichloroacetic acid soluble, non-iodine ^{125}I label. These counts represent ^{125}I-tyrosine and iodopeptides degradation of labeled LDL. Blank values were obtained by incubating flasks in the absence of cells

with the medium treated as above.

The specific receptor mediated degradation is the difference between the total amount of LDL degraded and that degraded in the presence of excess unlabeled LDL (representing internalization by bulk endocytosis). The measurement of maximally derepressed LDL receptor activity was done once during the study.

Receptor activity was also measured in freshly isolated mononuclear cells without the 66 hour derepression incubation. Twenty-five cm^2 flasks were seeded with 4.0 million cells in one milliliter of RPMI-1640 medium containing 30% LPDP, 2 mM glutamine, 100 µg/ml streptomycin and 100 u/ml penicillin (51). All the flasks received 25 µg/ml ^{125}I-LDL. Some flasks received an additional 500 µg/ml of unlabeled LDL as controls. The degradation of labeled LDL was determined as above. The activity of the freshly isolated cells is felt to reflect the in vivo activity at any given time.

RESULTS

Optimal Conditions for LDL Receptor Activity Measurement in Mononuclear Cells

Several experiments were done to assess optimal assay conditions for the determination of LDL receptor activity in freshly isolated mononuclear cells. LDL degradation was measured as a function of incubation time, cell number and substrate concentration. The effect of increasing calcium concentration was also investigated because LDL binding has been reported to be calcium dependant.

LDL degradation was found to be linear with cell number from one to twenty million cells per milliliter (fig. 1). As a function of time, degradation of LDL increased linearly with times over two hours to at least six hours (fig. 2). When the calcium concentration was varied from 0.3 mM to 1.8 mM the rate of LDL degradation decreased (fig. 3). LDL degradation was maximal at 0.3 mM calcium. The curve for LDL degradation vs. substrate concentration was biphasic (fig. 4). Overall, however, degradation increased with increasing LDL concentrations up to 200 µg/ml. This biphasic curve demonstrates the occurance of two binding processes. The steeper portion of the curve is mainly due to specific receptor mediated uptake of LDL. At an LDL concentration of about 25 µg/ml, the receptor pathway becomes saturated and the curve flattens out. The remaining slow increase in degradation is due to concentration dependant bulk phase endocytosis.

Derepression of cellular LDL receptors on mononuclear cells was achieved by incubating them for 18, 42, or 66 hours in RPMI-1640 supplemented with 10% human lipoprotein depleted plasma. As figure 5 illustrates, specific (receptor mediated) degradation of LDL increased

FIGURE LEGENDS

Fig. 1. Specific receptor mediated degradation of ^{125}I-LDL vs cell number. Total degradation was measured by incubating the indicated number of cells in 1 ml of medium containing 25 μg ^{125}I-LDL. Nonspecific degradation was measured after the addition of 500 μg of unlabled LDL. Specific degradation was determined by difference.

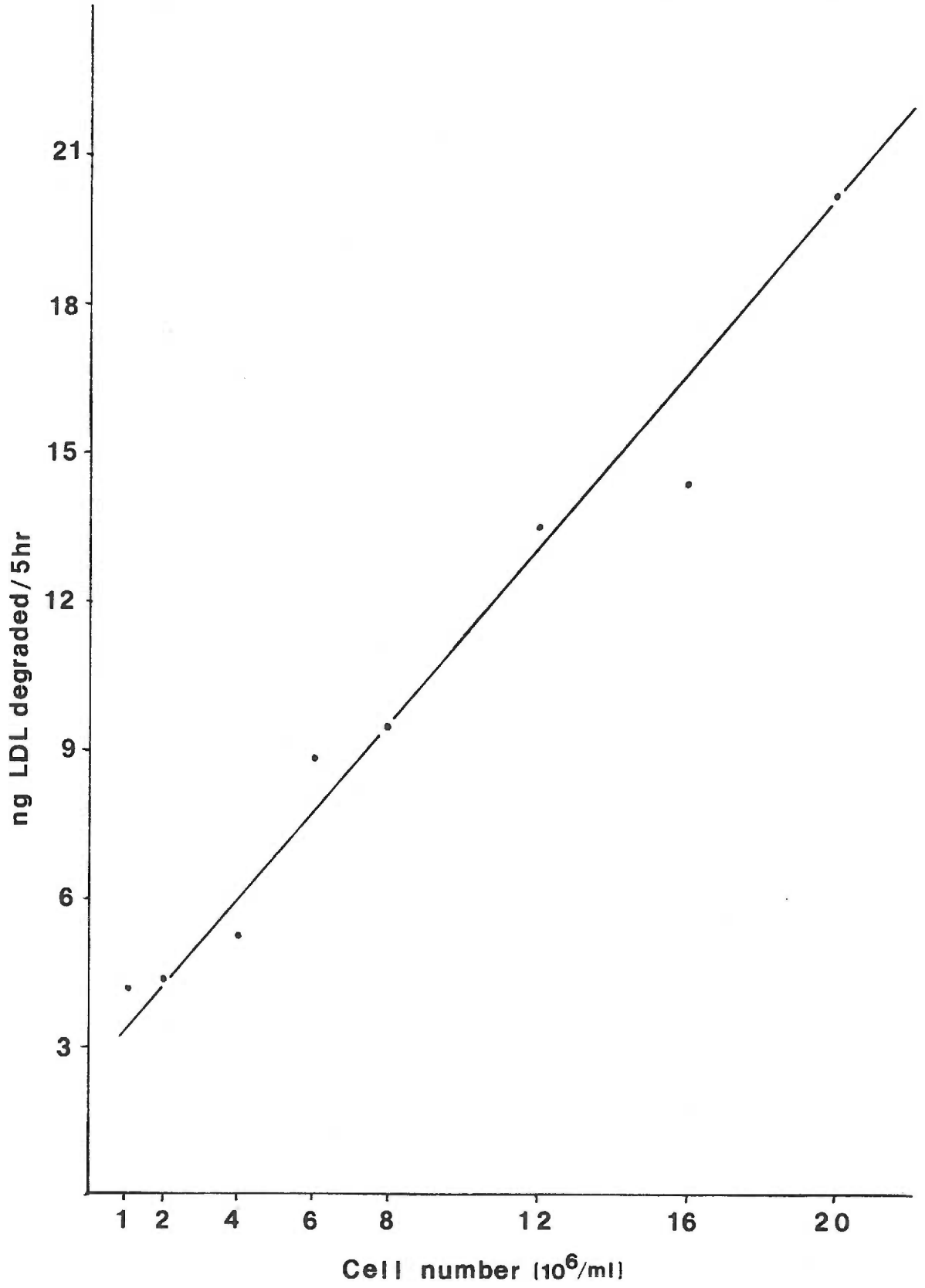

21
18
15
12
9
6
3
ng LDL degraded/5hr
1
2
4
6
8
12
16
20
Cell number (10^6/ml)

Fig. 2 Specific receptor mediated LDL degradation with time. After a two hour lag phase, degradation of LDL was linear with time for at least six hours of incubation. Specific degradaton was defined as the difference beween the total amount of LDL degraded (^{125}I-tyrosine produced) and the amount produced in the presence of a 20-fold excess of unlabeled LDL.

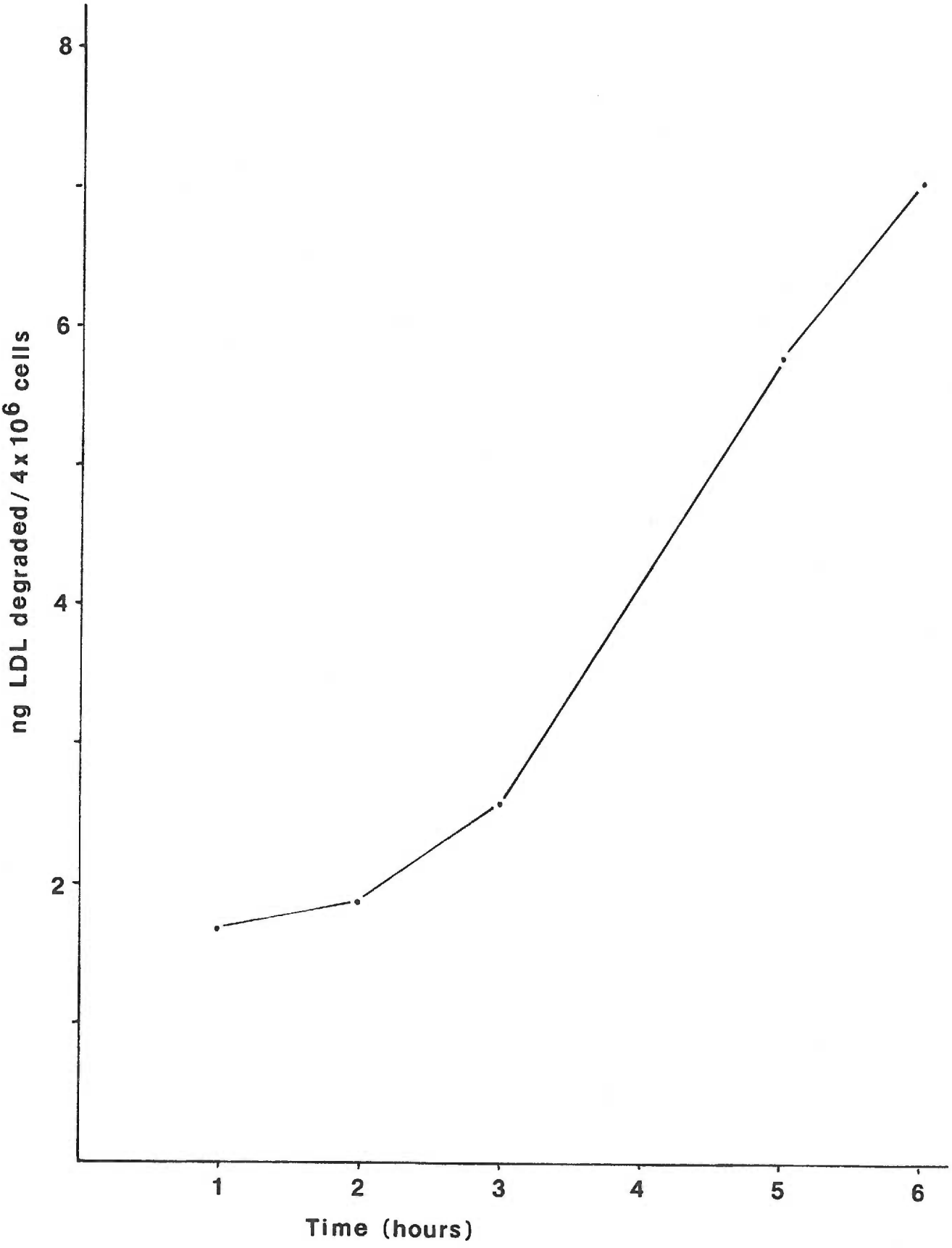

8
6
4
2
ng LDL degraded / 4 x 10^6 cells
1
2
3
4
5
6
Time (hours)

Fig. 3 Effect of calcium on specific degradation of LDL. Increasing amounts of calcium (as gluconate) were added to the incubation medium. Calcim concentrations greater than 1.8 mM produced a precipate in the medium, and thus could not be used. Concentrations less than 0.3 mM were not examined because this was the basal concentration of calcium in the RPMI-1640.

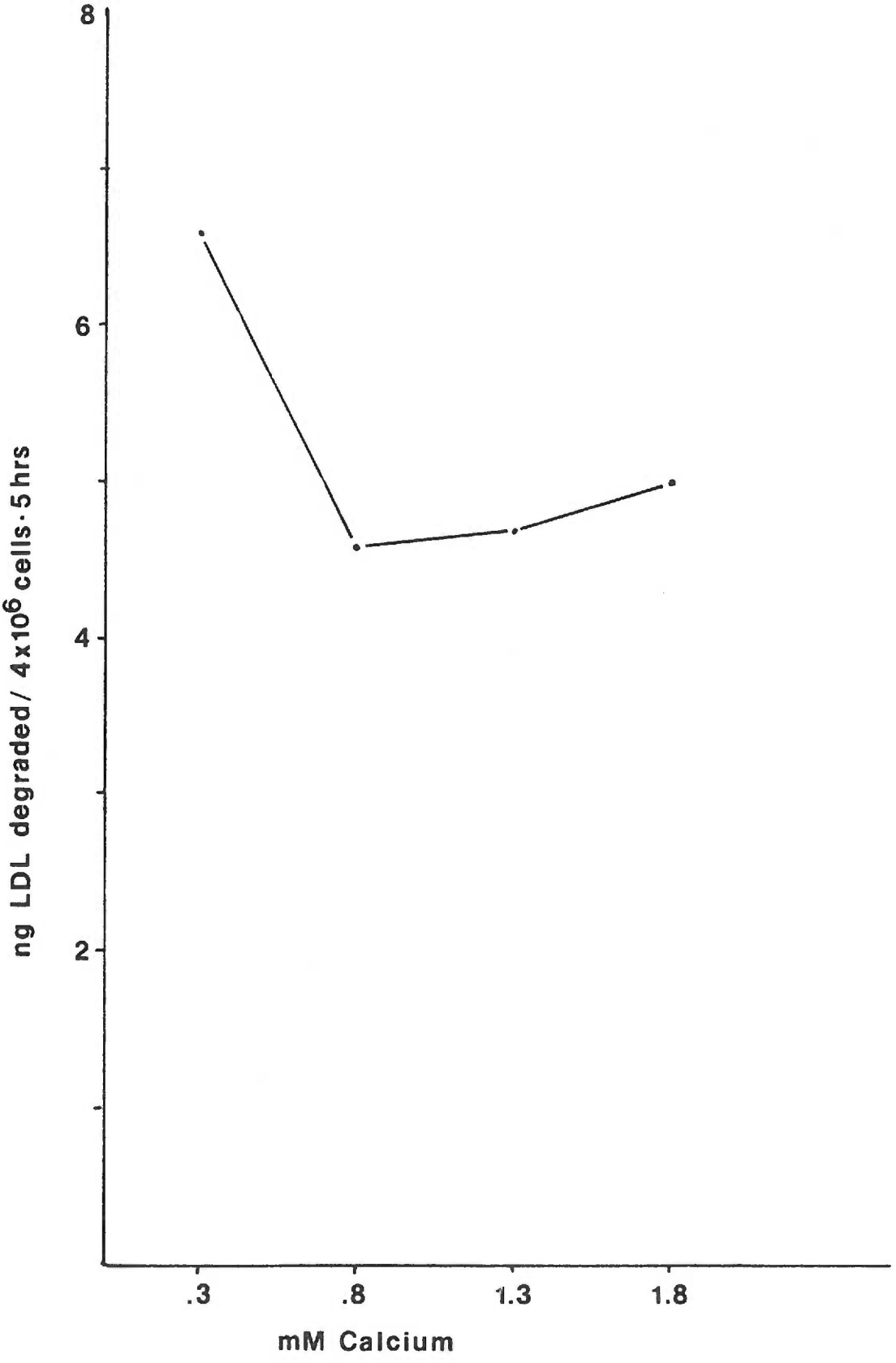

8
6
4
2
ng LDL degraded/ 4x10^6 cells·5 hrs
.3
.8
1.3
1.8
mM Calcium

Fig. 4 Specific degradation of LDL with increasing substrate concentratons. 4×10^6 cells were incubated in one ml of RPMI-1640 for 5 hours at 37°C in the presence of 0.75 to 200 µg/ml ^{125}I-LDL with and without 1000µg unlabeled LDL.

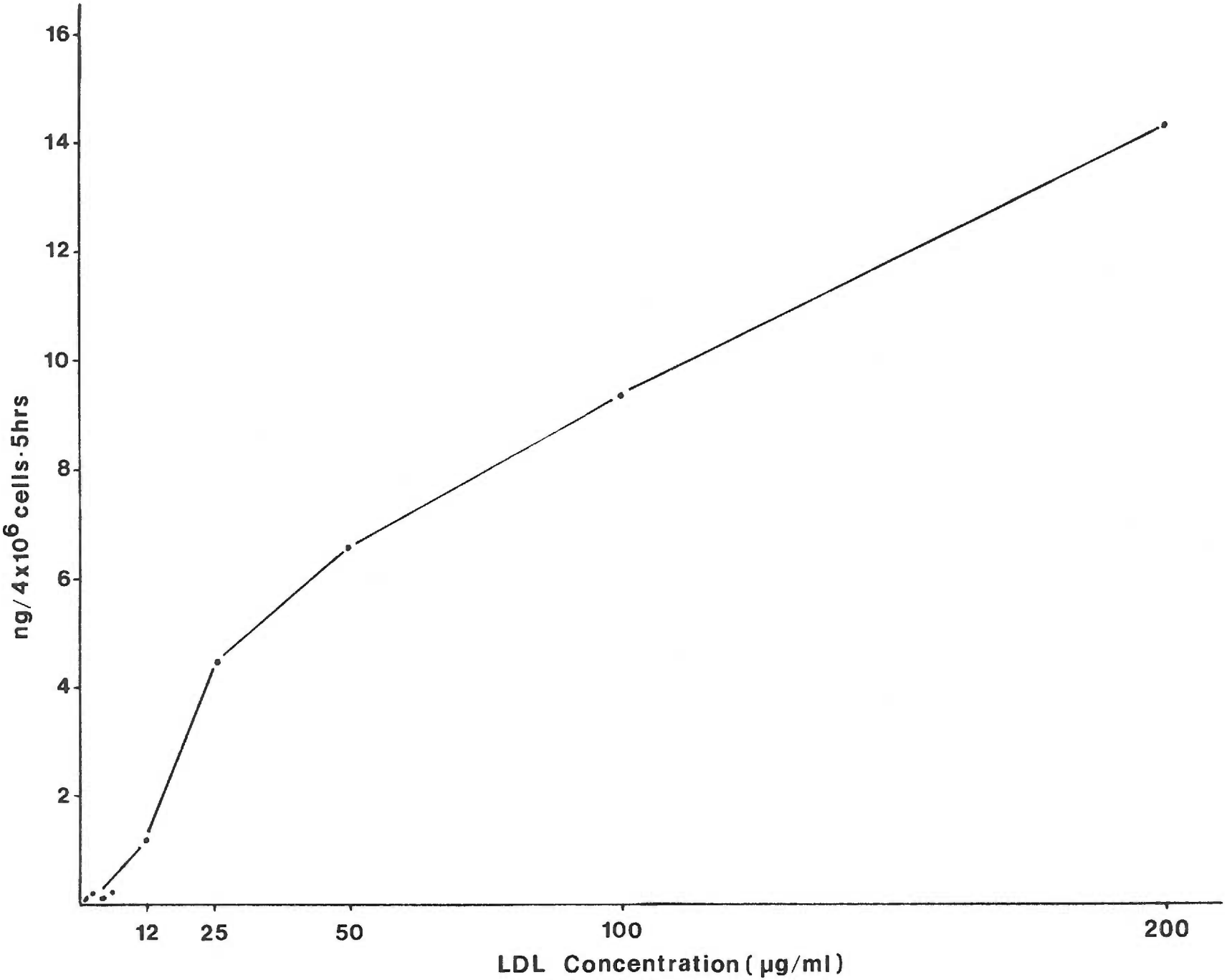
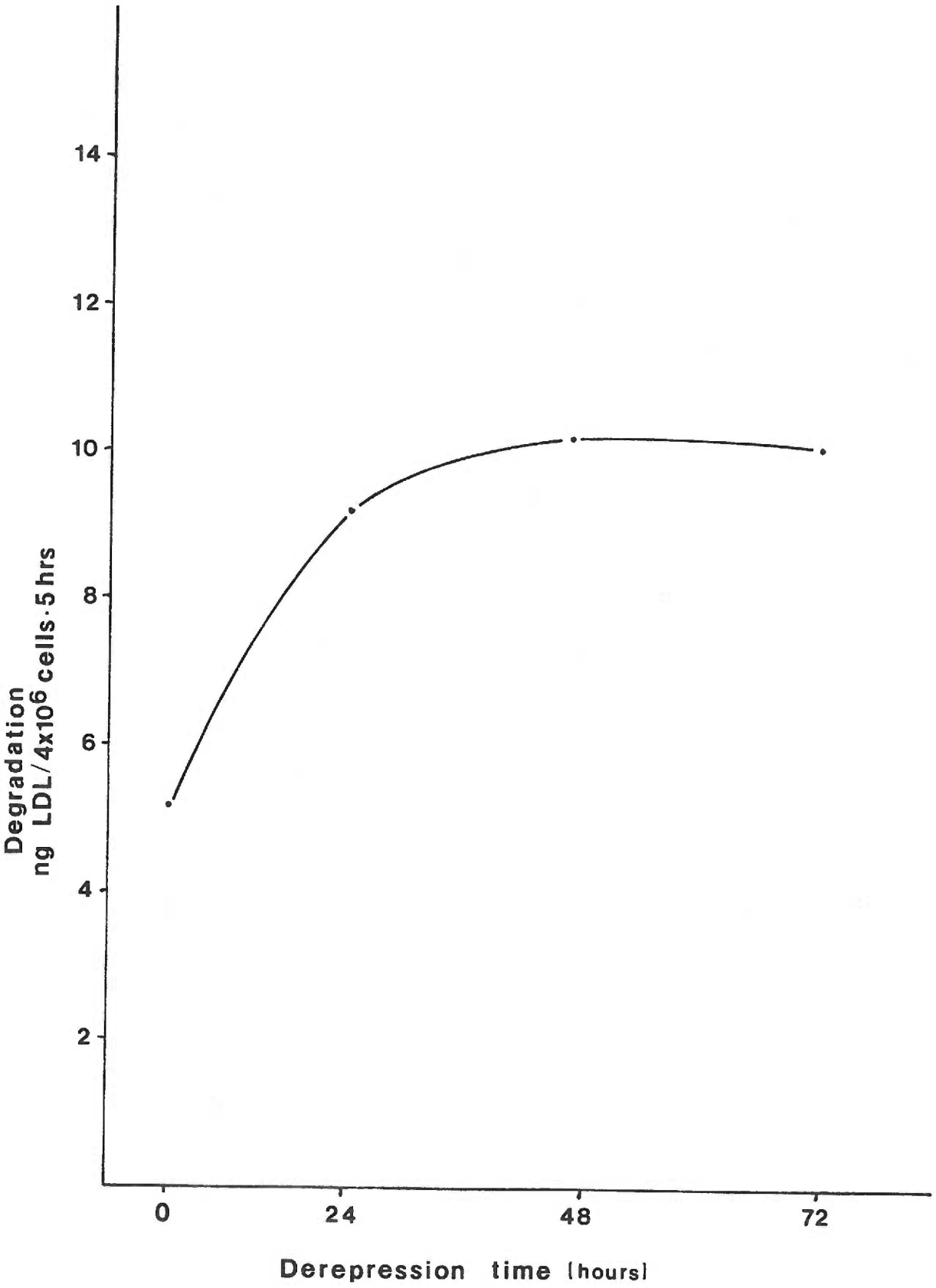

16
14
12
10
8
6
4
2
ng/4x10^6 cells·5hrs
12
25
50
100
200
LDL Concentration (µg/ml)

Fig. 5 Response of LDL receptor activity to derepression. Mononuclear cells (2×10^6/ml) were incubated to 18, 42, or 66 hours in RPMI-1640 supplemented with 10% LPDP. After this incubation, 12.5µg/ml of ^{125}I-LDL was added to replicate aliquots. Control aliquots had an additional 500 µg/ml unlabeled LDL added. Cells were then incubated for an additional five hours and processed in the same manner as freshly isolated cells. Specific degradation increased in a linear fashion for 66 hours of derepression. Beyond this length of derepression, there was a decrease in the number of viable cells and a decrease in the accuracy of the assay.


14
12
10
8
6
4
2
Degradation
ng LDL/4x10⁶ cells·5 hrs
0
24
48
72
Derepression time (hours)

linearly in response to longer times of derepression in the lipid free medium. Cell viability, measured by Trypan Blue exclusion, indicated that more than 90% of the cells were viable after 66 hours of incubation.

The results of these experiments established the standard conditions as outlined in the methods.

Optimal Conditions for Cholesterol and phospholipid synthesis measurement

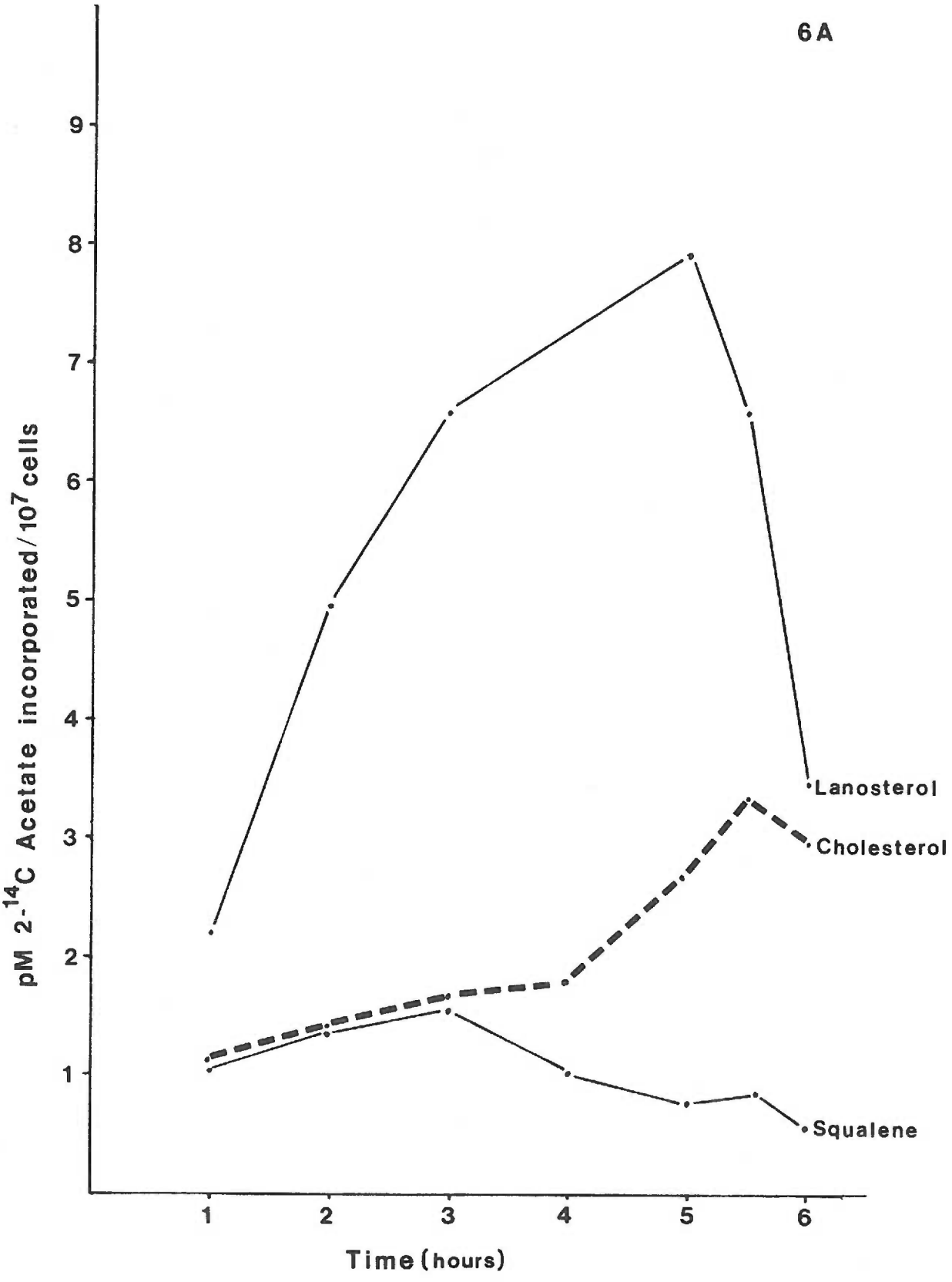
To establish appropriate assay conditions for measuring lipid synthesis in a mononuclear cell system, studies similar to those described above were conducted. One aim of these studies was to minimize differences between the *in vivo* environment in plasma and the *in vitro* conditions. With this in mind, the incubation medium was based on autologous plasma buffered to pH 7.4. The time dependent incorporation of 2-[^{14}C]-acetate into sterols and their immediate C-30 precursor, squalene, is shown in fig. 6a. The curves show typical precursor-product relationship with squalene peaking at 3 hours, lanosterol at 5 hours and cholesterol at 5 $\frac{1}{2}$ hours. The drop in cholesterol incorporation at 6 hours is probably due to impaired viability of the cells.

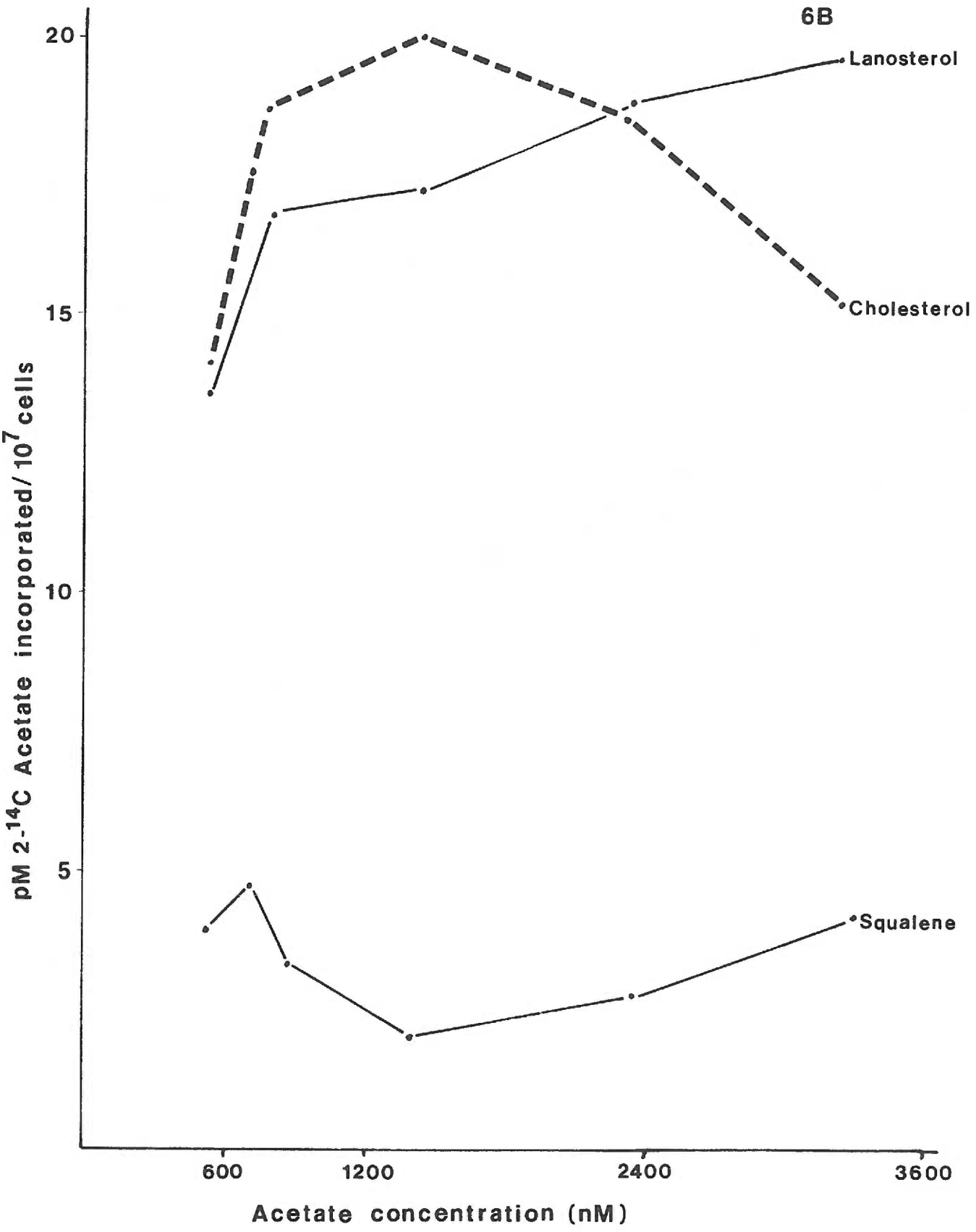
The substrate curve (fig. 6b) shows a linear increase in acetate incorporation into lanosterol, but maximal cholesterol labeling seems to occur at about 1300 nM acetate. The drop in synthesis observed at higher concentration of acetate may be due to saturating acetyl-CoA formation or to possible toxic effects from the high concentration of acetate.

Fig. 6. Acetate incorporation into sterols.

a. Cells were incubated for up to 6 hours with 1350 nM of 2-^{14}C acetate. Sterols were separated by TLC and incorporation of label into the fractions was measured.

b. Influence of acetate concentration on lipid synthesis. Ten million cells were incubated at 37°C for 5 hours with acetate concentration from 450nM to 3350nM.


6A
9
8
7
6
5
4
3
2
1
pM 2-14C Acetate incorporated/ 10^7 cells
Lanosterol
Cholesterol
Squalene
1
2
3
4
5
6
Time (hours)


6B
20
15
10
5
pM 2-14C Acetate incorporated/ 10^7 cells
Lanosterol
Cholesterol
Squalene
600
1200
2400
3600
Acetate concentration (nM)

Incorporation of [^{3}H]-choline into phosphatidyl choline increased with time (fig. 7a) and reached a plateau at 5 hours. A similar pattern of incorporation was also seen with lysolecithin. Choline incorporation into phospholipids was linear with substrate concentrations, up to 2mM (fig. 7B) at which point there was a tendency toward saturation. Phosphatidyl choline was the primary phospholipid labeled under all conditions and relatively small amounts of choline were incorporated into lysolecithin and sphingomyelin at any substrate concentration.

Plasma Lipids in Patients with FH on Different Medications

The lipid values for groups of adult patients with heterozygous FH on no lipid lowering drugs, on single drug therapy with either colestipol or nicotinic acid, or on combined drug therapy with colestipol plus nicotinic acid are shown in Table 1. These values represent a cross section and because of time constraints do not represent data from the same patients treated sequentially. Nonetheless, the observed differences agree quite closely with those seen in a recent sequential study (10).

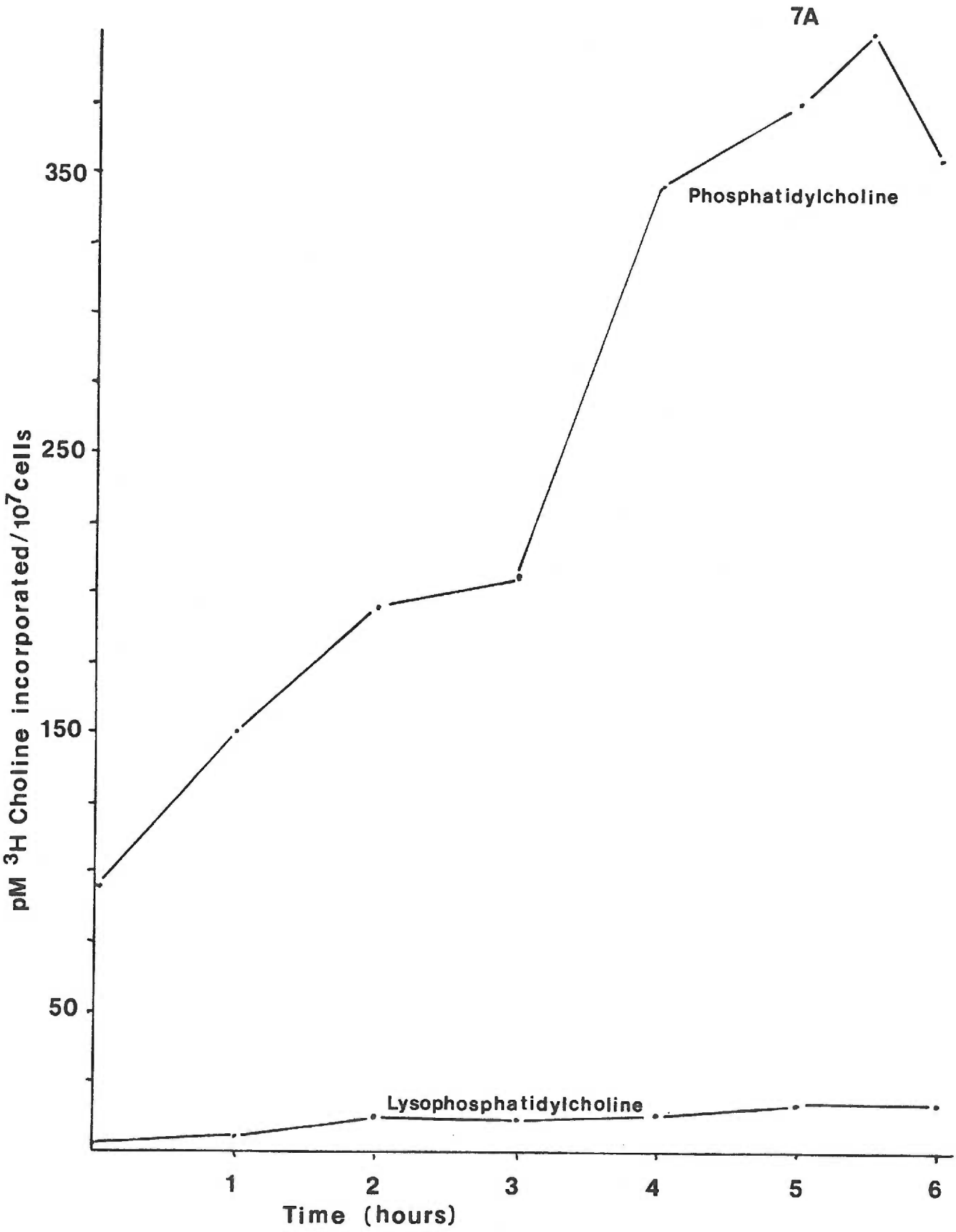
Plasma cholesterol values were 16.9% ($p < .005$) lower in patients on bile acid sequestrants alone than in the group on no medications. The addition of nicotinic acid increased the difference to 30.6% ($p < .001$). Nicotinic acid alone produced a drop of 13.0% ($p < .05$) (Table 1). There was no significant effects of any of the treatments on triglyceride levels.

Low density lipoprotein was isolated from plasma by ultracentrifugation as outlined in the methods. Table 2 shows the

Fig. 7 Choline incorporation into phospholipids.

a. Ten million cells were incubated for times up to 6 hours with 500nM of ^{3}H(Me) choline. Phospholipids were separated by TLC and incorporation of the label into the fractions was measured.

b. Influence of substrate concentration on ^{3}H-choline incorporation. Mononuclear cells were incubated 5 hours with concentrations of choline variying from 100 to 3000nM.


7A
350
250
150
50
pM ^{3}H Choline incorporated/10^{7}cells
Phosphatidylcholine
Lysophosphatidylcholine
1
2
3
4
5
6
Time (hours)

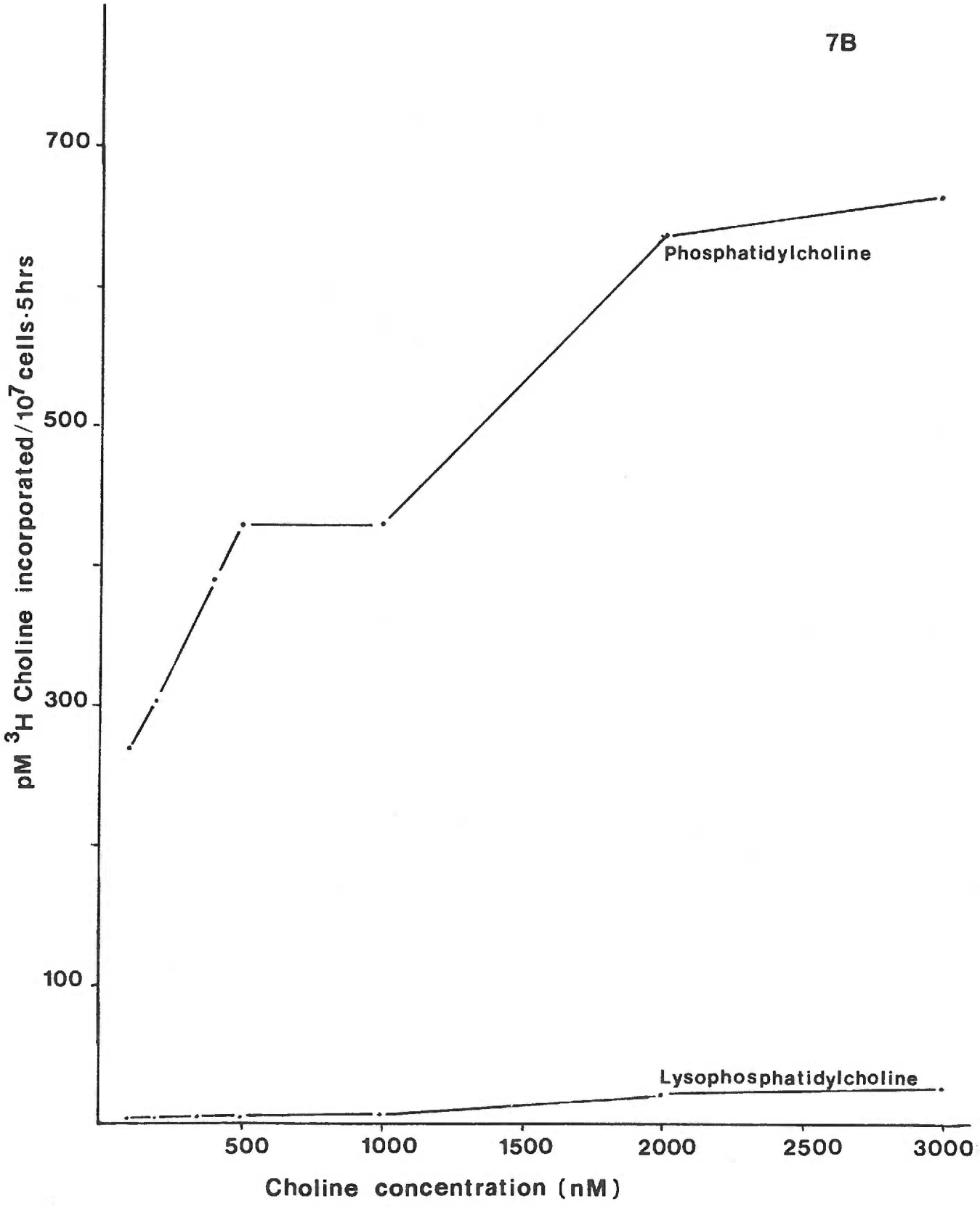

7B
700
500
300
100
pM ^{3}H Choline incorporated/10^{7} cells·5hrs
Phosphatidylcholine
Lysophosphatidylcholine
500
1000
1500
2000
2500
3000
Choline concentration (nM)

TABLE 1 PLASMA LIPID LEVELS IN THE STUDY POPULATIONS

	Plasma Cholesterol mg/dl		LDL Cholesterol mg/dl		Plasma Triglyceride mg/dl		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	No. Subjects
Normal	184.2+	13.5	113.4+	22.4	69.0	23.1	5
FH (no medication)	362.8	58.7	289.8	64.4	116.8	47.5	17
FH (Nicotinic acid)	314.2*	100.5	223.6*	76.5	129.4	111.2	9
FH (sequestrant only)	301.5**	49.7	232.2**	46.6	97.2	38.0	18
FH (sequestrant & Nicotinic acid)	287.3**	32.7	207.5**	35.5	121.3	44.0	12
Abetalipoproteinemia	25.7	2.1	-0-	-	5.3	4.5	3

* $p<.05$ vs. FH (No medications)
** $p<.005$ vs. FH (No medications)
\+ $p<.001$ vs. FH (No medications)

TABLE 2 LOW DENSITY LIPOPROTEIN COMPOSITION

The relative amounts of free cholesterol, cholesterol ester (expressed as oleate) protein, phospholipid, and triglyceride were measured in low density lipoprotein isolated from subjects in the indicated groups. Results are expressed as percent by weight. There were no significant differences between any of the groups in any of the fractions.

TABLE 2 LOW DENSITY LIPOPROTEIN COMPOSITION

	Free Cholesterol mean ± S.D.		Cholesterol ester mean ± S.D.		Protein mean ± S.D.		Phospholipid mean ± S.D.		Triglyceride mean ± S.D.		No. Subjects
Familial hypercholesterolemia no medication	8.5	0.9	39.2	2.1	18.2	2.3	29.5	5.3	5.0	2.3	11
Familial hypercholesterolemia sequestrant only	8.0	2.6	40.1	6.3	18.0	5.3	28.7	6.1	5.3	4.9	7
Familial hypercholesterolemia sequestrant + nicotinic acid	9.3	1.2	38.9	2.2	18.4	3.1	27.4	5.8	6.8	2.9	10
Normal subjects	7.9	2.3	40.5	9.5	20.3	7.9	25.8	5.4	5.1	4.8	7

relative amounts by weight of free cholesterol, cholesterol ester, protein, phospholipid and triglyceride in the LDL in each of the groups. There was a slight tendancy for the relative amounts of free cholesterol and triglyceride to be higher in the patients with FH, but there were no significant differences. Similarly, the trend of decreasing phospholipid content in the LDL from patients with FH after treatment was not significant.

The Effects of Hypolipidemic Drugs on Mononuclear Cell LDL Receptor Activity

Receptor mediated degradation of labeled LDL in freshly isolated mononuclear cells from patients with familial hypercholesterolemia (when not being treated with lipid lowering medications) was significantly lower than that of normal subjects (2.4 vs 5.3 ng/4 x 10^6 cells/5 hrs, p. <.001) as seen in Table 3. This difference is similar to that shown by prior studies (51). Treatment with bile acid sequestrants was associated with an increase in specific degradation rate of LDL to 5.6 ng/4 x 10^6 cells/5 hours (Table 3). This value is similar to that seen in normal subjects, but is significantly higher than that seen in untreated patients with heterozygous FH (Table 2). LDL degradation by mononuclear cells from patients treated with a sequestrant plus nicotinic acid was slightly higher (6.3 ng/4 x 10^6cells/5 hours), but this was not significantly different from the values in patients on bile acid sequestrants alone. Mononuclear cells from patients on bile acid sequestrants showed a significantly higher LDL receptor activity than cells from patients on nicotinic acid

TABLE 3 LIPID SYNTHESIS AND LDL RECEPTOR ACTIVITY IN FRESHLY ISOLATED MONONUCLEAR CELLS.

4×10^6 cells/ml were incubated in RPMI-1640 supplemented with glutamine and 30% LPDP for 5 hours at 37°C in 95% air/5% CO_2. 25 µg/ml of ^{125}I LDL was added to all flasks. Two flasks from each patient had an additional 500 µg/ml of unlabelled LDL added to suppress the receptor mediated pathway. Blank values were determined by incubations without cells Specific degradation is the difference between the total amount of ^{125}I LDL degraded and the amount degraded in the presence of excess unlabelled LDL.

TABLE 3 LIPID SYNTHESIS AND LDL RECEPTOR ACTIVITY IN FRESHY ISOLATED MONONUCLEAR CELLS

	Specific Degradation ng LDL/4x10^6 cells/5 hrs.			Lipid Synthesis pM label incorporated/10^7 cells/5 hrs.				
				Cholesterol Incorporation of 2-[^{14}C]-Acetate			Lecithin Incorporation of [^{3}H]-Choline	
	N	Mean	S.D.	N	Mean	S.D.	Mean	S.D.
Normal	15	5.3**	1.9	3	16.9	6.2	2206	916
FH- no medication	11	2.4*	1.5	4	32.2	10.4	5531	5177
FH- Nicotinic Acid	7	2.9*	1.2	0	N.D.		N.D.	
FH- Sequestrant only	9	5.6**	2.0	1	127.4+	---	2988	---
FH- Sequestrant and Nicotinic acid	6	6.3	7.2	6	70.3++	23.1	3448	1302
Abetalipoproteinemia	2	8.7**	2.2	4	111.6	78.0	5200	3818

N number of subjects
N.D. not determined
--- single patient, mean of replicate assays
* P<.001 vs normal subjects
** P<.001 vs FH on no medications
++ P<.01 vs FH on no medications, P<.01 vs normal subjects
\+ P<.05 vs normal, P<.001 vs FH on no medications

alone ($p < .01$). There were no significant differences between LDL receptor activity in cells from patients treated with nicotinic acid alone and those on no drug treatment.

After incubation of the mononuclear cells in a lipid free medium for a 66 hour period to derepress LDL receptor activity, differences in LDL receptor activity were still observed between patients on bile acid sequestrants and those on no therapy (Table 4). It is of particular note that the induction of LDL receptors by bile acid sequestrants persists through derepression. LDL receptor activity in derepressed mononuclear cells from patients with FH was half the level of that found in cells from the normal controls. This difference was significant ($p < .05$). Treatment with sequestrants or sequestrants and nicotinic acid increased the receptor activity to normal levels. Although the difference between sequestrants as single agents and combined therapy was not statistically significant, this may be due to the small sample numbers rather than a lack of drug effect. Nicotinic acid as a single agent had no consistant effect on receptor activity in derepressed cells.

The Influence of Hypolipidemic Drugs on Lipid Synthesis

Cholesterol synthesis from 2-[^{14}C] acetate was higher in the cells from patients with FH than the controls (Table 3). Incorporation of 2-[^{14}C] acetate into cholesterol rose from 16.9 pM/10^7cells / 5 hours to 32.2 pM in the FH patients on no medications, 70.3 pM for patients on combined therapy, and 127.4 pM for sequestrant therapy alone. Patients with abetalipoproteinemia incorporated 111.6 pM/10^7 cells / 5 hours.

TABLE 4 MONONUCLEAR CELL LDL RECEPTOR ACTIVITY FOLLOWING DEREPRESSION

Derepression of LDL receptor activity was achieved by incubating 2 x 10^6 cells/ml in RPMI-1640 supplemented with 10% LPDP for 66 hours at 37°C in 95% air/5% CO_2. Specific receptor mediated degradation of LDL was measured as the difference between the degradation of 12.5 µg/ml ^{125}I LDL in the presence and absence of 500 µg/ml unlabelled LDL. Blank values were determined by incubations in the absence of cells.

TABLE 4
MONONUCLEAR CELL LDL RECEPTOR ACTIVITY
FOLLOWING DEREPRESSION

	Specific Degradation of LDL ng/4x10^6 cells/5 hrs.		
	Mean	S.D.	N
Normal	6.1*	2.9	7
FH- no medications	3.2	1.9	6
FH- nicotinic acid	2.9	1.5	6
FH- sequestrant only	4.7	2.2	7
FH- sequestrant and nicotinic acid	8.0**	3.4	4
Abetalipoproteinemia	9.2***	0.5	2

* P<.05 vs FH on no medications
** P<.025 vs FH on no medications
*** P<.01 vs FH on no medications

There were no significant differences between the groups in incorporation of [^{3}H] choline into phospholipid which ranged from 2206 to 10161 pM/10^{7} cells/5 hours (Table 3).

Discussion

Hypercholesterolemia, the biochemical hallmark of heterozygous FH, has been attributed to a 50% or greater reduction in the number of specific cell surface receptors for LDL measured *in vitro*. Consistent with this, studies of ^{125}I-LDL turnover have demonstrated a reduced fractional catabolic rate (FCR) of LDL apo-B in FH heterozygotes (70,107,108). This has been associated with either an unchanged or increased synthesis rate for apo-B (70,108,108). In contrast, patients with other primary genetic causes for their hypercholesterolemia, such as combined familial hypercholesterolemia (CFH), show a normal FCR for LDL and normal levels of LDL receptors. These patients appear to have an inherent overproduction of VLDL and LDL as their primary abnormality (109,110).

There are several approaches to therapy for hypercholesterolemia. These can be categorized into mechanisms which reduce LDL synthesis, increase LDL catabolism, or result in physical removal of LDL from the plasma (plasmapheresis). The bile acid sequestrants colestipol and cholestyramine are the first line therapy for primary hypercholesterolemia. Despite structural differences between these drugs, their mode of action is the same. Both agents bind bile acids in the intestinal lumen thus preventing their reabsorbtion, enhancing their excretion, and increasing the hepatic synthesis of bile acids from cholesterol (82,111,112). This causes a reduction in the hepatic pool of cholesterol. The depletion of this pool results in an increased rate of hepatic cholesterol synthesis (113). Studies in which colestipol was given to dogs have shown that this drug may also increase the number of hepatic LDL receptors and thus promote the catabolism of

LDL (88). When dogs were given colestipol and mevinolin, a competitive inhibitor of cholesterol synthesis, LDL levels fell more than with either agent alone. This decrease was paralleled by greater increases in LDL receptor activity than occured with either single agent treatment. Cholestyramine has been shown to increase the FCR of ^{125}I-LDL (79,114), and inferentially from studies of cyclohexanedione modified LDL, it also increases receptor mediated uptake of LDL (79).

Decreases of 10-30% usually occur in LDL and apo-B levels during bile acid sequestrant therapy. These changes may be followed by small increases in VLDL (82,111,112,115,116,117), but little or no change in HDL levels. In the current study, there were no consistant changes in VLDL or HDL levels.

The magnitude of the hypolipidemic response in individual patients treated with bile acid sequestrants is both variable and unpredictable. One likely explanation is that inherent allelic differences in LDL receptor activity (either the number of receptors demonstrated in vivo or the maximum number expressed after in vitro derepression) exist. Variation of response to therapy may also be due to alterations in the LDL synthesis rate, catabolism, or to increases in the cholesterol synthesis rate which might compensate for the increased drain on the hepatic cholesterol pool.

The efficacy of nicotinic acid as a hypolipidemic agent in both single and combined drug regimen has been demonstrated (118,119). Studies of the hypolipidemic mechanisms of nicotinic acid indicate that its primary effect is to reduce free fatty acid mobilization from adipocytes. When used alone, an inhibition of endogenous cholesterol synthesis has been shown (120). When parameters of LDL metabolism were

examined in normal subjects and patients with type IIa hypercholesterolemia, both the biological half-life and the FCR of LDL were unchanged, but the total synthetic rate was reduced (114). The effects of nicotinic acid on VLDL metabolism have recently been examined in a heterogeneous group of patients with hyperlipidemia (121). VLDL synthesis in the group as a whole was decreased 21% by nicotinic acid therapy. This change was paralleled by small (not significant) increases in biliary cholesterol excretion, but no consistant changes in sterol balance. In theory, nicotinic acid by itself should be more effective in CFH (where the defect is overproduction of VLDL and LDL) than in FH, but there have been no comparative studies yet reported.

The combination of a bile acid sequestrant and nicotinic acid therapy seem to be effective in many cases where single drug therapy is inadequate (10,122). The synergism shown by these two agents is similar to that exhibited by the combination of mevinolin and colestipol in dogs (88). In the latter, the addition of mevinolin resulted in a decrease in LDL synthesis, an increase in FCR of LDL, and an increase in the number of LDL receptors on hepatic membranes. To date, no studies in man have examined the effect of bile acid sequestrants in combination with nicotinic acid on lipoprotein metabolism and whole body cholesterol synthesis. Similarly, the effect of these interventions upon cholesterol synthesis or LDL receptor activity in freshly isolated mononuclear leukocytes has not hitherto been examined.

Recent studies have indicated that freshly isolated mononuclear cells respond to many of the same factors which influence _in vivo_ cholesterol synthesis rates in liver or _in vitro_ rates of cholesterol synthesis in cultured cells. Young and Rodwell (45) have

shown parallel changes in HMG CoA reductase activity in freshly isolated leukocytes and hepatic tissue from rats fed cholesterol (decreased activity), or cholestyramine (increased activity). McNamara, et al (46) have shown similar effects in human cells. Cholesterol synthesis rates were twice as high in mononuclear cells from patients treated with cholestyramine as in control subjects or patients taking clofibrate. This is consistent with the changes observed in the livers of patients treated with cholestyramine (16). Cholesterol feeding has been reported to cause parallel decreases in HMG CoA reductase activity and LDL receptor activity in mononuclear cells freshly isolated from normal volunteers (49). There was no correlation between LDL cholesterol levels and the absolute LDL receptor number. However, there was a negative correlation observed between the increase in LDL cholesterol and the absolute number of receptors demonstrable on derepressed mononuclear cells. Taken together, these studies suggest that comparative changes in LDL receptor activity and cholesterol synthesis seen in mononuclear cells from patients on different drug or dietary regimens may parallel changes in other body tissues, especially the liver.

The mechanism by which the non-absorbable bile acid sequestrants may influence lipid synthesis and LDL receptor activity in mononuclear cells is unknown. McNamara, et al (46) found no relation between sterol synthesis rates in freshly isolated mononuclear cells and plasma LDL levels. Studies with other model systems, however, imply that reductions in cellular cholesterol concentration (125,126), or an increase in cell membrane sphingomyelin content (127) result in compensatory increases in cholesterol synthesis and usually (125,126),

but not always (127), in the number of LDL receptors. In mononuclear cells the rates of sterol synthesis may be influenced by many things, including the magnitude of change in LDL concentrations induced by drug therapy, variations in LDL composition (126), or changes in the FCR of LDL , as well as variations in the levels of hormones known to alter lipid metabolism. Contrary to the data of Witztum, et al (126), we found no evidence of a change in LDL composition as a response to drug tratment.

The drop in plasma cholesterol found in this study was slightly less than that previously reported (10). This is probably due, in part, to differences in experimental design. The studies of Kane, et al (122), and Illingworth, et al (10) were longitudinal, with each patient serving as his own control, whereas the present study was cross-sectional. Given that the changes in LDL cholesterol from this study are less than those previously reported, it is probable that the changes in LDL receptor activity demonstrated are, if anything, understated.

The predicted responses to drug treatment are that concomitant with the fall in LDL cholesterol, 1) an increase in mononuclear cell LDL receptor activity would occur during treatment with bile acid sequestrants, and 2) the cholesterol synthesis rate might increase if the cholesterol synthetic capacity was not at a maximum level. The elevations seen in freshly isolated mononuclear cell LDL receptor activity and cholesterol synthetic rate in response to bile acid sequestrants, suggest that mononuclear cells show metabolic responses similar to those previously reported for HMG CoA reductase in the hepatocytes (45), hepatic membrane LDL receptors (88), or the degradation of ^{125}I-LDL in the whole body (79). The higher rates of

cholesterol synthesis seen in patients with FH on no medications may be due to the low cholesterol diet being followed by these patients. The normal subjects were not on a low cholesterol diet.

The further addition of nicotinic acid to colestipol or cholestyramine therapy produces a further decrement in serum cholesterol as has been previously reported (10,124). A slight, but not significant, additional increase in the specific receptor mediated degradation also occured. The addition of nicotinic acid is believed to reduce the degree of increase in cholesterol synthesis produced by bile acid sequestrants. The effects of nicotinic acid may therefore be predicted to either decrease cholesterol synthesis (if there were a direct effect of the nicotinic acid upon the mononuclear cells) or increase cholesterol synthesis as a response to the drop in LDL cholesterol.

The one inexplicable effect of bile acid sequestrant therapy is that, as seen in Table 4, the increases in LDL receptor activity through derepression. This would be impossible if the effects on LDL receptor activity were due solely to a response to the drop in LDL cholesterol. One explanation for this seemingly inappropriate response is that stimulation of LDL receptor activity may occur during differentiation of the lymphoblast/monoblast into a mature cell and that this effect might persist after <u>in vitro</u> modification of the culture media. Nicotinic acid appears to have no such persistant effects. Further studies will be needed to clarify the mechanisms responsible for this persistant change in LDL receptor activity and to define a mechanism.

All of the previous studies, taken with the current data

validate freshly isolated human mononuclear leukocytes as a useful indicator of *in vivo* responses to dietary or drug induced changes in cholesterol metabolism. By extending these results to a longitudinal study of LDL receptor activity and cholesterol synthesis in freshly isolated mononuclear cells from patients treated sequentially (diet only), diet plus sequestrant or nicotinic acid, and diet plus both drugs) one should be able to see if any correlation exists between the magnitude of changes in plasma LDL and the changes in LDL receptor activity.

The assay of LDL receptor activity in fibroblasts has been established as the gold standard for the diagnosis of familial hypercholesterolemia. By definition, this is a maximally derepressed system. It has been suggested (6,60,61) that measurement of LDL receptor activity in freshly isolated mononuclear cells may offer an effective substitute for the fibroblast in facilitating the biochemical diagnosis of FH. Unfortunately, the data from the current study, suggest that the diagnosis may only be made using mononuclear cells from untreated subjects. There is also a considerable overlap between the receptor activity of individual normal subjects and individual patients with familial hypercholesterolemia, even though the overall mean values are significantly different. This individual variability may be due to other factors, such as hormone levels, in addition to allelic variation in receptor activity as suggested by Maartmann-Moe, et al (61). The determination of plasma concentrations of LDL, taken together with the family history, therefore remains the most practical and effective way of establishing the diagnosis of familial hypercholesterolemia in a given patient.

SUMMARY

Plasma cholesterol levels in patients with familial hypercholesterolemia dropped 16.9% with treatment using bile acid sequestrants. The addition of nicotinic acid to bile acid sequestrant therapy produced a total drop of 30.6%. Nicotinic acid alone provided a drop of 13.0%. Concomitant with these changes in plasma lipids, bile acid sequestrants produced an increase in LDL receptor activity from 2.4 to 5.6 ng LDL degraded/4 x 10^6 cells/5 hours. The addition of nicotinic acid to bile acid sequestrant therapy increased the LDL receptor activity slightly but not significantly from sequestrant treatment alone. Nicotinic acid as a single agent had no effect. These effects on LDL receptor activity were maintained through derepression.

Cholesterol synthesis rates were similarly increased by bile acid sequestrant therapy. This increase was blunted by the addition of nicotinic acid. None of the drug regimens had a consistant effect upon phospholipid synthesis.

Conclusions which may be drawn from this study are that: 1) changes in hepatic and whole body cholesterol synthesis and LDL receptor activity seem to be paralleled by similar alterations in freshly isolated mononuclear cells, 2) freshly isolated mononuclear cells may be an appropriate *in vitro* model system for investigating hypolipidemic drug induced changes in sterol metabolism, and 3) derepressed mononuclear cells are probably not suitable for the diagnosis of familial hypercholesterolemia in patients on hypolipidemic medications.

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