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# Cholesterol Synthesis and Sterol Esterification in Cultured Sitosterolemic Lymphocytes

by

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# ABSTRACT

Cholesterol synthesis and sterol esterification were evaluated in primary cultures of lymphocytes from two patients with sitosterolemia, a rare hereditary lipid storage disorder characterized by the abnormal absorption of plant sterols and reduced cholesterol synthesis. Freshly isolated sitosterolemic lymphocytes demonstrated significantly lower cholesterol synthetic rates than control cells, but upregulated cholesterol synthesis to control levels after 10 days of *in vitro* culture. This upregulation was concomitant with a 96% decrease in intracellular plant sterols. However, when sitosterolemic lymphocytes were preincubated in LDL, cholesterol synthesis remained 50% lower than in control cells. Sterol esterification was not significantly different in freshly isolated or cultured sitosterolemic lymphocytes than in control lymphocytes. When sitosterolemic lymphocytes were preincubated in LDL however, sterol esterification was 35% higher than in control cells. The abnormal responsiveness of sitosterolemic lymphocytes to exogenous LDL suggested that the primary defect in sitosterolemia may be at the level of sterol absorption.

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# BACKGROUND

## 1.0 INTRODUCTION

ß-sitosterolemia is a rare recessive hereditary lipid storage disease characterized clinically by tendon and tuberous xanthomas, recurrent arthralgias, and premature atherosclerosis .[1] The clinical manifestations of this disease have been attributed to the presence of unusually high concentrations of plant sterols in the plasma, and other tissues, of affected individuals.[1] Since plant sterols are not synthesized endogenously in humans, the high concentrations of sitosterol, stigmasterol and campesterol, are necessarily due to enhanced intestinal uptake.[20, 21, 27] Other biochemical abnormalities include 1) reduced hepatic secretion of cholesterol and the phytosterols.[5, 6, 21] 2) a small total body exchangeable cholesterol pool, with a disproportionately expanded pool of plant sterols (27) and, 3) decreased whole body cholesterol synthesis as a result of diminished quantities of microsomal HMG-CoA reductase protein and mRNA.

At the present time, the principal inherited defect in sitosterolemia has not been established with certainty. Clearly, the combination of decreased removal and increased absorption of phytosterols is linked to the pathogenesis of this disease. A key feature of sitosterolemia however, is a measurable reduction in cholesterol biosynthesis. This observation has led some researchers to speculate that the primary defect in this disorder may reside in the HMG-CoA reductase gene,[74] and that the indiscriminate uptake of sterols is simply a compensatory mechanism. Alternatively, the primary defect may be an

absorptive one, and low cholesterol biosynthesis may be an indirect result of the abnormal accumulation of plant sterols or some other abnormality. In the context of the latter hypothesis, Bhattacharya and Connor [1] have suggested that esterification may be the limiting factor in the uptake and storage of sitosterol. Which of these mechanisms is primary in this disorder, remains to be resolved.

Difficulty elucidating the primary defect underlying sitosterolemia, has been due in part, to an absence of suitable *in vitro* or *in vivo* models. Attempts to develop an animal model suitable for the study of sitosterolemia have been largely unsuccessful. [76 - 78] Accordingly, research has been restricted to evaluations of sitosterolemic patients and freshly isolated sitosterolemic tissues. As such, it has been difficult to separate the effects of phytosterols on the normal cholesterol biosynthetic and esterification pathways, from effects due to inborn metabolic errors.

A cell culture model is a potentially viable alternative to animal models, for the study of sitosterolemia. Plant sterol levels in sitosterolemic blood cells have been shown to be similar to levels found in other sitosterolemic tissues. [15, 19, 79] Furthermore, previous studies have demonstrated that the rate of sterol synthesis in peripheral blood mononuclear leukocytes accurately reflects whole body cholesterol synthesis, and is regulated by dietary and pharmacological agents that affect the rate of endogenous synthesis. [80 - 83] IL-2 dependent lymphocyte cultures can be maintained for up to 3 weeks, and IL-2 has been shown to stimulate rapid growth, facilitating a doubling of the cell population approximately every 24 hours. [88] This rapid growth would permit lymphocyte cultures to be initiated from small quantities of whole blood obtained from sitosterolemic and normal individuals, and would permit the performance of

enzymatic assays in both freshly isolated and cultured cells.

The capacity of sitosterolemic cells to regulate cholesterol synthesis in response to growth, sterol depletion and sterol loading has not previously been measured. Cholesterol synthesis, which has been shown to be deficient in sitosterolemic lymphocytes [72, 74, 75], is upregulated in response to mitogenic stimulation. IL-2 stimulated growth of sitosterolemic cells should permit an evaluation of the extent to which reductase activity can be upregulated in this disorder. Rapid IL-2 stimulated growth should also facilitate the removal of plant sterols from sitosterolemic cells, through dilution with endogenously and exogenously derived cholesterol. Conversely, it should permit the enrichment of normal cells with plant sterols using sitosterolemic LDL. This approach to the study of sitosterolemia would allow an evaluation of the effects of rapid growth and the culturing out of phytosterols on cholesterol homeostasis.

The rate of sterol esterification by acyl-CoA cholesterol acyl transferase (ACAT) has similarily not been previously measured in sitosterolemic cells. The lymphocyte tissue culture system would similarly permit an evaluation of ACAT activity in freshly isolated and IL-2 stimulated cells, in the presence and absence of exogenous cholesterol and plant sterols. The evaluation of ACAT activity may provide clues to both the abnormal absorption of plant sterols and the accumulation of sterol esters, which characterize this disease.

In summary, the primary objectives of this thesis were to evaluate 1) the rate of cholesterol synthesis and, 2) the rate of sterol esterification, in sitosterolemic cells under conditions stimulating rapid growth and the removal of plant sterols.

## 1.1 ß-SITOSTEROLEMIA

### 1.1.1 Clinical Manifestations

ß-sitosterolemia (or 'phytosterolemia') is a rare recessive hereditary lipid storage disorder ,[14] which was first described by Bhattacharyya and Connor in 1974 .[1] Since the original diagnosis, 34 cases of sitosterolemia, representing 21 different families, have been documented .[1-16, 200]

The disease is characterized clinically by the presence of large tendon and tuberous xanthomas, recurrent arthritis, accelerated atherosclerosis, and hemolytic episodes. [1-3, 5, 7, 16-19, 24-25] The onset of symptoms typically occurs in childhood [16] with the appearance of Achilles and extensor tendon xanthomas, in addition to xanthelasmas on the buttocks, ears, arcus corneae, and other regions of the body. Accordingly, sitosterolemia is frequently misdiagnosed as familial hypercholesterolemia.[15] Although hypercholesterolemia has been found to characterize a few cases of sitosterolemia,[18, 19, 23] and hyperapobetalipoproteinemia has been noted in several patients, [4, 14] most sitosterolemics have only modestly elevated serum cholesterol levels, or cholesterol levels well within the normal range .[20]

The mechanism for the accelerated atherosclerosis and xanthomatosis is not presently understood, but is apparently related to the accumulation of high concentrations of plant sterols (phytosterols) in the plasma and other tissues of affected individuals. In all sitosterolemic tissues, with the exception of brain, the unsaturated plant sterols sitosterol, campesterol and stigmasterol, comprise up to 25% of total sterols .[17, 21] Plasma sterol concentrations typically range from 3.99-

6.63 mmol/L (154-256 mg/dL) with phytosterols, predominantly ß-sitosterol, accounting for 0.34-1.86 mmol/L (13-72 mg/dL). [1, 4-10, 12, 14-18, 22-26] In contrast, less than 1% of tissue sterols in unaffected individuals are typically of plant origin, and more than 99% of total tissue sterol can be accounted for by cholesterol.[17, 21] The present clinical methodologies, a colourimetric assay that depends on a double bond between carbons 5 and 6, and an enzymatic method that detects the 3ß-hydroxy group, fail to differentiate the plant sterols from cholesterol. [17] Accordingly, a diagnosis of sitosterolemia is confirmed by the demonstration, using gas liquid chromatographic analysis, of abnormal levels of dietary phytosterols in the plasma or other tissues of a xanthomic patient. [22]

### 1.1.2 Phytosterol Absorption

Since phytosterols are not synthesized endogenously by humans, [28] the high concentrations of plant sterols which characterize sitosterolemia are necessarily due to increased intestinal uptake. Normally, the phytosterols are very poorly absorbed by humans, and less than 4% of ingested plant sterols are taken up from the intestine. [6, 21, 27-29] This uptake is about 1/10 the amount of cholesterol absorption. [31] In other animals, the percent absorption of ingested sitosterol varies, [31-36, 39] but a substantial difference between the proportion of cholesterol and sitosterol that is absorbed persists. Using the plasma dual-isotope ratio method, [30] after simultaneous intravenous and oral isotopic sterol dosing, Salen *et al.* determined that sitosterolemic subjects absorb up to 68% of ingested sitosterol. [27] Marginally enhanced cholesterol absorption (*ibid*) and the

abnormal absorption of shellfish sterols [21] have been similarly demonstrated in some sitosterolemic subjects. As such, the accumulation of plant sterols that characterizes sitosterolemia is likely due to failure of the normal mechanisms responsible for selectivity in the intestinal absorption of sterols. [20]

**a) Phytosterol structure**

From a structural perspective, the normal inability to absorb the plant sterols is most clearly related to methyl or ethyl substitutions (or their unsaturated counterparts) at carbon-24 on the sterol side chain, as exemplified by campesterol (24-methyl), sitosterol (24-ethyl), stigmasterol (Δ24,24-ethyl) and fucosterol (24-ethylidine). [36] Sterols are comprised of three connecting 6-carbon rings, joined to a fourth 5-carbon ring. Methyl groups at C10 and C13, in addition to a ß-hydroxyl group at C3, and a side chain at C17, [15] are characteristic (**Figure 1.1**). Efficient absorption of a sterol requires the presence of the 3ß-hydroxyl group, the double ring bond in the Δ5 position, the absence of additional double bonds in ring B, and the absence of a substituent at carbon-24. [20] Kuksis and Child determined that, from an eqimolar mixture of 7-dehydrosterols, uptake into jejeunal villus cells, brush border membranes, and erythrocytes decreased with an increasing number of carbon atoms at C-24 of the sterol side chain, in a manner identical to that observed for the parent Δ5-sterols. [39] The increased molecular weight of the plant sterols is likely not an important factor in the selective process, as indicated by the relatively greater uptake of ß-sitosterol by the rat intestine compared to its unsaturated counterpart, stigmasterol, which is two mass units lighter. [35, 38] Since stigmasterol, campesterol and sitosterol can be considered branch analogues of cholesterol, the bulk and configuration of the

side chain would appear to be the primary determinant of sterol absorbability.

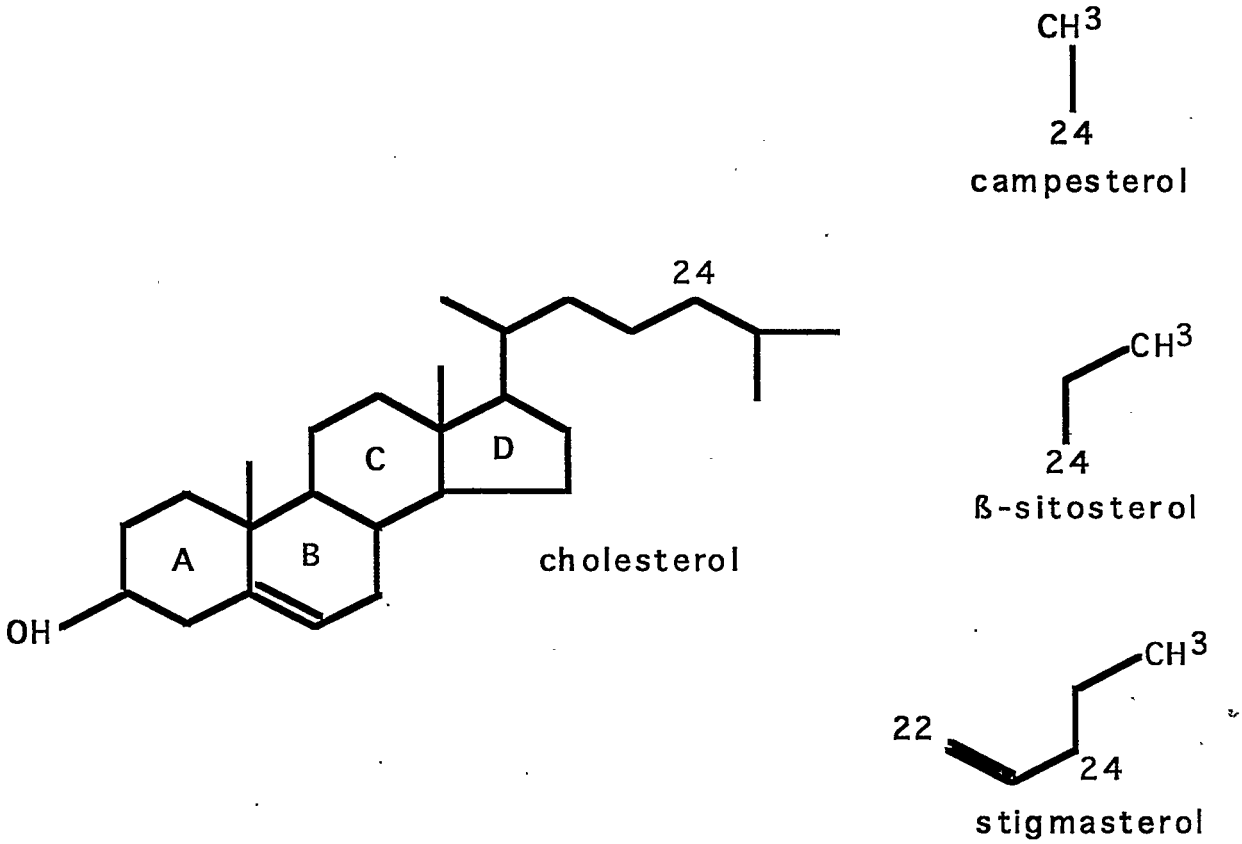


**Figure 1.1** Sterol structure (cholesterol and the 3 plant sterols, campesterol, ß-sitosterol and stigmasterol)

**b) Micellular solublization**

Several potential sites for the mechanism of discrimination between cholesterol and the structurally similar phytosterols have been proposed. Absorbable and non-absorbable sterols apparently demonstrate different affinities for the bile salt micelle. Slota *et al.* demonstrated that sitosterol was less soluble than cholesterol in a mixed micellular solution. [63] As such, the rate of delivery of sitosterol to the intestinal cell surface may differ. Armstrong and

Carey however, noted that sitosterol is less hydrophilic than cholesterol and has been found to have a lower capacity, but higher affinity, than cholesterol for binding to cholic acid containing micelles. [40] Furthermore, sitosterol has been shown to displace cholesterol from micellular solution, and can actually be used therapeutically to inhibit cholesterol absorption. [36, 40-42] Ikeda *et al.* noted however, that absorbable sterols transferred to sterol free triolein at 2-6 times the rate of nonabsorbable sterols in an *in vitro* model system. This differential transfer of sterols from the bile salt micelle was dependent upon sterol solublization, and was affected significantly by micellular bile salt type. [36] Collectively, these observations suggest that while bile salt micelles have some capacity to solublize phytosterols, these sterols are not as readily transferred to the brush border, tending instead to be retained in the intestinal micelles.

**c) Brush border uptake**

The observation that micellular solubilization does not ensure the uptake of phytosterols from the intestine, suggests that the brush border itself may be directly involved in the discrimination of absorbable and nonabsorbable sterols. Ikeda *et al.* noted that *in vitro* brush border sterol uptake from micellular solutions containing only cholesterol, was 3.5 fold greater than uptake from micelles containing only sitosterol. [40] Furthermore, sterol absorption is known to take place primarily in the proximal third of the small intestine, [64] and both cholesterol and sitosterol have been reported to bind to isolated rat brush border membranes, [37] to the brush border membranes of rat jejeunal loops in situ, [45] to isolated rat jejeunal villus cells, [37] and to rat proximal small intestine 2 hr after the *in vivo* administration of both sterols in a lipid meal. Neither Borgstrom [43] nor

Ikeda *et al.* [39] however, observed any accumulation of sitosterol in the intestinal wall. Kuksis [37] concluded that the brush border membrane possesses the capability to bring about the absorptive recognition of cholesterol that characterizes the intestinal wall *in vivo*, and that the inability of ß-sitosterol to enter this structure depends upon features that may be common to all plasma membranes. Hingson and Diamond [44] have suggested that structural features of plasma membranes which act to increase the degree of order within the acyl chains of the membrane phospholipids would enhance the selection against non-electrolytes with alkyl branch points, relative to non-branched analogues, during uptake from aqueous solutions. Since campesterol, sitosterol and stigmasterol are all considered to be branched analogues of cholesterol, a similar argument may apply in the discrimination of dietary phytosterols during the absorptive process.

**d) Intestinal esterification**

A third possible site of discrimination is at the level of intestinal sterol esterification. Although cholesterol is absorbed exclusively in the unesterified form, most cholesterol appearing in the lymph (80-85%) is esterified by the addition of various long chain unsaturated fatty acids. [46] Moreover, the mass of cholesterol esters in lymph increases in direct proportion to the amount of cholesterol absorbed. [47] As such, re-esterification of cholesterol necessarily occurs following the uptake of dietary sterol into the mucosal cell. Micelles cross the unstirred water layer, and transfer free sterol to the brush border of the mucosa, where the sterol is esterified to cholesterol esters by the microsomal enzyme ACAT (acyl-CoA cholesterol acyltransferase). [46] The resulting sterol

esters are then incorporated into chylomicrons, which are secreted into the lymph (*ibid*). The inhibition of ACAT activity in rats [61] and rabbits [62] has been correlated with the inhibition of dietary cholesterol absorption. Furthermore, the ability of sterols to be esterified by ACAT declines sharply with either reduction or extension of the side chain at carbon-24. [48] Alkylation at C-24, as found in the phytosterols, reduces esterification by at least 80%, [49] and numerous *in vitro* studies have demonstrated that sitosterol is less well esterified than cholesterol by intestinal ACAT. [50, 51, 52] Bhattacharyya and Connor, after describing two sisters with sitosterolemia, suggested that esterification may be the limiting factor in the absorption of plant sterols, [1] and Salen *et al.* reached a similar conclusion following a study of ß-sitosterol metabolism in man. [28] While phytosterols are poorly esterified in the gut wall, they can nonetheless be transferred to chylomicrons , and sitosterol which does appear in the lymph of experimental animals and humans is largely unesterified. [15, 33, 35, 53] As such, the relevance of intestinal sterol esterification in the absorptive process remains unresolved, and further understanding of the role of ACAT in the discrimination of absorbable and nonabsorbable sterols is necessary. Whatever the mechanism of differentiation between structurally similar sterols, tissue recognition has clearly been lost in sitosterolemia.

### 1.1.3 Sterol Turnover

The atherosclerosis and xanthomatosis arising from the enhanced absorption of plant sterols in sitosterolemia, is apparently exacerbated by

decreased hepatic removal. Normally, the liver preferentially secretes phytosterols, and relative to plasma cholesterol/sitosterol levels, there is a 3-fold enrichment of sitosterol in human bile. [28] In sitosterolemia however, not only is less cholesterol secreted into the bile, but sitosterol appears in the same or lower proportion relative to cholesterol. [1, 2, 6, 7, 54] While no unusual biliary bile acids have been detected, and bile acids are secreted in quantities sufficient to prevent steatorrhea, [1, 2, 5, 6, 55] the conversion of sitosterol to primary bile acids in affected individuals remains to be established. Shefer *et al.* have reported low cholesterol 7∂-hydroxylase activity in sitosterolemic subjects. [55] This rate-determining enzyme for bile acid synthesis is thought to be competitively inhibited by the large quantities of sitosterol and cholestanol in the hepatic tissue of sitosterolemics (*ibid*) As such, inhibition of this enzyme may explain the characteristic decreased bile acid production. The sitosterolemic liver has evidently lost both the capacity to recognize sitosterol, and the ability to preferentially secrete the 24-ethyl sterol into bile. [17] The resulting decreased hepatic turnover, coupled with increased absorption, are thought collectively to cause the massive tissue deposition of sterols which is so characteristic of this disease.

## 1.1.4 Treatment of Sitosterolemia

### a) Dietary therapy

Currently, there are two general approaches to the treatment of sitosterolemia. The first approach involves dietary sterol restriction. The

average daily North American diet contains approximately 400-700 mg. of cholesterol. [56, 57] An additional 200-300 mg. of sterol are ingested, which are of plant origin. [58] Of the plant sterols, sitosterol comprises an estimated 65%, campesterol 30%, and stigmasterol 5%. [59] As such, the prevalence of sitosterol in the tissues of sitosterolemics likely reflects the dietary predominance of this particular plant sterol. The phytosterols are principally found in vegetable oils, whole grains, nuts, and lipid-rich vegetables or fruits, where they exist as free sterols and sterol esters or glycosides. [60] Dietary therapy in the treatment of sitosterolemia conventionally restricts not only phytosterol intake (<100-150 mg/day), but cholesterol consumption as well (<300 mg/day). [15] While dietary therapy has been shown to have a profound effect on plasma sterol levels in a few sitosterolemic individuals, [11] dietary restriction more typically produces only modest improvements, [15, 16, 65, 54] or has no significant effect whatsoever. [54, 66, 24] In one case where plasma sterol levels were reported to be highly responsive to dietary manipulation, both the patient's cholesterol intake, and serum cholesterol levels before therapy, were noted to be abnormally high. [66] After a 3 month period of restricted sterol intake (<30% of calories obtained from fat), reductions in the plasma sterol levels of sitosterolemics typically range from 0-25%. [15, 16, 24, 54, 65, 66] The conventional food composition data base however, has been demonstrated to seriously underestimate the plant sterol content of foods. [15] This observation suggests that prescribed diets, even assuming patient compliance, are probably not as low in phytosterols as anticipated. Furthermore, the massive sterol pools characteristically found in sitosterolemic tissues [25, 27] likely limit the ability of short-term dietary therapy to lower serum sterol levels. To date, the efficacy of long-term dietary therapy (> 6 months) in the treatment of

sitosterolemia remains to be evaluated.

**b) Drug therapy**

The second conventional therapeutic approach involves the use of hypocholesterolemic agents. There are two general categories of cholesterol lowering drugs which are commonly administered in the treatment of hypercholesterolemia. Curiously, sitosterolemics respond atypically to both kinds of therapeutic agent.

**i) HMG-CoA reductase inhibitors**

The first category of drugs target 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA réductase), the rate controlling enzyme in the cholesterol synthetic pathway. This enzyme is a transmembrane glycoprotein of the endoplasmic reticulum [69] which catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate. [67] Drugs such as lovastatin (mevinolin), pravastatin, simvastatin and compactin are structural analogues of HMG-CoA. These drugs competitively inhibit HMG-CoA reductase, effectively lowering whole body cholesterol synthesis. [68] Reduced cholesterol synthesis in turn, stimulates the expression of LDL receptors and accelerates the clearance of plasma sterols. [70] In hypercholesterolemic subjects, plasma sterol levels typically decline by approximately 30% when 40-80 mg of lovastatin are administered daily. [71] Lovastatin has been shown however, to be suprisingly ineffectual in the treatment of sitosterolemia [66, 72] Nguyen *et al.* noted that plasma cholesterol levels actually increased by approximately 2% in 1 sitosterolemic patient following 20 weeks of lovastatin therapy, while plasma plant sterol levels

evidenced no significant change. [72] Paradoxically, degradation of $^{125}$I-LDL by monocytes freshly isolated from this patient, was diminished, rather than enhanced, by lovastatin therapy (*ibid*). Accordingly, HMG-CoA reductase inhibitors are not recommended in the treatment of sitosterolemia.

**ii) Bile acid sequestering resins**

The second category of cholesterol lowering agents used in the treatment of hypercholesterolemia, are the bile acid sequestering resins. Bile acids, metabolites of cholesterol, are normally reabsorbed in the jejeunum with about 95% efficiency. [68] Agents such as cholestyramine and colestipol bind bile acids in the intestinal lumen and prevent their reabsorption. [73] This bile acid sequestration enhances the secretion of bile by up to 10 fold, leading to increased hepatic cholesterol catabolism (*ibid*). The upregulation of bile acid synthesis in turn, correlates with stimulated expression of the LDL receptor, and the subsequent clearance of plasma sterols. [70] In the treatment of patients with heterozygous familial hypercholesterolemia, these resins produce approximately 20% reductions in LDL cholesterol levels. [68] Although a failure to respond to cholestyramine has been reported in one patient, [16] the treatment of sitosterolemia with this drug (15-24 g/day), has been shown to induce reductions in LDL sterol of up to 75%. [11, 15, 24, 66] Subsequent normalization of total plasma sterol levels, and complete regression of xanthomas have been reported in a number of patients. [11, 15, 24] Currently, cholestyramine is the hypocholesterolemic agent of choice in the treatment of this hereditary disorder.

### 1.1.5 Reduced Cholesterol Synthesis

The unusual potency of bile acid sequestering resins in the treatment of sitosterolemia, is thought to be due to diminished cholesterol synthesis in affected individuals. Normally, when bile acid sequestering resins are prescribed, HMG-CoA reductase activity is stimulated. [74] Reihner *et al.* noted that cholestyramine-treated gallstone patients evidenced a 5-fold increase in hepatic microsomal HMG-CoA reductase activity, compared to untreated controls, after 3 weeks of drug therapy (*ibid*). Nguyen *et al.* noted a similar increase (60%) in cholesterol synthesis in normal mononuclear leukocytes following treatment with colestipol. [72] This enhanced activity has been attributed to increased quantities of reductase protein in the microsomes of treated individuals. [74] The compensatory enhancement of endogenous synthesis, when the absorption of cholesterol is inhibited, tends to limit the efficacy of the bile acid sequestering resins. In sitosterolemic subjects however, neither cholesterol synthesis nor HMG-CoA reductase activity upregulate significantly following the administration of cholestyramine [72] or colestipol. [66] Furthermore, in the absence of drug therapy, both cholesterol synthetic rates and HMG-CoA reductase activity in sitosterolemic leukocytes and hepatic microsomes are less than half that of normal controls. [72, 74] Nguyen *et al.* demonstrated a deficiency in both reductase mRNA and reductase protein in the liver of 2 sitosterolemic individuals, [74] although the catalytic efficiency of the enzyme was determined to be somewhat higher [74] when compared to controls. This reduced endogenous cholesterol synthesis is apparently coupled to increased receptor-mediated LDL catabolism. Beil *et al.* reported that the fractional catabolic rate of LDL was 40% greater in a

sitosterolemic subject than in 3 controls. [75] Nguyen *et al.* confirmed this observation, but noted that cholestyramine and lovastatin therapy stimulated only small increases in LDL receptor activity when compared to controls. [66] Collectively , these observations suggest that subnormal cholesterol synthesis in sitosterolemia is compensated for an increase in LDL receptor function, and that bile acid sequestration produces a greater than expected decline in plasma sterol levels because of a failure to upregulate the subnormal endogenous cholesterol synthesis. [66]

## 1.1.6 Hypothesized Causes of Sitosterolemia

### a) Defective cholesterol synthesis

The subnormal cholesterol synthesis which evidently characterizes sitosterolemia , has led some researchers to postulate that the primary defect in this disorder is a genetic abnormality involving the cholesterol biosynthetic pathway . [74, 183] The indiscriminate uptake of sterols then, would be secondary to the reduced HMG-CoA reductase activity. This hypothesis requires subnormal cholesterol synthesis to somehow stimulate enhanced plant sterol absorption. The suppression of HMG-CoA reductase activity however, has not been shown to induce indiscriminate sterol uptake. Phytosterol levels in normal individuals receiving HMG-CoA reductase inhibitors for example, are not significantly different from those found in untreated individuals. [66] Moreover, Sviridov *et al.* demonstrated that treatment of small intestine organ cultures with an HMG-CoA reductase inhibitor stimulated cholesterol, but not sitosterol uptake in a dose-

dependent manner. [188] There is overall, very little evidence to suggest that intestinal sterol uptake is systemically regulated. [20]

There is similarly no evidence to confirm that sitosterolemic HMG-CoA reductase protein is abnormal. Nguyen *et al.*, noted that both control and sitosterolemic reductase protein migrated at 200,000 Mr, and that sitosterolemic microsomal HMG-CoA reductase reacted similarly immunologically, and exhibited the same catalytic efficiency as enzyme obtained from normal hepatic tissue.[74] To date, the only evidence in support of a hypothetical molecular defect, is the reduction in HMG-CoA reductase protein and mRNA which has been repeatedly measured in freshly isolated sitosterolemic tissues.

**b) Absorptive defect**

Alternatively, the primary defect in sitosterolemia may be an absorptive one, and low cholesterol biosynthesis may be an indirect result of the accumulation of plant sterols. Several mechanisms are known to be available to the body for adjusting to changes in the dietary uptake of sterol. [20] Most notably, sterols and several products of sterol metabolism have been shown to be directly involved in the regulation of cholesterol homeostasis. HMG-CoA reductase is subject to negative feedback inhibition by both cholesterol and oxygenated sterols, as well as non-sterol mevalonate-derived metabolic intermediates. Furthermore, this regulation has been shown to occur at the transcriptional, post-transcriptional, and post-translational levels [92] While sitosterol has not been demonstrated to be an effective feedback inhibitor of HMG-CoA reductase, [93, 94,183] the $\Delta 5$ double bond, which is common to both plant sterols and cholesterol, has been shown to be important in the downregulation of HMG-CoA reductase

by dietary sterols. [171] Furthermore, plasma cholestanol, which has been shown to be increased in sitosterolemia [26] has been shown to effectively downregulate HMG-CoA reductase activity. [171] Unfortunately, the poor absorption of this sterol by normal tissues and cells has rendered studies of the effects of phytosterols on cholesterol homeostasis difficult to evaluate. The complex regulation of this enzyme certainly raises the possibility that reduced cholesterol biosynthesis in sitosterolemia is secondary to an absorptive defect.

If the primary defect in sitosterolemia is an absorptive one, regulation of ACAT, which is responsible for the intracellular esterification of sterols, may be similarly abnormal. Bhattacharyya and Connor, [1] after describing two sisters with sitosterolemia, suggested that esterification may well be the limiting factor in the absorption of plant sterols. Gregg *et al.* proposed that in sitosterolemia, there may be a loss of recognition of the structure of different sterols which leads to intracellular esterification. [21] The retention of esterified sterols could account for the atherosclerosis and xanthomatosis which are so characteristic of this disease. Cholesteryl esters are a major component of atherosclerotic plaques in both hypercholesterolemic and sitosterolemic individuals, [25, 94] and increases in ACAT activity have been shown to accompany the development of atherosclerotic lesions. [95] Although this enzyme is widely believed to be regulated by cellular sterol pools,[95] ACAT activity has never been evaluated in sitosterolemia. The possibility that ACAT is involved in a putative absorptive defect, or in the pathogenesis of this disease, is pure speculation at this point, but certainly merits consideration.

## 1.2 HMG-COA REDUCTASE

### 1.2.1 Introduction

The key regulatory enzyme in the cholesterol biosynthetic pathway is 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase. [96] HMG-CoA reductase catalyzes the reduction of HMG-CoA to mevalonic acid, which is required for the production of cholesterol and other isoprenoids (**Figure 1.2**).

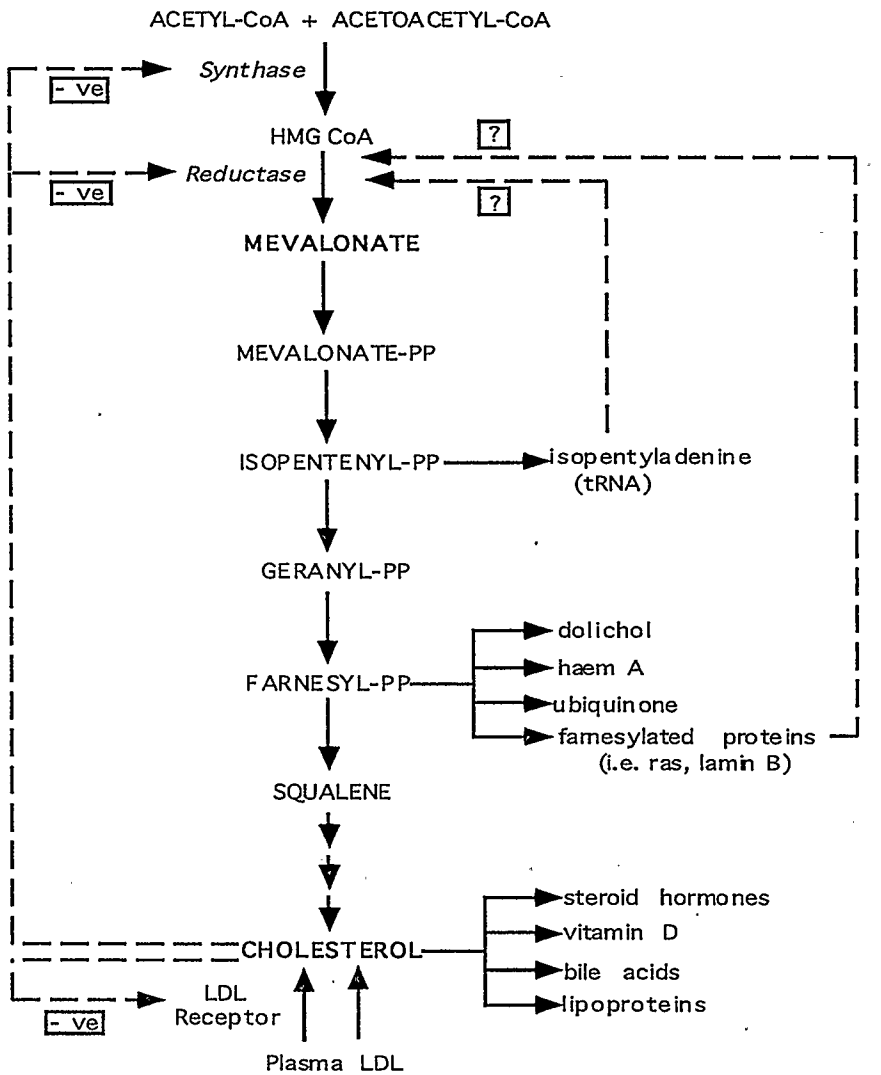


**Figure 1.2** Cholesterol synthetic pathway (adapted from Goldstein and Brown 1990) [97]

This enzyme is a 97 kDa glycosylated integral membrane protein of the endoplasmic reticulum. [69, 98] The catalytic domain lies in the cytoplasm, and the remainder of the protein spans the endoplasmic reticulum seven, [99] or possibly eight [100] times. This enzyme has been described as one of the most highly regulated proteins in nature, [97] and is subject to regulation by endogenous and dietary cholesterol, in addition to oxygenated sterol metabolic intermediates as well as mevalonate-derived metabolic intermediates. Regulation of this enzyme has been demonstrated at the transcriptional, translational, and post-translational levels.

### 1.2.1 Transcriptional regulation

Work in several laboratories has shown that HMG-CoA reductase transcription can be modulated *In vitro* by incubation under conditions in which cellular cholesterol content is increased or decreased. Within the cell, there is thought to be a regulatory pool of cholesterol which monitors free cholesterol content, and which regulates HMG-CoA reductase activity. The amount of free cholesterol in this pool is determined by the balance between utilization, synthesis, and lipoprotein uptake. [101] For the regulatory actions of LDL to be elicited in cultured cells, there must first be receptor-mediated uptake of LDL, followed by the hydrolysis of LDL bound cholesteryl esters in cell lysosomes, and the release of free cholesterol from the lysosome. [97] The input of free sterol into the regulatory pool via the LDL receptor pathway plays a predominant role in the regulation of reductase transcription.

**a) Oxysterols**

There is compelling evidence to suggest that cholesterol itself is not the intracellular sterol molecule directly responsible for regulating HMG-CoA reductase activity. Kandutsch *et al.* noted that reductase activity in cultured mouse hepatic cells and fibroblasts, was not suppressed by cholesterol when an antioxidant was present in the medium. [102] This observation has led to postulation that cholesterol liberated from lysosomes might be converted to the more biologically active oxysterols prior to exerting regulatory effects.

While oxygenated precursors of cholesterol have not yet been established as the physiological regulators of HMG-CoA reductase transcription, two separate lines of evidence support oxysterol mediated repression of this enzyme. Gupta *et al.* showed that the suppression of reductase by LDL in cultured intestinal cells could be prevented by specific inhibitors of cytochrome P450, a mixed function oxidase, suggesting that the inhibitory effects of LDL are mediated by P450-dependent oxysterol formation. [103] Cyclohexamide has been demonstrated to stabilize HMG-CoA reductase mRNA in the presence of oxysterols, suggesting that continuous synthesis of a short-lived protein regulator is required for oxysterol mediated post-transcriptional suppression of HMG-CoA. [203] Sinensky *et al.* isolated CHO cells in which HMG-CoA reductase was resistant to inhibition by oxysterol and LDL cholesterol. This inhibition was attributed to the absence of a specific oxysterol binding protein (OBP) which was determined to be essential for the inhibitory effects of both sterols in culture. Oxysterol binding protein was shown to bind cholesterol poorly or not at all, suggesting that LDL cholesterol must be converted to an oxygenated derivative in order to exert its effects. [104] Oxygenated derivatives of cholesterol, such as 25-

hydroxycholesterol and 7-ketocholesterol, have consistently proven to be more potent inhibitors of cholesterol synthesis than cholesterol itself. These sterols are however,much more water soluble than cholesterol and can achieve higher concentrations in the cytosol, which may account for their enhanced potency. [97] To date, there is no direct evidence that oxysterol binding protein participates in sterol-mediated regulation, and the role of oxysterols in the regulation of HMG-CoA reductase activity remains to be defined.

**b) Sterol regulatory element (SRE)**

The transcriptional regulation of HMG-CoA reductase by sterols and their oxygenated precursors has been traced to a single 8-nucleotide consensus sequence in the 5' flanking region of the reductase promoter, called the 'sterol regulatory element' (SRE-1). The SRE-1 acts as a conditional positive element that enhances transcription in the absence of sterols, but not when they are present. [106] The sequence is capable of conferring sterol regulation when it is incorporated into the promoter for the herpes simplex virus gene for thymidine kinase. [69, 105] When CHO cells were transfected with fusion genes in which the 5' flanking region of the reductase gene was placed upstream of a reporter gene, the fusion genes were transcribed in the absence of sterols and were repressed when sterols accumulated. [113] The SRE may be similarly involved in the active repression of reductase transcription. Osborne *et al.* noted that cells transfected with an SRE-1 in which 6 base pairs had been mutated, demonstrated constitutively high transcription in the presence or absence of sterols. [107] SRE consensus sequences are similarly found in the promoter regions of the LDL receptor gene (1 copy), the HMG-CoA synthase gene (2 copies), and the

farnesyl diphosphate synthetase gene (4 copies).[97] As such, the SRE probably facilitates the coordinate regulation of all four of these proteins, in the maintenance of cholesterol homeostasis.

**c) Transcription Factors**

Coordinate regulation of transcription through the SRE, is thought to be achieved by the binding of a sterol-dependent protein that represses transcription in the presence of sterols.[107] Rajavashisth *et al.* isolated a full cDNA clone from a HepG2 library, that encoded a 177 amino acid protein that bound specifically to the octanucleotide sequence conferring sterol responsiveness, and have suggested that this cellular nucleic acid binding protein (CNBP) may participate in the sterol mediated repression of HMG-CoA reductase by binding to the SRE.[108] CNBP in turn, may act by disrupting the binding of positive transcription factors to adjacent sites in the reductase promoter. Gil *et al.*, using affinity chromatography with specific sequences of the reductase promoter coupled to sepharose, isolated a 34 kD protein from hamster nuclei. This protein, reductase promoter factor (RPF), has been shown to bind to the reductase promoter.[109] RPF is similar in structure to nuclear factor-1 (NF-1), a protein that acts as a positive transcription factor for several eukaryotic and viral gene.[113] Recently, Wang *et al.* cloned and expressed the cDNAs for two proteins belonging to the leucine zipper family of transcription factors, designated sterol-regulatory-element-binding proteins 1 and 2 (SREBP-1 and SREBP-2). These proteins have been shown to bind to the SRE with a nucleotide specificity that precisely matches the requirement for sterol regulated transcription.[204] SREBP-1 is synthesized as a 125 kD precursor that is bound to the ER membrane and the

nuclear envelope as an integral membrane protein. In sterol depleted HeLa cells, this protein is cleaved proteolytically to release a 68 kD species from the membrane which enters the nucleus to stimulate transcription of genes containing the SRE sequence.[184] Gasic has speculated that cholesterol-mediated changes in the thickness of the ER membrane may trigger the cleavage of the SREBP-1 precursor by rendering it more susceptible to proteolysis. [195] The sterol regulated proteolysis of membrane bound transcription factors may provide a novel mechanism by which SRE-mediated transcription can be regulated. [184]

Positive or negative transcriptional elements which exert their effects by binding to the SRE, may themselves be subject to regulation by phosphorylation/dephosphorylation mechanisms. Conditions which result in an increase in intracellular calcium have been reported to stimulate transcription of the reductase gene. [110, 111] Wilkin and Edwards noted that the treatment of THP-1 and HepG2 cells with the calcium ionophore A23187, stimulated 2-15 fold increases in HMG-CoA reductase mRNA. [111] The addition of phorbol ester to THP-1 cells has similarly been shown to stimulate transcription of the reductase gene, suggesting that protein kinase C may also be involved in this regulatory process. [112] Sterol-dependent regulation of reductase transcription via the SRE then, may involve the phosphorylation or dephosphorylation of various trans-acting proteins by either protein kinase C or the calcium/calmodulin-dependent protein kinases. The sterol dependent mechanism of gene regulation, and its role in the maintenance of cholesterol homeostasis, continues to be the subject of much speculation.

### 1.2.2 Translational Regulation

Changes in the activity of HMG-CoA reductase cannot be attributed solely to changes in reductase mRNA. Studies have demonstrated that translational control also plays a significant role in the regulation of reductase activity.

**a) Role of mevalonate**

The exact mechanism of translational control is currently unknown, but a non-sterol mevalonate-derived metabolic intermediate in cholesterol biosynthesis is thought to be involved. [114] Peffley and Sinensky, using a CHO cell line unable to synthesize mevalonate (Mev-1 cells), noted that the addition of mevalonate to cells in which reductase transcription had been fully repressed by 25-hydroxycholesterol, produced a further 50 fold decrease in the synthesis of HMG-CoA reductase and that this decrease occurred without any apparent changes in reductase transcription. [115] Nakanishi *et al.* similarly noted that CHO cells incubated with sterols and high concentrations of compactin, a competitive inhibitor of HMG-CoA reductase, evidenced a decrease in reductase protein following the addition of mevalonate. This decline was attributed to an 80% decrease in the translation of reductase mRNA. [114] Nakanishi *et al.* subsequently concluded that the sterol-mediated suppression of reductase transcription is incomplete, and that non-sterol metabolites of mevalonate bring about further reductions in enzyme synthesis by inhibiting translation of the reductase gene (*ibid*).

The rate of translation of reductase mRNA is apparently dictated by the cell's demand for non-sterol isoprenoids. In the presence of high concentrations

of sterol, enough mevalonate must still be synthesized to sustain the production of non-sterol metabolites such as dolichol, haem A, ubiquinone, and isopentenyl-adenine. [97] When mevalonate is saturating and these needs are satisfied, reductase activity is reduced by repressed translation of the reductase message.

**b) Isopentenyl-tRNA and farnesylated proteins**

The regulation of reductase translation is thought to be achieved through the generation of variably sized reductase mRNA's. The reductase gene lacks the TATA sequence that determines the transcription initiation site in most genes, and transcription is evidently initiated from multiple sites, [116] thus producing mRNA's with 5' untranslated regions of variable length. [117] Cells grown in the absence of sterol, produce an increased proportion of the shorter transcripts (*ibid*). Mevalonate is known to give rise to isopentyladenine, which is associated with the anticodon loop of isopentenyl-tRNA. [97] It is possible, but not yet proven, that translation of certain mRNA transcripts is regulatable when isopentenyl-tRNA becomes limiting. [101]

Alternatively, translational regulation could be mediated by the farnesylation of proteins. Farnesyl acetate and ethyl farnesyl ether, two analogues of farnesyl diphosphate, have been shown to reduce the translation of HMG-CoA reductase, and to enhance the degradation of reductase protein. [201] Farnesylated proteins include members of the Ras and Lamin B families of proteins which contain cystein residues and which are processed by the attachment of a farnesyl group. [118, 97] These small farnesylated proteins have been shown to regulate cell growth. [119] Farnesyl diphosphate is derived directly

from mevalonate. When mevalonate is unavailable, these proteins accumulate in an unfarnesylated form. [120] These proteins could conceivably regulate HMG-CoA reductase translation by interacting with the 5' untranslated region of reductase transcripts. [118] Currently, post-transcriptional regulation of the reductase gene is poorly understood, and potential mechanisms of translational regulation are the subject of ongoing research.

### 1.2.3 Post-translational Regulation

#### a) Phosphorylation-dephosphorylation

Rapid post-translational modulation of reductase activity is known to be mediated by a well characterized bicyclic phosphorylation cascade. The ATP-dependent inactivation of reductase was first observed by Beg *et al.*, [128] and has since been determined to be due to the covalent phosphorylation of one or more serine residues in the linker region of the reductase protein. This phosphorylation is catalyzed by an AMP-activated kinase (AMP-PK, formerly called reductase kinase) which itself, undergoes reversible phosphorylation. AMP-PK is phosphorylated by a cAMP independent cytosolic kinase, formerly called reductase kinase kinase, and is dephosphorylated by protein phosphatase. Unlike reductase, AMP-PK is active in the phosphorylated form. Reactivation of phosphorylated HMG-CoA reductase is due to the removal of phosphate by a fluoride-sensitive phosphoprotein phosphatase. This protein phosphatase is in turn, is inhibited by a cytosolic protein, possibly protein kinase A. [101, 129] Collectively, this series of phosphorylation-dephosphorylation reactions are

thought to mediate short-term changes in cholesterol synthesis and to coordinate the regulation of cholesterol synthesis with other metabolic pathways.

A number of hormones have been shown to mediate changes in the proportion of reductase activated by phosphorylation. Both adrenaline and glucagon inhibit cholesterol synthesis, and insulin stimulates cholesterol synthesis, through the cAMP dependent activation of a protein thought to be PKA. [129] This protein exerts its regulatory effects by inhibiting the protein phosphatase which activates HMG-CoA reductase. The AMP activated protein kinase which phosphorylates HMG-CoA reductase, also phosphorylates and inactivates acetyl-CoA carboxylase [130] and hormone-sensitive lipase/cholesterol esterases.[131] As such, coordinate regulation of both fatty acid synthesis and HMG-CoA reductase activity may be facilitated by this phosphorylation-dephosphorylation cascade. [129]

There is general agreement that changes in the phosphorylation state of reductase make at least some contribution to the rapid changes in reductase activity that occur in response to mevalonate and LDL cholesterol. Kennelly and Rodwell suggested that the initial response of cells to changes in the demand for mevalonate or sterol, is rapid modulation of the phosphorylation state of HMG-CoA reductase. [132] These short-term changes in activity however, are followed within 1-2 hours by changes in the amount of enzyme due to subsequent modulation of synthesis or degradation. Parker *et al.* have shown that the phosphorylation of rat liver reductase increases its susceptibility to proteolysis, which may be one mechanism by which long term changes in reductase activity are coordinately regulated with changes in the phosphorylation state of the enzyme. [127] Unless special precautions are taken to inhibit dephosphorylation

during the preparation of microsomes, reductase activity assayed *in vitro* will not accurately reflect *in vivo* activity. As such, *in vivo* regulation of HMG-CoA reductase activity by phosphorylation-dephosphorylation has been difficult to prove. Myant in his review of this aspect of regulation concluded that in liver, and possibly other tissues where there might be sudden demands for cholesterol, reductase probably exists in a partially phosphorylated state which is modulated *in vivo* by a variety of physiological and non-physiological stimuli. [101]

**b) Enzyme degradation**

Post-translational control of HMG-CoA reductase occurs through a degradative process that is accelerated by both sterols and mevalonate. Reductase has a relatively short half-life (2-3 hrs), and the rate of enzyme degradation has been shown to vary in parallel with the free cholesterol content of cells. [101] Orci *et al.* demonstrated that the addition of LDL to the growth medium of UT-1 cells, which contain massive amounts of reductase protein, increased the cholesterol content of the ER membrane and resulted in the complete disappearance of the crystalloid ER in which the enzyme was located. [121] Mevalonate and 25-hydroxycholesterol have also been shown to accelerate reductase degradation. [122] While the stimulation of reductase degradation by mevalonate might be mediated by the formation of endogenous cholesterol, evidence suggests that a non-sterol isoprenoid, as well as sterol, is required. In CHO cells that were incubated in such high concentrations of compactin that mevalonate production was completely blocked, sterols alone did not accelerate the degradation of reductase. Accelerated degradation required the addition of exogenous mevalonate. [114] HMG-CoA reductase inhibitors, such as compactin,

actually stabilize reductase protein, and this stabilization can reduce enzyme degradation by up to 7 fold.[123] The mechanism for enzyme stabilization, whether through direct binding of the inhibitor to the enzyme or depletion of sterol or mevalonate pools, is unknown.

The mechanism of accelerated degradation is similarly not known, but apparently requires the membrane domains of HMG-CoA reductase. [124] Gil *et al.* noted that the cytoplasmic catalytic domain of the enzyme was needed to restore normal growth to a HMG-CoA reductase deficient line of CHO cells, but degradation of this truncated enzyme was not accelerated by the presence of sterols. [124] Chun and Simoni demonstrated that replacement of membrane spans 5 or 6 with a transmembrane sequence from bacteriorhodopsin, resulted in a reductase protein whose degradation was not accelerated by mevalonate, LDL, or 25-hydroxycholesterol, suggesting that very specific transmembrane domains are likely involved in the sterol-mediated degradation of HMG-CoA reductase. [125]

The intracellular site of reductase degradation has not been ascertained, but reductase is thought to be degraded within the ER membrane itself , or to be vesicularized and transported to lysosomes for degradation. Parker *et al.* noted that monensin, an inhibitor of lysosomal activity, blocked mevalonic acid stimulated degradation of reductase in rat hepatocytes *in vivo*. [127] Chun *et al.* however, demonstrated that Brefeldin A, which blocks the exit of proteins from the ER, did not affect mevalonate-dependent accelerated degradation, suggesting that the ER membrane is the site of posttranslational regulation. [126] The observation that mevalonate regulated degradation is abolished by cyclohexamide treatment, and by protease inhibitors, has led researchers to suggest that a short-lived protein may be involved in the degradative process. [126]

Perturbation of cellular Ca++ has similarly been shown to attenuate the rapid degradation of HMG-CoA reductase [122] suggesting that the putative protein signalling pathway may be calcium dependent. While collective evidence favours proteolytic degradation of reductase in the ER membrane itself, this hypothesis remains to be confirmed.

**c) Membrane fluidity and thickness**

Introduction of cholesterol has two principle effects on a lipid bilayer. Fatty acyl chains adjacent to the cholesterol ring system become more ordered and tightly packed thereby reducing bilayer permeability. This causes a measurable reduction in membrane fluidity. Cholesterol-ordered segments of the acyl side chains also lie more perpendicular to the bilayer, thereby increasing membrane thickness by as much as 20%.[195] The activity of some, but not all, membrane bound enzymes has been demonstrated to change in response to altered membrane microviscosity [48] or possibly thickness. [184] Changes in membrane cholesterol content might exert these regulatory effects by altering the conformation of transmembrane proteins, or by rendering them more susceptible to activation or degradation. [133, 184]

Mitropoulos and Venkatesan have suggested that unesterified cholesterol may modulate reductase activity *in vivo* by modifying the fluidity of the ER membrane in the vicinity of reductase protein. [133] The cholesterol/phospholipid ratio in the ER membrane is very low (0.06) relative to the plasma membrane (0.85), [134] which suggests that this membrane might be particularly sensitive to perturbations in cholesterol content. Gasic noted that the low cholesterol content of the ER membrane would render small changes in free cholesterol far easier to

detect than at the cholesterol-rich plasma membrane. [195] Gasic further suggested that a small change in the cholesterol content of the relatively cholesterol-poor ER membrane may cause the bilayer to expand by several angstroms, possibly shielding proteolytic sites on integral membrane proteins (*ibid*). Rothblat *et al.* have demonstrated the existence of cholesterol rich and cholesterol poor domains in plasma membranes. [135] The existence of similar heterogeneous pools of cholesterol in the ER membrane might further facilitate regulation by cholesterol content.

While there is considerable implicative evidence in support of this mode of regulation, changes in membrane fluidity and thickness have not been conclusively demonstrated to alter reductase activity. Changes in lymphocyte and erythrocyte membrane microviscosity have been shown to correlate with plasma lipid levels in humans, [136, 137] and aortic microsomes isolated from atherosclerotic rabbits evidenced a significant decrease in membrane fluidity . [139] An increase in membrane sphingomyelin content has been shown to inhibit cholesterol synthesis. [140] Furthermore, oxysterols have been demonstrated to insert perfectly into lipid bilayers effectively reducing membrane fluidity, [138] and Richert *et al* has noted that HMG-CoA reductase activity could be correlated with oxysterol-induced changes in membrane viscosity in cultured hepatoma cells. [141] Venkatesan and Mitropoulos demonstrated that when the free cholesterol content of liver microsomes was increased following incubation with human serum, a phase change in the membrane lipids was coincident with a decrease in HMG-CoA reductase activity. [142] Hashimoto *et al.* however, failed to find any change in reductase activity when hepatic microsomes were enriched with cholesterol. [143] While the experimental data is very suggestive, it has proved

difficult to distinguish between effects mediated by changes in membrane fluidity or thickness, and effects due to cholesterol itself.

## 1.3 ACYL-CoA CHOLESTEROL ACYLTRANSFERASE

### 1.3.0 Introduction

In addition to being present within the cell as free sterol, cholesterol is found esterified to long chain fatty acids. The percent of total sterol present as sterol ester varies from <1% to as high as 85%. [48] In general, the concentrations of sterol esters are greatest in those tissues which play a major role in the synthesis and metabolism of cholesterol (*ibid*). Cholesterol esters cannot replace free cholesterol in cells, but are the primary storage and transport form of excess sterol.

Acyl-CoA cholesterol acyltransferase (ACAT) is widely believed to be the enzyme responsible for the intracellular esterification of cholesterol. This enzyme is an integral membrane protein of the rough endoplasmic reticulum, and is found in most mammalian cells. [143, 98] ACAT catalyzes the transfer of fatty acyl-CoA from an acyl donor, such as triglyceride or phospholipid, to free cholesterol (**Figure 1.3**). By the action of this enzyme, free cholesterol is thought to be removed from the ER and localized to cytoplasmic lipid droplets. [134] While other sterols can be esterified by ACAT, esterification declines sharply with either reduction or extension of the $C^{24}$ side chain. The percent esterification of campesterol by ACAT, relative to cholesterol, is approximately 23%, and is <5%

for sitosterol and stigmasterol. [144] This substrate specificity is similar for most eukaryotic cells, suggesting a conservation of enzyme structure during evolution [48]

Relatively little is known about the structure of this enzyme. Chang *et al.* however, recently isolated a 1.7 kB cDNA encoding an integral membrane protein comprising 550 amino acid residues, and thought to encode human macrophage ACAT. [205] Protein homology analysis has suggested that this protein may possess fatty acid ligase activity in addition to acyltransferase activity (*ibid*). Chang et al. have suggested that this polypeptide alone may not comprise the ACAT holoenzyme, but that the peptide may form homo or heterodimers (*ibid*). The cloning of ACAT should facilitate a more comprehensive understanding of structure-function relationships, protein-sterol interactions, and modes of regulation of this enzyme.

### 1.3.1 Regulatory Mechanisms

The level of cholesterol in the cellular substrate pool appears to be the primary regulator of ACAT activity. The cholesterol content of a cell is increased by two primary mechanisms, LDL hydrolysis and endogenous synthesis. Both exogenously, [146, 206] and endogenously [147, 206] derived cholesterol, in addition to oxygenated sterols such as 25-hydroxycholesterol [206] have been correlated with increased ACAT activity. *In vivo*, the substrate pool for ACAT is thought to be limiting, permitting direct regulation by intracellular sterol levels. [48]

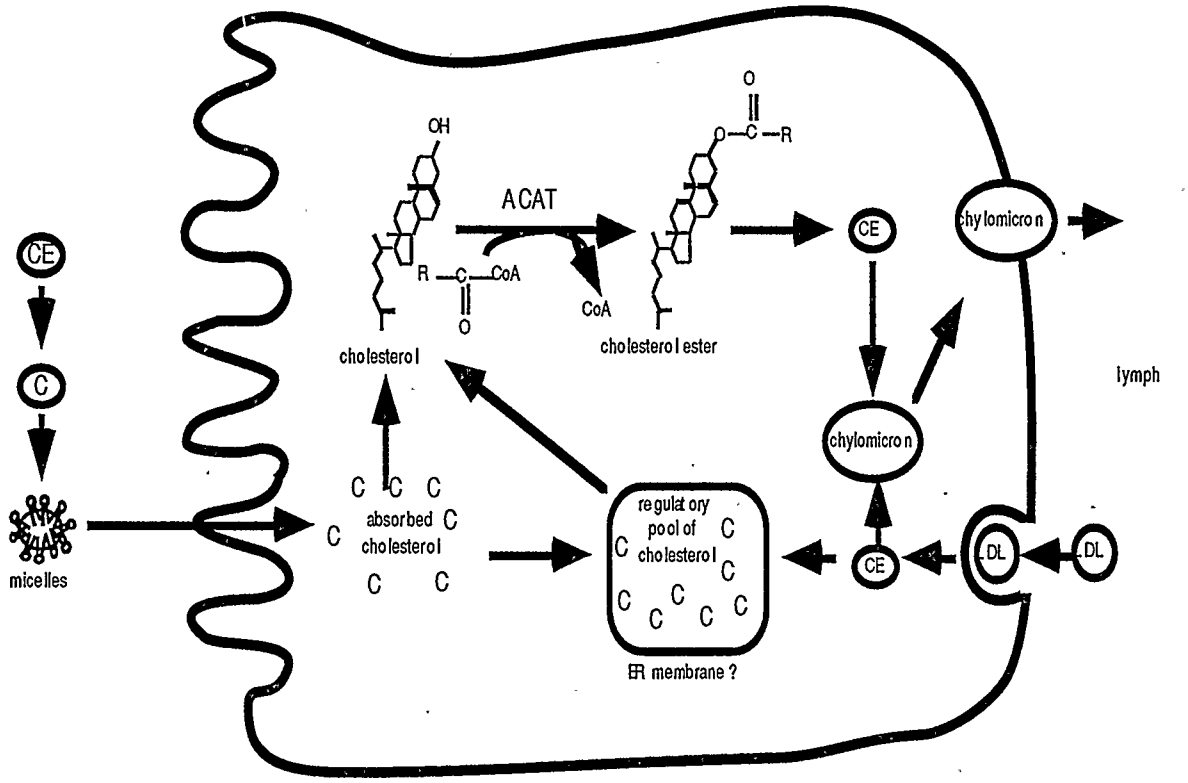


**Figure 1.3** Schematic representation of intestinal mucosal cell adapted from Sliskovic and White (CE, cholesterol ester; C, free cholesterol; ACAT, acyl-CoA cholesterol acyltransferase)

Additional factors may contribute to the regulation of ACAT activity either by affecting enzyme activity directly, or by affecting the supply of cholesterol to the substrate pool. Regulation by phosphorylation remains unsubstantiated, as both increases [148] and decreases [149] in ACAT activity have been reported when hepatic microsomes were incubated with ATP/Mg++. Several investigators have demonstrated a 6-8 fold increase in ACAT activity following cyclohexamide treatment however, [155, 156] which suggests the involvement of a short-lived protein inhibitor of ACAT. This factor may inhibit the enzyme directly or may work by preventing cholesterol delivery to the enzyme. The observation by

work by preventing cholesterol delivery to the enzyme. The observation by Tabas and Boykow that the cyclohexamide effect was ameliorated by endogenous cholesterol, has led to the suggestion that this protein may act by preventing the entry of cholesterol into the substrate pool. [156] The compound U1866A which inhibits the cellular transfer of LDL cholesterol from the plasma membrane to intracellular membranes, has been shown to inhibit ACAT activity. [175] Sterol carrier protein 2 (SCP-2) however, does not appear to be required for the stimulation of ACAT activity that follows LDL uptake. [173] Direct regulation of this enzyme by mechanisms other than cholesterol concentration remain to be conclusively demonstrated.

ACAT is evidently very sensitive to membrane perturbations, and a variety of lipophilic compounds, such as anesthetics and detergents, have been found to affect ACAT activity. [48] As such, membrane microviscosity is thought to possibly be involved in the regulation of esterification. Plasma membrane cholesterol has been demonstrated to be accessible to ACAT, [154] and Hashimoto reported a 7-fold increase in ACAT activity following the enrichment of hepatic microsomes with cholesterol.[143] Curiously, a similar enrichment of microsomes with sitosterol did not appear to affect ACAT activity. [157] Microsomes prepared from the aortas of normal rabbits which were enriched with exogenous cholesterol to achieve the same viscosity as microsomes prepared from atherosclerotic rabbits, retained an 8-fold lower level of ACAT activity, suggesting that membrane fluidity was not likely responsible for the observed difference. [153] Gillies *et al.* have suggested that there may be a correlation between ACAT activity and membrane fluidity at non-saturating concentrations of cholesterol (*ibid*). More recently, cell membrane sphingomyelin content has been shown to be correlated with ACAT activity. [172]

[175] Cellular sphingomyelin, which increases the capacity of cell membranes to accomodate excess cholesterol, has been shown to decrease ACAT activity in macrophages by increasing the threshold at which ACAT is stimulated by lipoprotein delivery of cholesterol. [172] Degradation of plasma membrane sphingomyelin by neutral sphingomyelinase (N-SMase) in cultured fibroblasts has been shown to induce translocation of plasma membrane sterols and to stimulate ACAT activity. [202, 175] As such, the regulation of ACAT activity by membrane sterols may be mediated by changes in cholesterol trafficking within the cell. [172]

### 1.3.2 Role in Atherosclerosis

Interest in ACAT has stemmed primarily from its potential significance in the development of atherosclerosis. An increase in ACAT mediated esterification of cholesterol, and the subsequent accumulation of cholesterol esters, are well documented events in atherosclerosis. Whether the increase in ACAT activity constitutes an etiological event in atherosclerosis is controversial. The accumulation of cholesterol ester may simply be a consequence of increased cholesterol availability, in which case the increased ACAT activity which accompanies lesion development is unlikely to be of significance. [48] Murine J774 macrophages however, which accumulate large amounts of cholesterol ester, have been demonstrated to have 10-30 fold higher ACAT activity when incubated in the same concentrations of LDL as mouse peritoneal macrophages. [158] Furthermore, ACAT inhibitors have demonstrated potent anti-atherosclerotic

effects, [46] which suggests that the regulation of ACAT activity may play a central role in atherogenesis.

### 1.3.3 Role in Intestinal Absorption of Sterols

Although the mechanism of cholesterol uptake at the brush border membrane is unknown, cholesterol esterification appears to play a role in the absorption of dietary sterols (**Figure 1.3**). ACAT activity has been shown to parallel cholesterol absorption along the length of the intestine. The activity of intestinal ACAT is higher in cells of the villi than in crypt cells, [206] and is higher in the segment of the jejunum from which cholesterol is absorbed than in any other segment of the intestine. [207] Treadwell and Vahouny reported that 70-90% of the cholestrol secreted into the lymph as chylomicrons, following assembly in the brush border, is esterified. [169] Moreover, the supression of ACAT activity by ACAT inhibitors has been shown both *in vitro* and *in vivo*, to decrease cholesterol absorption by intestinal cells. [46, 48, 61, 62, 174] In recognition of the role of ACAT in both atherosclerosis and dietary sterol absorption, a variety of ACAT inhibitors are currently under clinical or pre-clinical evaluation. The utility of these inhibitors for either lowering plasma cholesterol or reducing atherosclerotic lesions in humans however, remains to be determined.

## 1.4 SUMMARY

The primary defect in sitosterolemia remains to be resolved. The absence of an endogenous mechanism for the regulation of intestinal sterol uptake, suggests that reduced cholesterol synthesis in this disorder may be secondary to the indiscriminate absorption of plant sterols. The clear structural similarities between the phytosterols and cholesterol, and the ubiquitous regulation of cholesterol homeostasis by sterols, similarly favour this hypothesis. The development of a cell culture model which will permit the separation of effects due to inherent genetic defects, from effects due to high phytosterol concentrations, should facilitate an understanding of the underlying disorder in sitosterolemia.

# CHAPTER 2
# METHODS

## 2.0 INTRODUCTION

The primary objective of this study was to evaluate cholesterol synthesis and sterol esterification in cultured lymphocytes obtained from individuals with the lipid disorder ß-sitosterolemia. Lymphocytes were isolated from whole blood, and cultured in media containing phytohemagglutinin (days 1-4) and IL-2 (days 5-10). The rate of cholesterol synthesis and the rate of sterol esterification was evaluated on day 0 and day 10 of culture, using radiolabelled substrates. These values were used as a measure of HMG-CoA reductase activity and ACAT activity respectively, and enzyme activity was evaluated both in the presence and absence of exogenous LDL cholesterol. Patient values were compared to those of normolipidemic controls using lymphocytes cultured under identical conditions.

The secondary objective was to determine if the plant sterols could be cultured out of the patient lymphocytes under conditions stimulating rapid growth , and to determine what effect, if any, this reduction in plant sterol content had on the rate of cholesterol synthesis and the rate of sterol esterification. Conversely, cholesterol synthesis and sterol esterification were evaluated in normal control lymphocytes incubated in the presence of patient LDL containing plant sterols. The lipid fraction was extracted from patient lipoproteins and from patient lymphocytes, and sterol content was quantitated

using gas liquid chromatography.

In this chapter, the two ß-sitosterolemic patients and the control subjects who participated in this study are described. This chapter outlines the general techniques used in the experiments, and details both specific methodologies and the verification of these methodologies. The methodologies are sequential, and are presented in the order in which they were performed.

## 2.1 EXPERIMENTAL SUBJECTS

### 2.1.1 Sitosterolemic Patients

Two patients with sitosterolemia, one male (G.S.) and one female (A.S.), participated in this study.

**a) Patient #1 (G.S.)**

G.S. is a 56 year old man of Argentinian origin. He was diagnosed in his teens with familial hypercholesterolemia, on the basis of the presence of tendonous xanthomas. At age 23, he had xanthelasmas removed from both eyelids, and was treated with an unknown medication for hypercholesterolemia. Following his immigration to Canada in 1980, he was investigated by a family physician for hyperlipidemia, based on a 5 year history of intermittent chest heaviness and a 7 year history of intermittent claudication in both legs, in addition to the presence of xanthomas, xanthelasmas, thickened Achilles tendons, bilateral arcus cornealis, and a significant family history of heart

disease. He subsequently underwent dietary treatment for 7 years, until the confirmation of a diagnosis of sitosterolemia in 1987. He has since continued dietary therapy, and has received both diltiazem (60 mg PO tid), and cholestyramine (24.0 g / day). The cholestyramine therapy resulted in a marked lowering of his serum sterol levels, complete regression of his xanthomas / xanthelasmas, and the disappearance of his claudication. Cholestyramine therapy was ongoing at the time of the tissue culture experiments.

**b) Patient #2**

The second patient (A.S.) is the younger sister of G.S. A.S. is a 42 year old female who was diagnosed with sitosterolemia in 1990, subsequent to G.S.'s diagnosis. In 1987 she developed xanthelasmas on the nasal bridge, and there was evidence of xanthomas over the extensor surfaces of the knuckles, elbows, popliteal fossa, and Achilles tendons. She had no significant cardiovascular symptoms, but based on her family history subsequently underwent both dietary and cholestyramine (12.0 g / day) therapy, resulting in complete regression of her xanthomas/xanthelasmas. Cholestyramine therapy was similarly ongoing at the time of the tissue culture experiments.

**c) Family history**

The parents of G.S. and A.S. are apparently healthy and have no symptoms of sitosterolemia. There is however, a significant history of heart disease among their 7 children **(Figure 2.1)**. In addition to G.S. and A.S., who have both been diagnosed with sitosterolemia, one brother died at age 30 of myocardial infarction. A second brother (age 53) underwent bypass surgery at

38 years of age. A third brother (age 45) has similarly been diagnosed with cardiac disease. A younger sister (age 40) has been diagnosed with a lipid disorder of unknown origin. The youngest sibling (age 34) remains healthy. G.S. has 3 children (aged 23, 24 and 28) and A.S. has 2 children (aged 6 and 8), none of whom display any physical or biochemical evidence of ß-sitosterolemia.

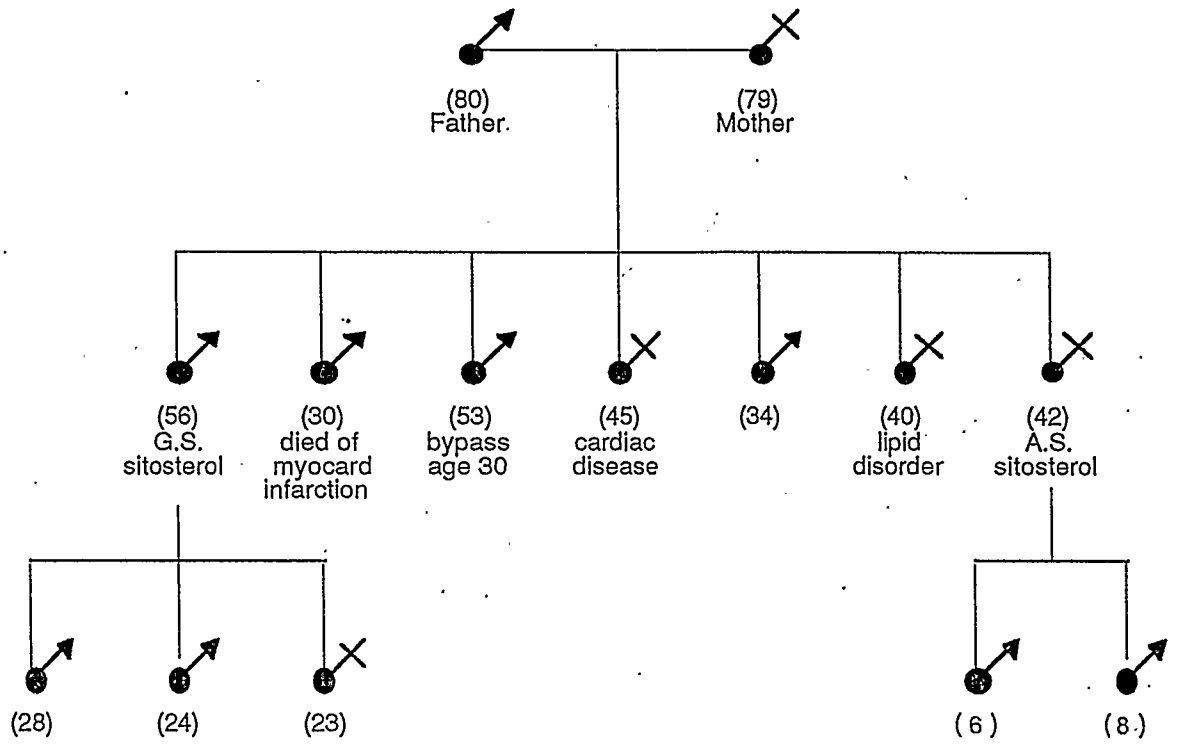


**Figure 2.1** Family history of the two sitosterolemic patients (ages shown in brackets)

## 2.1.2 Control Subjects

Lymphocytes obtained from a total of 13 control subjects were used in the various tissue culture experiments. The controls were healthy normolipidemic

males (n=7) and females (n=6) ranging in age from 21 to 65 years (mean age 39.9 ± 14.9 yr.). Total serum cholesterol levels for the control group ranged from 2.32 to 5.76 mmol / L (mean 4.63 ± 1.00 mmol / L), and none of the controls had any history of heart disease.

## 2.2 TISSUE CULTURE

### 2.2.1 Isolation of Lymphocytes

Blood samples (50 - 80 mls) were drawn from both controls and patients before 10 AM, following an overnight fast of at least 12 hours duration. Blood was drawn into 7 or 10 ml vacutainers containing 0.1% EDTA (Becton-Dickinson). Anticoagulant-treated whole blood was then diluted 1 : 1 (v/v) with sterile DPBS (Dulbecco's phosphate buffered saline ; Sigma Chemicals) containing 8.00 g / L sodium chloride, 1.15 g / L sodium phosphate dibasic, 0.20 g / L potassium chloride, and 0.20 g / L potassium phosphate. Diluted blood samples were carefully layered over 10 ml of sterile Ficoll-Paque (Pharmacia) at room temperature, in 50 ml sterile conical polystyrene tubes (Falcon). The blood was then centrifuged at 400g and 18 - 20° C, for 35 min. After centrifuging, the upper layer (plasma) was removed using a sterile pasteur pipette, leaving the lymphocyte layer ('buffy coat') undisturbed at the interface. The lymphocyte layer was transferred to a 15 ml sterile conical polystyrene tube, and washed 3X with 3 volumes of DPBS (100 g, 5 min). Sterile conditions were maintained throughout the isolation procedure.

If red blood cell contamination was evident , red blood cells were lysed by gently vortexing the cell pellet for 10 sec in 1 ml of sterile double distilled water. Isotonicity was restored by rapid resuspension in an additional 9 ml of concentrated DPBS. This procedure was repeated a second time if necessary. Monocytes were removed from the cell pellet by resuspending the cells in 10 mls of RPMI 1640 (Gibco), and incubating the cell suspension horizontally in a 25 $mm^2$ culture flask, at 37° C and 5% $CO_2$ in humidified air, for 1 hr. During this incubation period, the monocytes would adhere to the bottom of the culture flask. The lymphocytes, which remained suspended, were re-pelleted and brought up in 1 ml of fresh RPMI 1640 for counting purposes. The mean yield of lymphocytes following this procedure was $0.67 \pm 0.17 \times 10^6$ cells / ml of whole blood (n =15).

## 2.2.2 Counting Cells

Cells were counted using a Coulter Counter (Coulter Counter Channelyzer 256). Ten microlitres of the cell suspension was resuspended in 20 ml of 'Isoton' (Baxter Canlab isotonic counting solution without azide) in a Coulter counting vial. Each 10 µl sample was counted 3X, and this procedure was repeated twice for each cell pellet. Within sample variability was ± 1.85 % (n=10), and between sample variability was ± 4.59 %. The total number of cells per ml was determined from the mean of duplicate counts, following correction for the dilution factor (X4000).

### 2.2.3 Cell Viability

Cell viability was assessed using the trypan blue dye exclusion method. [219] Ten microlitres of the cell suspension, prepared as described above, was diluted with 90 µl of DPBS, and 100 µl of 0.1% trypan blue dye solution (Sigma Chemicals). The number of cells excluding trypan blue dye was quantitated using a Neubauer hemocytometer and a phase contrast microscope. Each sample was counted twice, and a minimum of 500 cells were assessed in each count. Mean cell viability following the isolation procedure was 97.1 ± 2.9% (n=10).

### 2.2.4 Cell Culture

Lymphocytes were initially cultured in sterile RPMI 1640 (Gibco), containing 15% fetal bovine serum (FBS, Gibco), 2.0 mmol / L glutamine, 1.0 mmol / L sodium pyruvate, 2.0 g / L sodium bicarbonate, 100 U / ml penicillin, 100 µg / ml streptomycin, and 1% phytohemagglutinin M (PHA, Gibco). Media was adjusted to pH 7.4, and filter sterilized by passage through a 0.2 µ millipore filter, before addition to cells.

Cultures were initiated in 25 $mm^2$ culture flasks (Corning), at a density of 1 X $10^6$ cells / ml, in 8 - 10 ml of culture medium. The open flasks were incubated without shaking, in the horizontal position, at 37° C in 5% atmospheric $CO_2$ in air. On day 4 of culture, cell clumps were gently dispersed by repeated

passage through a sterile wide-bore pasteur pipette. The cells were counted using a Coulter counter, and subcultured to a density of 0.5 X $10^6$ cells / ml in RPMI 1640 containing 10 U / ml of interleukin-2 (IL-2, Amgen Biologicals). This concentration of IL-2 gave the most consistent rate of rapid growth, when growth was compared in cultures containing 0 - 20 U / ml of IL-2 (**Figure 2.2**) Cultures were routinely maintained at a density of 0.3 - 2.0 X $10^6$ cells / ml by appropriate subculture. Under these conditions, a constant rate of rapid growth was sustainable for up to 12 days. After approximately 2 weeks, the growth rate invariably declined, and cultures typically ceased growing altogether within 3 weeks of lymphocyte isolation (**Figure 2.3**).

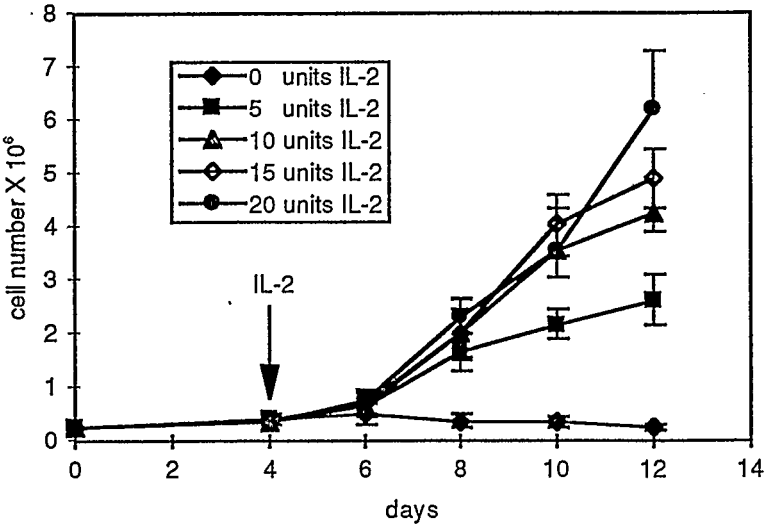


**Figure 2.2** Growth of IL-2 stimulated lymphocytes using various concentrations of IL-2 (n=4; mean ± SD)

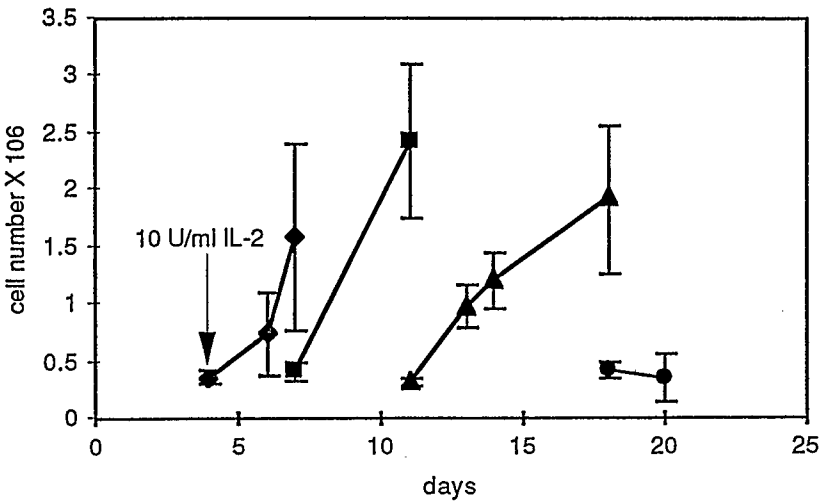


**Figure 2.3** Growth of IL-2 stimulated lymphocytes in 10 U/ml IL-2. Cells were subcultured every 2-4 days to maintain a cell density of 0.3 - 2.5 X $10^6$ cells/ml. Under these conditions, rapid growth was sustainable for 10-14 days. After 2 weeks of culture, the growth rate declined and after 3 weeks of culture cells ceased to grow. (n=4 ; mean ± SD)

## 2.3 ISOTOPE STUDIES

### 2.3.1 HMG-CoA Reductase Assay

The rate of incorporation of [$^3$H]-sodium acetate ($CH_3$*COONa) into the cholesterol fraction of whole cells, was used as a measure of HMG-CoA reductase activity in cultured lymphocytes. A stock solution of [$^3$H]-sodium acetate in RPMI 1640 was prepared by drying 280 µl of [$^3$H]-sodium acetate in ethanol (10 mCi / ml, Dupont - NEN) under nitrogen gas. The [$^3$H]-sodium acetate was then redissolved in 2.22 ml of RPMI 1640, and an additional 2.78 ml of unlabelled 10 mM sodium acetate solution in RPMI 1640. Following vortex

mixing, this solution was filter sterilized by passage through a 0.2 μ millipore syringe filter. For each assay, 100 μl of this stock solution was added to 400 μl of a cell suspension containing 0.5 X $10^6$ lymphocytes in RPMI 1640 supplemented with 10% lipoprotein deficient fetal bovine serum (LPDS - Sigma). This yielded a final cell density of 1.0 X $10^6$ cells / ml in 2.5 μM sodium acetate (100 dpms / pmol).

The assays were carried out in small (12 X 75 mm) sterile polystyrene culture tubes. Cell suspensions were incubated without shaking at 37° C in a humidified atmosphere of 5% $CO_2$ in air, for 6 hours. Under these conditions, sodium acetate was not limiting (**Figure 2.4**), and the incorporation of isotope was linear for up to 8 hours (**Figure 2.5**).

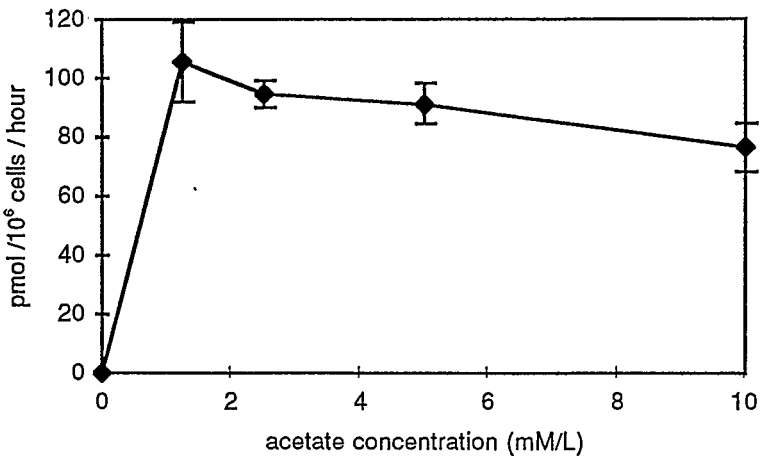


**Figure 2.4** [$^3$H]-Na-acetate incorporation into the cholesterol fraction of IL-2 stimulated lymphocytes at various concentrations of acetate. (n=3 ; mean ± SD)

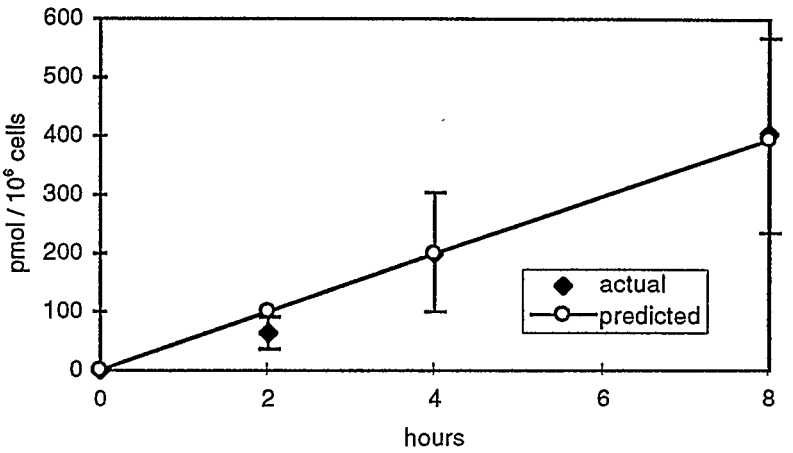


**Figure 2.5** Incorporation of [$^3$H]-Na-acetate (2.5 µM Na-acetate) into the cholesterol fraction of IL-2 stimulated lymphocytes over an 8 hour period. Predicted line calculated by linear regression analysis ( r2 = 0.97). ( n=3, mean ± SD)

The reaction was terminated by washing the cells 3X with ice cold DPBS, and then adding 1.0 ml methanol. Total lipids were extracted from the lymphocytes by the method of Folch *et al.* [217] and were separated by thin layer chromatography (as described in Sections 2.5.1 and 2.5.2). Incorporation of [$^3$H]-sodium acetate into the cholesterol band was expressed as pmol / $10^6$ cells / hr, and was used as a measure of HMG-CoA reductase activity. Assays carried out using [$^{14}$C]-sodium acetate (Dupont-NEN) and [$^3$H]-cholesterol (Amersham) as a recovery standard, demonstrated consistently high recovery of labelled cholesterol using this method (mean recovery = 93.4 ± 11.8%, n =20).

## 2.3.2 ACAT Assay

The rate of incorporation of [$^3$H]-oleic acid [$CH_3$ ($CH_2$) $_7$CH* = CH* $CH_2$)$_7$COOH] into the cholesterol ester fraction of cultured lymphocytes was used as a measure of acyl-CoA cholesterol acyltransferase (ACAT) activity. A stock solution of [$^3$H]-oleic acid was prepared by combining 225 µl of [3H]-oleic acid (50 mCi / ml ; Dupont - NEN) with 250 µl of 100 mM unlabelled oleic acid (Sigma Chemicals) in methanol. This mixture was dried completely under nitrogen gas. To facilitate solublization, a potassium salt of the oleic acid was prepared by the addition of 250 ul of 0.12 M KOH in ethanol (a 20% molar excess of K+). After gentle vortexing, this mixture was dried down under nitrogen gas to yield slightly damp crystals. The oleic acid was complexed to albumin by the addition of 4% fatty acid free bovine serum albumin (BSA) in RPMI 1640. The oleic acid / BSA solution was vortexed gently at regular intervals over a period of 1 hour, and then filter sterilized by passage through a 0.2 µ millipore syringe filter.

For each assay, 100 µl of the stock solution (prepared as described above) was added to 400 µl of a cell suspension containing 0.5 X $10^6$ lymphocytes in RPMI 1640 + 10% LPDS. This yielded a final cell density of 1.0 X $10^6$ cells / ml in 0.5 mM oleic acid (100 dpms / pmol oleic acid), and a ratio of fatty acid : BSA of 4 : 1. Assays were carried out in small (12 X 75 mm), sterile, polystyrene culture tubes (Falcon). Cell suspensions were incubated without shaking at 37° C in a humidified atmosphere of 5% $CO_2$ in air for 6 hours. Under these conditions, oleic acid was not limiting (**Figure 2.6**), and the

incorporation of radioisotope was linear for up to 8 hours (**Figure 2.7**).

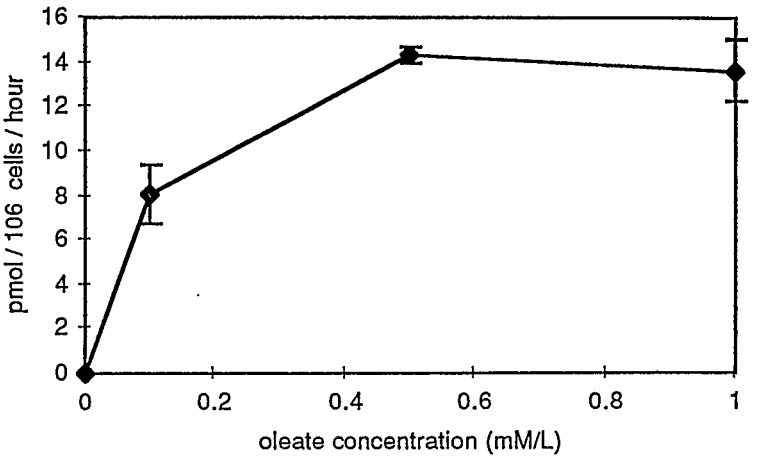


**Figure 2.6** Incorporation of [$^3$H]-oleate into the cholesterol ester fraction of lymphocytes using various concentrations of oleate (n=3 ; mean ±SD)

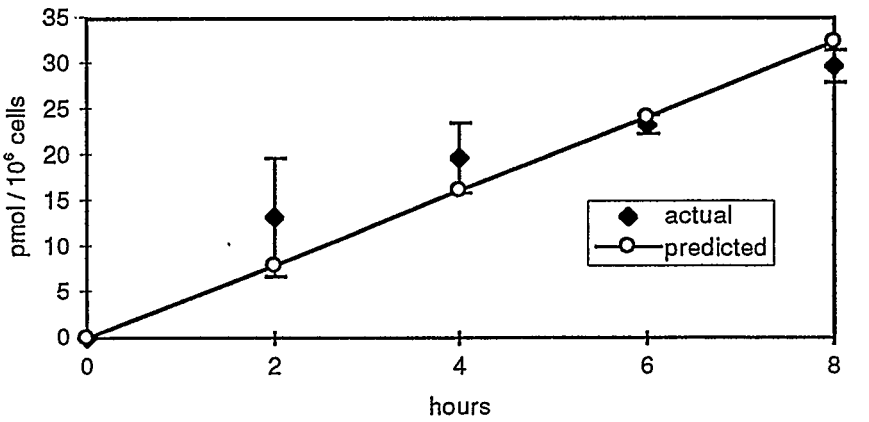


**Figure 2.7** Incorporation of [$^3$H]-oleate (0.5 mM oleate) into the cholesterol ester fraction of lymphocytes over an 8 hour period. Predicted line calculated by linear regression analysis of the data ($r^2$ = 0.89). (n=3; mean ± SD)

All assays were similarly performed in the presence of exogenous cholesterol supplied in the form of human LDL. In experiments using patient LDL and comparing its effects to control LDL, this LDL was prepared from patient and

control plasma, as described in Section 2.4.1. Otherwise, human LDL supplied by Sigma Chemical Co. was used for cholesterol loading. ACAT assays comparing the Sigma LDL to the LDL prepared in the lab, yielded comparable results. Low density lipoprotein (LDL) was added to the culture media at a concentration of 50 µg LDL protein / ml. This concentration of LDL was determined to be saturating when [$^3$H]-oleic acid incorporation was evaluated at various LDL concentrations (**Figure 2.8**).

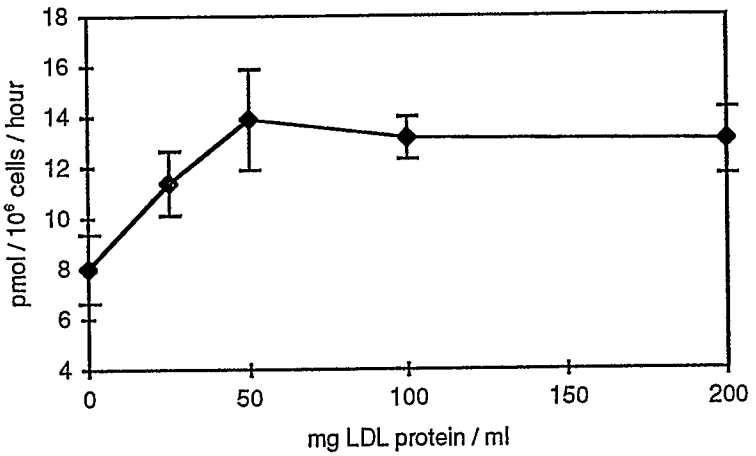


**Figure 2.8** Effect of varying LDL concentration on the incorporation of [$^3$H]-oleate into the cholesterol ester fraction of lymphocytes (n=3 ; mean ± SD)

Cells were incubated in culture medium containing 50 µg/ml LDL protein for a total of 24 hours. This period of 'cholesterol loading' was shown to maximize [$^3$H]-oleic acid incorporation into the cholesterol ester fraction of lymphocytes (elicit maximal ACAT activity), when various incubation periods were compared (**Figure 2.9**).

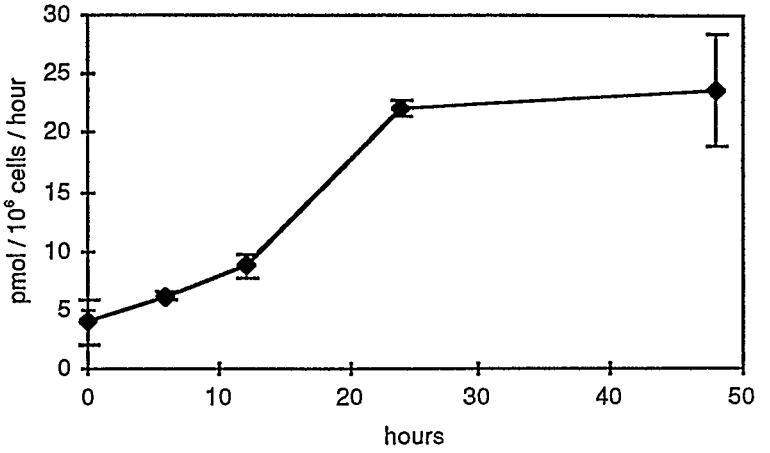


**Figure 2.9** Upregulation of [$^3$H]-oleate incorporation into the cholesterol ester fraction of lymphocytes with incubation in 50 mg/ml LDL protein. (n=3 ; mean ± SD)

[$^3$H]-oleic acid was added to the lymphocyte cultures for the last 6 hours of the 24 hours of incubation in LDL, and the assay was carried out as described above. Leaving the LDL in the culture during the 6 hour isotope pulse vs. removing the LDL prior to the addition of isotope, was demonstrated not to affect overall [$^3$H]-oleic acid incorporation, as shown in **Figure 2.10.**

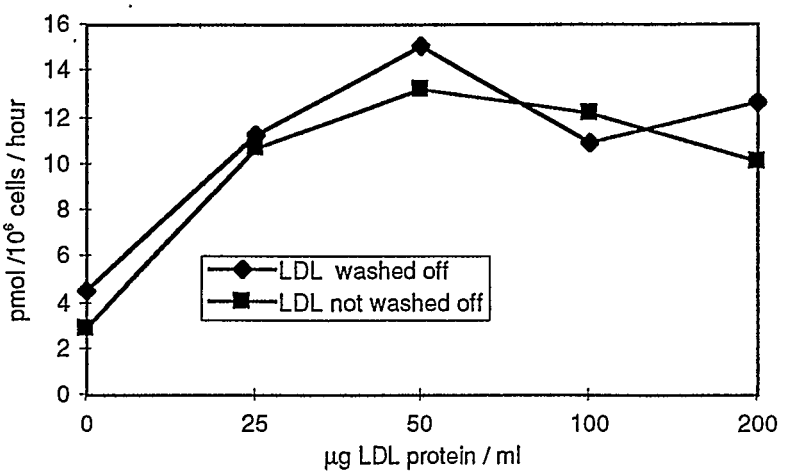


**Figure 2.10** Effect of removing the LDL prior to the addition of isotope on the incorporation of [3H]-oleate into the cholesterol ester fraction of lymphocytes. (n=2)

The reaction was terminated by washing the cells 3X with ice cold DPBS, and then adding 1.0 ml of methanol. The neutral lipids were extracted from the lymphocytes by the method of Folch *et al.* [216] and were separated by thin layer chromatography, as described in Sections 2.5.1 and 2.5.2. Incorporation of [$^3$H]-oleic acid into the cholesterol ester band was expressed as pmol / $10^6$ cells / hour, and was used as a measure of ACAT activity. Assays carried out using [$^{14}$C]-oleic acid (Dupont - NEN) and [$^3$H]-cholesterol ester (Amersham) as a recovery standard, demonstrated relatively high recovery of labelled cholesterol ester using this method (mean recovery = 81.3 ± 11.0%, n = 25).

### 2.3.3 Scintillation Counting

Isotope incorporation was measured by beta scintillation counting in a Beckman Beta Counter. The appropriate lipid band was scraped directly from the TLC plate into a scintillation counting vial. One ml of methanol was added to each sample to facilitate solublization, before the addition of 10 ml of scintillation fluid (Cytoscint, ICN Biomedicals). Each sample was counted for 5 min. Counting efficiency consistently ranged from 33 - 37%. Radioactivity was measured as (dpms - background). Background radiation was determined by processing samples (as described above, in Sections 2.3.1 and 2.3.2) at time 0, immediately after the addition of isotope. Background radiation in each lipid fraction was calculated separately for every experiment, and was expressed as the mean of triplicate samples.

## 2.4 LIPOPROTEIN PREPARATION

### 2.4.1 Isolation of Lipoproteins

Lipoproteins were isolated from plasma by density gradient ultracentrifugation using the method of Terpestra *et al.* [220] Lipoproteins were isolated for two purposes: 1. to provide LDL for ACAT assays (as described in Section 2.3.2), and 2. to quantitate plant sterols and cholesterol in the various lipoprotein fractions of patient and control blood samples.

Fasted whole blood obtained as described previously (Section 2.2.1), was spun at 500 g for 15 min to pellet blood cells. The volume of plasma obtained was subsequently measured, and the density of the plasma was adjusted to 1.25 kg / L by the addition of potassium bromide (0.385 g / ml plasma) and sucrose (0.025 g / ml plasma). The plasma was then vortexed to completely dissolve the potassium bromide and sucrose. Two ml of plasma was transferred to a precalibrated 14 X 89 mm (12.5 ml) ultracentrifuge tube, and overlayered with 2.0 ml of solution #1 (d = 1.225 kg / L), containing 11.42 g / L sodium chloride, 315.54 g / L potassium bromide, and 0.1 g / L ethylenediaminetetraacetic acid (EDTA). A perfusion pump and the capillary gravity technique were used to ensure discontinuous layering in the density gradient. Using the same technique, solution #1 was overlayered with 4.0 ml of solution #2 (d = 1.10 kg / L), containing 11.42 g / L sodium chloride, 133.48 g / L potassium bromide, and 0.1 g / L EDTA. Solution #2 was then overlayered with 4.0 ml of double distilled water (d = 1.006 kg / L). The ultracentrifuge tubes were then spun at 40,000 rpm

(SW41Ti swinging bucket rotor, Beckman L8 70M Ultracentrifuge) at 4° C for 20 hr. Following ultracentrifugation, the lipoprotein bands were visible as yellowish bands at d > 1.006 (VLDL), 1.0193 < d < 1.063 (LDL), and 1.10 < d < 1.21 (HDL), as illustrated in **Figure 2.11.**

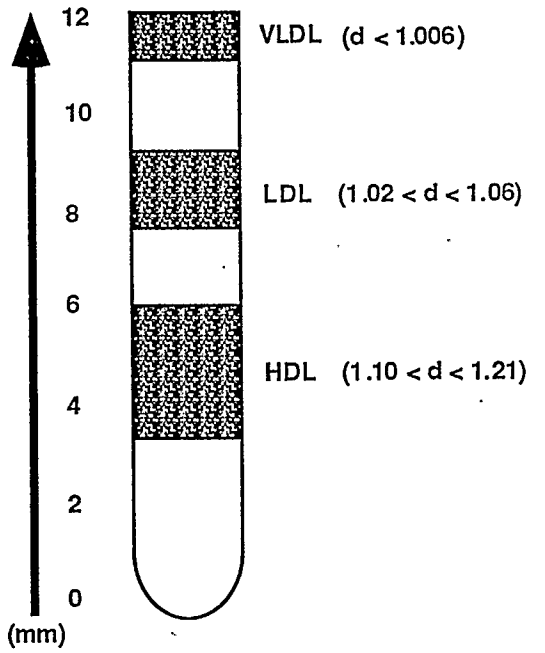


**Figure 2.11** Distribution of lipoprotein fractions (VLDL, LDL and HDL) in a sucrose/KBr density gradient following ultracentrifugation

Plasma samples stained with 10 µl of Coomassie Blue solution (0.015 g / ml distilled water) and 10 ul of Sudan Black solution (0.004 g / ml polyethylene glycol 400) were used as visual reference standards to assist in the identification process. A perfusion pump was used to transfer each lipoprotein band to a separate glass test tube for either the determination of sterol content (as described in Sections 2.5.1 to 2.5.5), or for subsequent purification and addition

to cell cultures prior to performing enzyme assays (as described in Section2.4.2).

## 2.4.2 LDL Preparation

LDL was isolated from control and patient plasma by density gradient ultracentrifugation, as described in Section 2.4.1. The LDL fraction was dialyzed against 150 mM sodium chloride and 0.5 mM EDTA, at pH 7.4. Dialysis took place in a 4 L volume of dialysis solution, which was circulated continuously, and which was maintained at 4° C. After 48 hr of dialysis and three changes of the dialysate, the LDL solution was transferred to microconcentrator tubes (Microsep 50K, Filtron Technology Corp.). The microconcentrator tubes were centrifuged at 3000 g in a fixed angle rotor, with periodic removal of the filtrate, until 10-12 mls of LDL solution had been concentrated to a total volume of approximately 500 µl. The concentrated LDL was then sterilized by passage through a 0.2 µ syringe filter. Protein and cholesterol content were measured before addition of the LDL to cell culture media as described in Sections 2.4.3 and 2.4.4 respectively.

## 2.4.3 Assay for Protein

LDL protein was assayed by the Coomassie brilliant blue G250 dye binding method of Bradford [218] using commercially produced Bradford reagent (Bio-Rad). Fatty acid free bovine serum albumin was used as a standard.

Briefly, 10 µl of concentrated LDL was added to 1590 µl of double distilled water, and 400 µl of freshly filtered Bradford reagent. After thorough vortex mixing, absorbance at 595 nm was measured against a reagent blank, using a spectrophotometer (Beckman Du-50). The sensitivity of the assay was determined by dilution of the BSA standard. The standard curve was linear to 80 µg of LDL protein and all measurements were confined to this range.

### 2.4.4 Assay for Cholesterol

Total cholesterol in serum samples and in LDL was measured using a commercially produced enzymatic-colourimetric assay kit (Sigma Diagnostics). This method is a modification of the method described previously by Allain *et al.* .[216] In principle, cholesterol esters are hydrolyzed by cholesterol ester hydrolase to free cholesterol and fatty acids. Free cholesterol in turn, is oxidized to choles-4-en-3-one and hydrogen peroxide by cholesterol oxidase. The hydrogen peroxide produced in this reaction is then coupled with the chromogen 4-aminoantipyrine and p-hydroxybenzenesulphonate in the presence of peroxidase, to yield a red coloured quinoneimine dye. This dye has an absorbance maximum of 500 nm, and the intensity of colour produced is directly proportional to the total cholesterol concentration. This method does not distinguish cholesterol from the plant sterols, and measurements of total cholesterol in serum or LDL include that proportion of the sterols attributable to ß-sitosterol, stigmasterol and campesterol. Stabilized aqueous solutions of bovine lipoprotein cholesterol (Sigma Diagnostics) were used as standards. Briefly, 10

µl of serum or LDL solution was added to 1.0 ml of the assay reagent. After thorough vortex mixing, and incubation at 37° C for 15 min, absorbance at 500 nm was measured against a reagent blank, using a spectrophotometer (Beckman Du-50). The standard curve was linear to 600 mg / dL, and all measurements were confined to this range.

## 2.5 LIPID ANALYSIS

### 2.5.1 Extraction of Total Lipids

Total lipids were extracted from cells and from lipoproteins by the method of Folch *et al.* .[216] An 8 : 4 : 3 (v/v) chloroform : methanol : water ratio was used in all extractions, and all solvents used in this and subsequent procedures, were either pesticide or HPLC grade (Fisher or BDH Chemicals). A total of 12 ml of this solvent mixture was used to extract lipids from pelleted cells. Lipoprotein lipids were extracted by the addition of 16 ml chloroform, 8ml methanol and 4 ml water to each ml of lipoprotein solution obtained following density gradient ultracentrifugation (as described in Section 2.4.1), thereby maintaining the requisite 8 : 4 : 3 solvent ratio. Acid-washed glassware was used in all extractions, and the extraction tubes were capped tightly. Following vigorous vortex mixing, the extraction mixture was placed in a 4° C fridge, and allowed to separate into two phases overnight. The lower lipid phase was collected using a double-pipette method. A narrow 9" glass pipette, inserted into a wider 7" glass pipette, was used to withdraw the chloroform layer to a separate glass tube. The

lower phase, containing the lipid fraction, was then dried down under nitrogen gas, and resuspended in 200 µl of chloroform : methanol (2 : 1, v/v). Extraction efficiency was evaluated by measuring the recovery of [$^3$H]-cholesterol and [$^3$H]-cholesterol ester (Amersham) in a separate experiment. This method of extraction recovered 97.3 ± 3.2% of the cholesterol, and 91.2 ± 3.8% of the cholesterol ester as shown in **Figure 2.13.**

## 2.5.2 Separation of Lipid Fractions

Neutral lipids in the lipid extract were separated using one-dimensional thin-layer chromatography (TLC). Lipid extracts, prepared as described previously (Section 2.5.1), were spotted onto pre-activated 20 X 20 cm, 250 µ silica gel G plates (Analtech). The plates were developed for 45 min in a sealed tank containing hexane : diethyl ether : acetic acid (80 : 30 : 1, v/v). Neutral lipid bands were visualized in iodine vapour, and identified by response factor ($R_f$). $R_f$ values were calculated using known lipid standards which were run in a separate lane of each G plate according to the following formula:

$$R_f = \frac{\text{distance moved by lipid class}}{\text{distance moved by solvent}}$$

A representative standard run is shown in **Figure 2.12.**

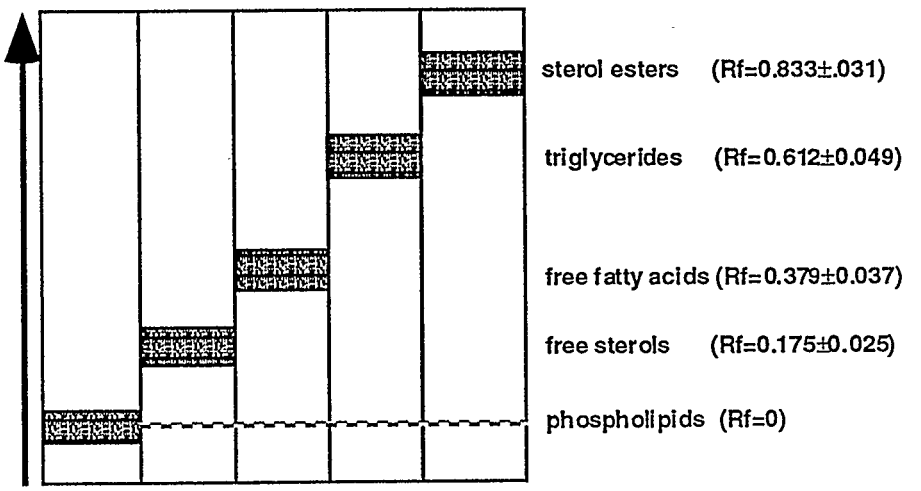


**Figure 2.12** Separation of lipid fractions by TLC on a silica gel G plate using a hexane : diethyl ether : acetic acid (80 : 30 : 2) solvent system

In a separate experiment using [$^{3}$H]-cholesterol and [$^{3}$H]-cholesterol ester (Amersham), this method of TLC was shown to recover 97.9 ± 0.8% of the cholesterol and 92.8 ± 1.4% of the cholesterol ester present after Folch extraction (**Figure 2.13**)

Radioactively labelled neutral lipids extracted for the assay of HMG-CoA reductase or ACAT activity (see Sections 2.3.1 and 2.3.2, respectively) were separated by TLC as described above, with the following modification. The Folch extract of the cultured lymphocytes was supplemented with 20 µl of a lipid mixture containing 1 mg / ml each of unlabelled cholesterol, cholesterol oleate, oleic acid, phosphotidyl choline, and tripalmitate, to act as cold carrier in the separation process.

## 2.5.3 Extraction of Lipids from Silica Gel

Sterols and sterol esters separated by TLC as described above, were extracted from the silica gel of each TLC plate for subsequent quantification. The sterol band and the sterol ester band from each lane were scraped into separate acid-washed glass extraction tubes. The silica gel was extracted 3X with 3 ml of pure chloroform. In a separate experiment using [$^3$H]-cholesterol and [$^3$H]-cholesterol ester (Amersham), the extraction efficiency of three different solvent mixtures was compared: chloroform : methanol (2 : 1, v/v), chloroform : methanol (4 : 1, v/v), and pure chloroform, respectively. The latter method of extraction was the most efficient, and resulted in the recovery of 92.8 ± 1.4% of the cholesterol and 88.4 ± 2.6% of the cholesterol ester from the silica gel (**Figure 2.13**). The chloroform extracts were pooled and dried down under nitrogen gas. Lipids extracted from the sterol band were resuspended in hexane for GLC analysis. Lipids extracted from the sterol ester band underwent saponification, as described in Section 2.5.3, before GLC analysis.

## 2.5.4 Saponification of Sterol Esters

The sterol ester fraction obtained following TLC, was saponified to remove the fatty acid component. Briefly, 1.0 ml of alcoholic KOH (94 ml ethanol + 6 ml 50% KOH) was added to the sterol ester extracts. After vortex mixing, the tubes were capped and heated in a dry heating block (Model 145 Isotemp Dry Bath, Fisher) at 50$^{o}$ C. Saponification efficiency was evaluated at four different time

points: 30 min, 60 min, 90 min, and 120 min. The 60 min time period gave the greatest recovery of free sterol (92.4 ± 6.8%). After 60 min of heating, the reaction was terminated by placing the glass tubes on ice. Free sterols were extracted with 3.0 ml chloroform + 1.5 ml 40% ethanol. After vigorous vortex mixing, the tubes were centrifuged to separate the upper and lower phases. The upper phase was aspirated, and the lower phase was extracted two more times with 1.5 ml of 40% ethanol. The chloroform layer was then dried down under nitrogen gas, and the sterols were resuspended in hexane for GLC analysis.

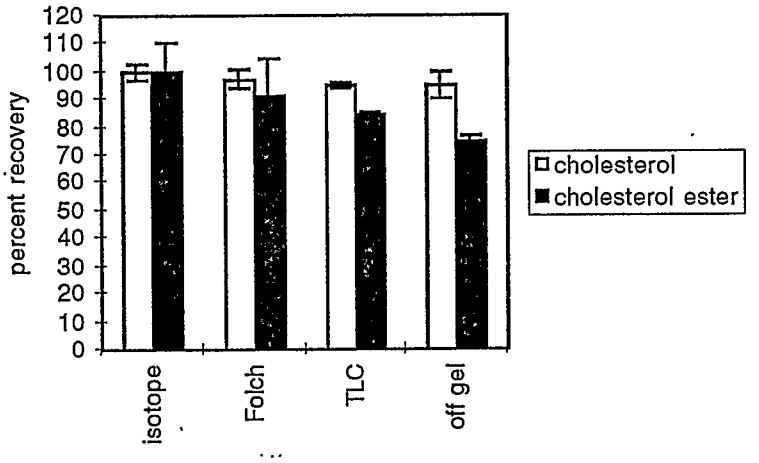


**Figure 2.13** Recovery of cholesterol and cholesterol ester at various steps during the separation procedure (n=3. mean ± SD)

## 2.5.5 Gas Liquid Chromatography Analyses

Plant sterols and cholesterol content in whole cell extracts and in lipoprotein particles, were quantitated using gas liquid chromatography (GLC). Cellular sterols were extracted and separated as described previously. Following the addition of 10 ug of 5∂-cholestane (Sigma Chemicals), as an internal

standard, each sample was dried down under nitrogen gas, redissolved in hexane, and injected onto the GLC column. A Varian 3600 model GLC (Varian Instruments) fitted with a SAC-5 capillary column (Sulpelco), was used to analyze the lipid samples. A representative sterol run is shown in **Figure 2.14.** Operating conditions for the GLC are outlined in **Table 2.1.**

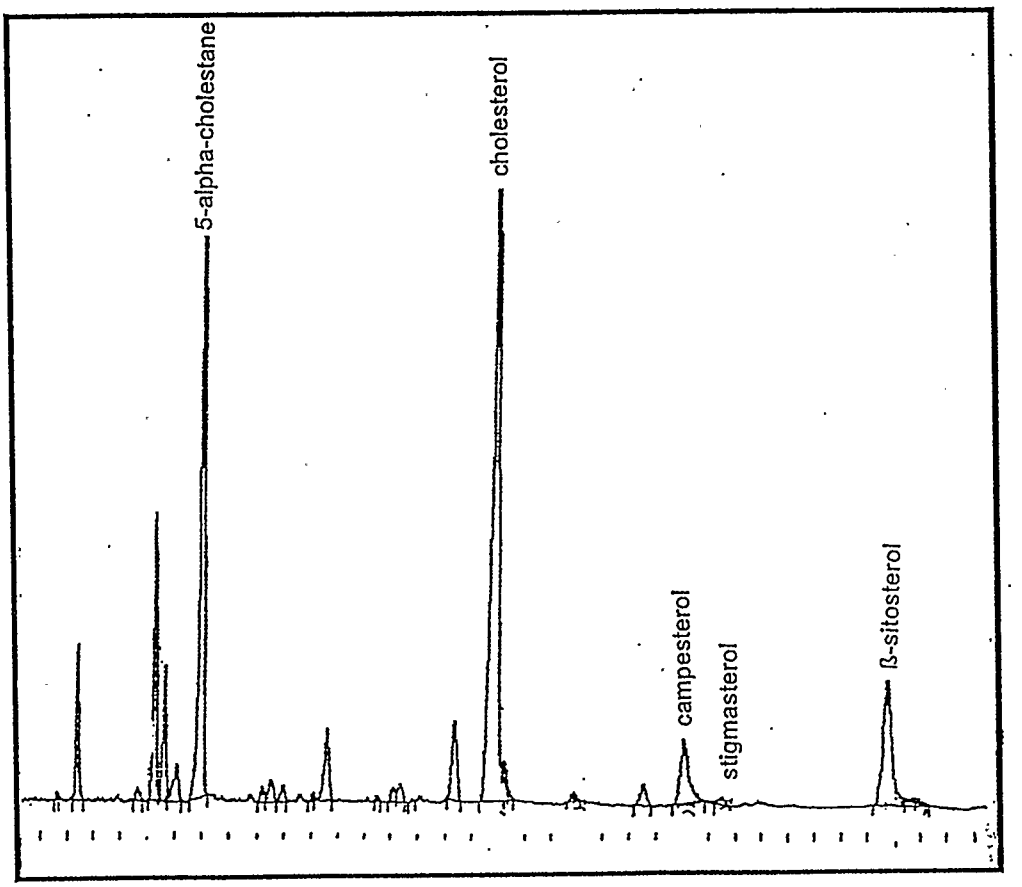


**Figure 2.14** Gas liquid chromatographic analysis of sitosterolemic serum (G.S.)

| | |
|---|---|
| **capillary column** | SAC-5 (Sulpelco) |
| **column dimensions** | 30 m X 0.25 mm ID<br>0.25 μm bonded film thickness |
| **column flow** | 1.25 ml / min |
| **carrier gas** | helium |
| **injector type** | isothermal , splitless |
| **injection port temperature** | 300° C |
| **sample volume injected** | 2.0 μl (Varian autosampler) |
| **detector type** | flame ionization |
| **detector temperature** | 320° C |
| **programmed column run** | |
| **initial column temperature** | 130° C<br>(hold time 0 min) |
| **step #1** | 10° C / min to reach 280° C<br>hold time 0 min |
| **step #2** | 7.5° C / min to reach 300° C<br>(hold time 7.0 min) |
| **total run time** | 22.6 min |

**Table 2.1** Operating conditions used for the gas chromatograph in the separation and quantification of sterols.

Sterols were identified by retention time. Retention times ($R_t$) for cholesterol, sitosterol, stigmasterol and campesterol were determined from purified standards (plant sterols, Sulpelco; cholesterol, Sigma Chemicals). A response factor ($R_f$) was calculated for each sterol, using 5∂-cholestane as the standard. Retention times and response factors are summarized in **Table 2.2.**

| sterol | mol. weight gm/mol | retention time mins (mean ± SD) | response factor mean ± SD |
|---|---|---|---|
| cholestane | 414.69 | 17.24 ± 0.06 | 1 |
| cholesterol | 412.67 | 19.34 ± 0.08 | 1.03 ± 0.01 |
| campesterol | 400.66 | 20.52 ± 0.10 | 1.08 ± 0.04 |
| stigmasterol | 412.67 | 20.85 ± 0.15 | 0.99 ± 0.02 |
| ß-sitosterol | 414.68 | 21.63 ± 0.14 | 1.05 ± 0.06 |

**Table 2.2** Retention times and response factors for the various sterols separated by gas chromatography (5∂-cholestane used as the internal standard)

Peak areas were computed by electronic integration (Varian Star Workstation, Varian Instruments), and sterols were quantitated using the following calculation:

$$\frac{A \times Cstd \times Rf}{Astd}$$

**Astd** = area of internal standard peak
**A** = area of sterol peak
**Cstd** = conc. of internal standard (ug)
**Rf** = response factor for sterol

Each value was expressed in ug, and represented as the mean of duplicate samples.

## 2.6 STATISTICAL ANALYSIS

The statistical significance of differences between mean values obtained for controls and patients was estimated using a one-tailed student's t-test (two sample for means assuming unequal variance). A two-sample paired student's t-test was performed to compare values obtained at different points in culture, and

to compare values obtained using different kinds of LDL. Linear regression analysis was performed by the least-squares method. All calculations were made in Microsoft Excel, version 5, and a $p < 0.05$ was considered to be statistically significant.

# CHAPTER 3
# RESULTS AND DISCUSSION

## 3.1 LIPID PROFILES

Lipoproteins isolated from the serum of the two sitosterolemic patients were used to quantify cholesterol and the plant sterols, ß-sitosterol, campesterol and stigmasterol. Free and esterified sterols in each of the lipoprotein fractions were quantitated separately in order to evaluate the extent to which plant sterols were esterified in sitosterolemic serum.

### 3.1.1 Lipoprotein Sterol Content

Patient serum lipid profiles were determined annually over the study period, both in the hospital using conventional diagnostic procedures and in the lab using the methods outlined in sections 2.4.1 and 2.5.1-2.5.5. Conventional clinical methodologies for serum sterol analysis, a colourimetric assay that detects a double bond between carbons 5 and 6 and an enzymatic method that detects the 3ß-hydroxy group, fail to differentiate the plant sterols from cholesterol. Accordingly, measurements of total serum cholesterol, HDL cholesterol, and LDL cholesterol determined by these methods reflect combined plant sterols and cholesterol. Using conventional diagnostic methods, the mean total serum sterol for the two patients was determined to be 3.40 ± 0.06 mmol/L. Mean HDL sterol was 1.36 ± 0.19 mmol/L, mean LDL sterol was 1.51 ± 0.09 mmol/L, and mean triglycerides were 1.61 ± 0.19 mmol/L (**Table 3.1**)

| | total sterol (mmol/L) | HDL sterol (mmol/L) | LDL sterol (mmol/L) | triglycerides (mmol/L) |
|---|---|---|---|---|
| patient #1 | 3.46 ± 0.34 | 1.17 ± 0.18 | 1.60 ± 0.22 | 1.61 ± 0.19 |
| patient #2 | 3.34 ± 0.30 | 1.55 ± 0.07 | 1.42 ± 0.40 | 0.97 ± 0.04 |
| mean | 3.40 ± 0.06 | 1.36 ± 0.19 | 1.51 ± 0.09 | 1.29 ± 0.32 |
| reference range | (3.20 - 6.20) | (0.90 - 2.49) | (1.60 - 4.10) | (0.50 - 2.30) |

**Table 3.1** Serum sterol analysis for the 2 sitosterolemic patients (using clinical methodologies; mean ± SD of triplicate values). Normal reference ranges are indicated in brackets.

Total serum sterol levels determined in the lab by gas liquid chromatography (GLC) yielded similar results. This method however, facilitated the quantitation of plant sterols in the various lipoprotein fractions. Using GLC to measure sterol levels, the mean total serum sterol level for the two patients was determined to be 3.71 ± 1.65 mmol/L. Mean HDL sterol was 0.97 ± 0.58 mmol/L, mean LDL sterol was 1.68 ± 0.79, and mean VLDL sterol was 1.07 ± 0.28 mmol/L. The plant sterols ß-sitosterol, campesterol, and stigmasterol were present in all three lipoprotein fractions, and the relative proportions of cholesterol and the various plant sterols were similar in the HDL, LDL and VLDL fractions (**Table 3.2**). Overall, cholesterol accounted for 2.72 ± 1.25 mmol/L (73.21%) of total serum sterol. ß-sitosterol was the most prevalent of the plant sterols, accounting for 0.63 ± 0.03 mmol/L (17.28%) of the serum sterols, while campesterol accounted for 0.27 ± 0.11 mmol/L (7.28%) and stigmasterol for 0.90 ± 0.02 mmol/L (2.51%).

| | cholesterol (mmol/L) | campesterol (mmol/L) | stigmasterol (mmol/L) | sitosterol (mmol/L) | total sterols (mmol/L) |
|---|---|---|---|---|---|
| LDL | 1.240 ± 0.644 (73.90) | 0.119 ± 0.047 (7.09) | 0.0342 ± 0.002 (2.04) | 0.286 ± 0.100 (17.04) | 1.678 ± 0.793 |
| HDL | 0.650 ± 0.366 (67.22) | 0.079 ± 0.055 (8.17) | 0.027 ± 0.013 (2.79) | 0.207 ± 0.144 (21.41) | 0.967 ± 0.578 |
| VLDL | 0.826 ± 0.242 (77.20) | 0.0724 ± 0.004 (6.77) | 0.0315 ± 0.004 (2.94) | 0.139 ± 0.025 (13.00) | 1.070 ± 0.276 |
| serum total | 2.716 ± 1.252 (73.21) | 0.270 ± 0.106 (7.28) | 0.093 ± 0.019 (2.51) | 0.632 ± 0.025 (17.04) | 3.710 ± 1.647 |

**Table 3.2** Quantities of plant sterols and cholesterol (mmol/L) in patient lipoprotein fractions (mean ± SD for 2 patients; duplicate or triplicate samples). Values indicated in brackets represent the quantity of each sterol as a percentage of total sterols.

## discussion

There was good agreement between mean values obtained by GLC and those determined by conventional diagnostic methods. Although the mean HDL sterol concentration estimated by GLC was somewhat lower, and the mean total serum sterol value somewhat higher, than hospital laboratory values, both concentrations were within the range of values obtained in the hospital laboratory over the study period (0.98 to 1.60 mmol/L for HDL, 3.04 to 3.80 for total serum sterol. The total serum, HDL, and LDL sterol concentrations for both sitosterolemic patients were well within the reference ranges for normolipidemic individuals of similar age. This observation suggests good patient compliance with respect to both dietary restrictions and cholestyramine therapy. Furthermore, it is consistent with previous reports which have indicated significant normalization of serum sterol levels in sitosterolemic patients treated with bile acid sequestering resins. [11, 15, 24] The serum sterol values obtained in this study are among the lowest recorded for sitosterolemic patients. Serum

sterol levels in adult sitosterolemic patients treated with either cholestyramine or cholestipol for a period of 2-3 months, have been shown to range from 5.08 to 6.35 mmol/L [11,24] Long-term efficacy in the treatment of sitosterolemia has not however, been addressed in the literature. Cholestyramine therapy in the two patients participating in the present study has exceded 5 years duration. As such, the low sterol levels noted in these two patients suggest that significant reductions in plasma sterol concentrations are not only sustainable, but may continue to decline with long term therapeutic compliance. These observations also suggest that regular monitoring of plasma sterol levels should be ongoing in sitosterolemic patients treated with bile acid sequestering resins.

While serum sterol levels have apparently been normalized by cholestyramine therapy, the high proportion of plant sterols in sitosterolemic serum persists. Plant sterols in these two patients accounted for approximately 26.8% of total serum sterols. While the relative amounts of the various plant sterols were consistent with previous reports, this value is somewhat higher than typically recorded in cases of sitosterolemia. Reported values for total plant sterol concentrations in sitosterolemic serum have ranged from 8.03 to 26.1%. [1, 4-10, 14-18, 22-26] The relatively high proportion of plant sterols noted in the present study may simply reflect individual differences or differences in GLC methodologies. Alternatively, this observation may be attributable to decreased hepatic turnover of plant sterols . Previous studies have shown significantly slower incorporation of plant sterols into bile acids in sitosterolemic patients than in controls. [1, 2, 6, 7, 54] This problem could conceivably be exacerbated by the long term use of bile acid sequestering resins, and might lead to proportionally higher levels of plant sterols despite overall reductions in serum sterol concentration.

**conclusions**

Plant sterols were found in similar proportions in all lipoprotein fractions analyzed. Patient lipid profiles revealed that plant sterols accounted for approximately 27% of total serum sterols, despite long-term cholestyramine therapy and complete normalization of serum sterol concentration. This observation indicated that the efficacy of cholestyramine treatment is high in the treatment of sitosterolemia, and that reductions in serum sterol concentration are sustainable. Cholestyramine therapy does not however, appear to reduce the overall proportion of plant sterols in the plasma of sitosterolemic patients.

## 3.1.2 Lipoprotein Sterol Esterification

The proportion of cholesterol and plant sterols which was esterified in each of the three lipoprotein fractions was also determined (**Table 3.3**).

| | cholesterol (%) | campesterol (%) | stigmasterol (%) | sitosterol (%) |
|---|---|---|---|---|
| LDL | | | | |
| free | 48.98 ± 0.22 | 58.46 ± 15.12 | 77.66 ± 2.34 | 36.55 ± 1.2 |
| esterified | 51.02 ± 0.22 | 41.54 ± 15.12 | 22.34 ± 2.34 | 63.45 ± 1.2 |
| HDL | | | | |
| free | 28.13 ± 2.3 | 36.33 ± 21.26 | 34.98 ± 8.17 | 21.02 ± 4.07 |
| esterified | 71.87 ± 2.3 | 63.67 ± 21.26 | 65.02 ± 8.17 | 78.98 ± 4.07 |
| VLDL | | | | |
| free | 29.51 ± 6.18 | 45.92 ± 10.65 | 59.28 ± 13.61 | 40.06 ± 17.25 |
| esterified | 70.49± 6.18 | 54.08 ± 10.85 | 40.72 ± 13.61 | 59.94 ± 17.25 |
| serum total | | | | |
| free | 42.23 ± 0.49 | 52.86 ± 17.11 | 60.05 ± 12.92 | 35.43 ± 7.50 |
| esterified | 57.76 ± 0.49 | 47.14 ± 17.11 | 39.95 ± 12.92 | 64.54 ± 7.58 |

**Table 3.3** Percent esterification of plant sterols and cholesterol in each lipoprotein fraction (mean ± SD of 2 patients; duplicate or triplicate samples)

Overall, cholesterol and ß-sitosterol were esterified to a similarly high extent (62.05 ± 0.49% and 64.54 ± 7.58% respectively), while both campesterol and stigmasterol were esterified to a lesser extent (52.86 ± 17.11% and 47.1 ± 12.92% respectively) (**Figure 3.1**).

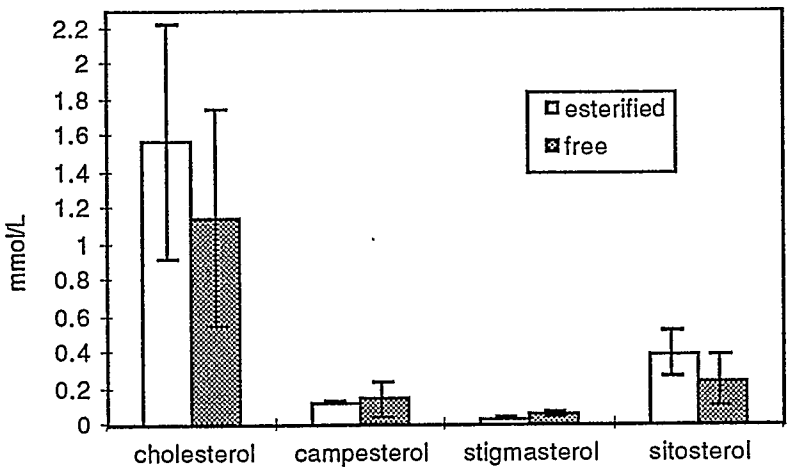


**Figure 3.1** Quantity of esterified and free sterol in sitosterolemic serum (mean ± SD of 2 patients; duplicate samples)

## discussion

The esterification levels noted for plant sterols in this study were similar to those reported previously for cases of sitosterolemia. Kuksis *et al.* demonstrated an esterification level of approximately 60% for most plant sterols, [22] while Nguyen *et al.* indicated that approximately 70% of the ß-sitosterol was esterified [79] in sitosterolemic plasma. Stigmasterol has been reported to have the lowest level of esterification of the plant sterols, [22] which is consistent with the observation that less than 50% of the stigmasterol was esterified in the serum of the two patients studied. Bhattacharya *et al.*[1] and Kuksis *et al.*[22] have

suggested that plant sterol esters in sitosterolemic plasma likely originate from both synthesis in plasma via lecithin-cholesterol acyltransferase (LCAT), and in tissues via ACAT. LCAT has been shown to be capable of esterifying ß-sitosterol *in vitro*, although at a significantly slower rate than cholesterol. [160, 161] ACAT is present in the liver, which has been shown to take up plant sterols and to return them to the plasma as components of lipoproteins. [159] ß-sitosterol appearing in the lymph of experimental animals and humans unaffected by sitosterolemia however, has been reported to be largely unesterified. [33, 35, 53] The high rate of plant sterol esterification noted in sitosterolemic patients may simply reflect the high levels of plant sterols present in the plasma of these individuals. Conversely, it may suggest indiscriminate esterification by either LCAT or ACAT.

**conclusions**

Overall, cholesterol and ß-sitosterol were esterified to a similar extent(approximately 60%) in the plasma of the sitosterolemic subjects. Campesterol and stigmasterol were esterified to a lesser degree (53% and 47%, respectively). This observation indicates that plant sterols can be esterified by LCAT and possibly by ACAT.

## 3.2 CELL CULTURE

Lymphocytes were initially isolated to determine whether or not sitosterolemic cells could be successfully cultured *in vitro*, and to determine if the

growth rate of these cells was the same as that of normal lymphocytes. IL-2 stimulation was used to facilitate rapid long-term *in vitro* growth, and plant sterol content was measured periodically to determine whether or not the plant sterols could be 'cultured out' of these cells.

### 3.2.1 Cell Growth

Sitosterolemic lymphocytes cultured in RPMI 1640 containing 10% FBS and 10 U/ml IL-2 demonstrated a growth rate which was not significantly different from that of control cells cultured under the same conditions (**Figure 3.2**). In all experiments, growth of sitosterolemic cells parallelled that of normal cells. Cell density doubled approximately every 2 days, and this rate of growth was sustainable with appropriate subculture, for up to 2 weeks. In the absence of subculture, growth typically declined after day 8 of culture.

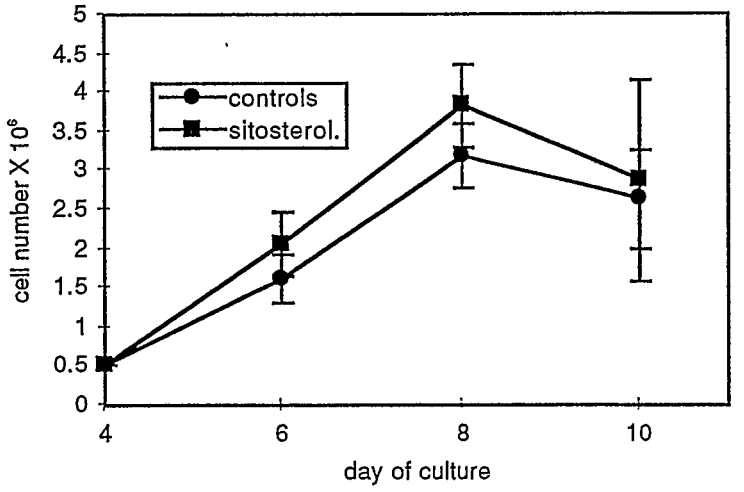


**Figure 3.2** Growth of control (n=4) and sitosterolemic (n=2) lymphocytes in RPMI 1640 + 10%FBS + 10U/ml IL-2 (no subculture). Values represent mean ± SD.

One attempt was also made to grow both sitosterolemic and control cells in lipoprotein deficient medium (IMDM supplemented with 0.5% BSA, 10.0 μg/ml monothioglycerol, 50 μg/ml human transferrin, and 10 U/ml IL-2) after an initial 4 days of culture in RPMI containing 10% FBS and PHA. After day 4, growth of both control and sitosterolemic cells was poor. There was however, no significant difference in growth rate between the two cell populations (**Figure 3.3**).

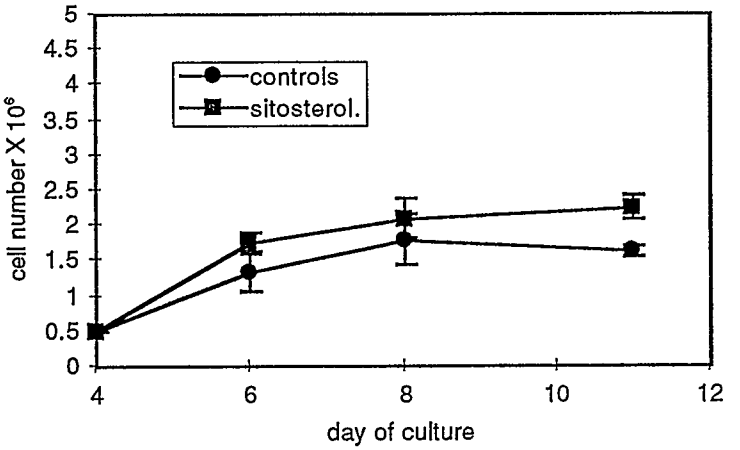


**Figure 3.3** Growth of control (n=4) and sitosterolemic (n=2) lymphocytes in lipoprotein deficient medium supplemented with 10U/ml IL-2 (no subculture)

## discussion

Long-term culture of sitosterolemic cells has not been previously described. Reduced cholesterol synthesis however, has been reported in short-term *In vitro* experiments using sitosterolemic lymphocytes, [72, 74] and cholesterol is widely known to be an absolute requirement for cell membrane biogenesis. Furthermore, previous work has also shown that the inhibition of cholesterol synthesis can diminish mitogen-induced lymphocyte responsiveness. [162] There was however, no significant difference in growth rate between sitosterolemic and

normal lymphocytes cultured under IL-2 stimulation for periods of up to 10 days.

While cholesterol is essential for cell growth, it may be obtained either by endogenous synthesis or by exogenous uptake. Sterol biosynthesis has been shown to be upregulated in cultured lymphocytes even when the cells are maintained in medium containing cholesterol, [163] and to precede DNA synthesis prior to cell division. [162,163] The suppression of endogenous sterol synthesis by oxgenated sterols in human lymphocytes stimulated by PHA and cultured in the presence of exogenous cholesterol however, does not prevent either cell growth or DNA synthesis. [163] Melzner *et al.* observed that as little as 50 μg/ml of exogenous cholesterol supplied as complete serum or as cholesterol / phosphatidylcholine liposomes, was sufficient to suppress cholesterol synthesis in human peripheral lymphocytes to approximately 20% that in cells cultured in lipoprotein deficient medium, while still permitting cell division. [164] Mevalonate however, must be synthesized for cells to enter the S phase of the cell cycle, prior to cell division. [97] While the requirement for mevalonate is reduced when cholesterol is supplied as LDL, it is not eliminated. [97] While the growth of sitosterolemic lymphocytes in medium containing serum would not necessarily be affected by reduced cholesterol synthetic activity, a modest level of reductase activity must necessarily be present in these sitosterolemic cells.

Although *de novo* cholesterol synthesis may not be required for lymphocyte growth in media containing exogenous cholesterol, cholesterol synthesis is essential for growth in media lacking cholesterol. [97] Sitosterolemic cells also demonstrated a growth rate similar to that of control cells, when they were cultured in lipoprotein deficient medium. This observation strongly suggests that sitosterolemic cells have some capacity to upregulate cholesterol

synthesis, and that these cells are not entirely dependent on exogenous cholesterol for normal growth. Moreover, these observations strongly suggest that the rate-limiting step in the cholesterol synthetic pathway is not defective.

**conclusions**

Sitosterolemic lymphocytes demonstrated a normal growth rate both in medium supplemented with FBS and in lipoprotein deficient medium, when stimulated with PHA and IL-2. This observation suggested that sitosterolemic lymphocytes have some capacity to upregulate cholesterol synthesis, despite the reduced HMG-CoA reductase activity that has been reported to characterize this lipid disorder.

## 3.2.2 Cell Sterol Levels

Sterols were extracted from freshly isolated peripheral blood mononuclear cells, and quantitated by GLC. Mean cholesterol content in control cells was 0.383 ± 0.165 $\mu g/10^6$ cells, and plant sterols were undetectable. Mean cholesterol content in sitosterolemic cells was 0.351 ± 0.051 $\mu g/10^6$ cells, and plant sterols (predominantly ß-sitosterol and campesterol) accounted for an additional 0.235 ± 0.070 $\mu g/10^6$ cells (**Table 3.4**).

| | cholesterol ($\mu g / 10^6$ cells) | plant sterols ($\mu g / 10^6$ cells) | total sterols ($\mu g / 10^6$ cells) |
|---|---|---|---|
| controls | 0.383 ± 0.165 | ND | 0.383 ± 0.165 |
| sitosterolemic | 0.351 ± 0.051 | 0.235 ± 0.070 | 0.602 ± 0.108 |

**Table 3.4** Sterol content of lymphocytes freshly isolated from controls (n=4) and sitosterolemics (n=2) (mean ± SD; single samples for controls, duplicate samples for sitosterolemics)

Plant sterols accounted for 39.88 ± 1.87 % of the sterols present before culture. By day 6 of culture, plant sterols accounted for 12.81 ± 7.99 % of total sterols, and by day 10 of culture this proportion had declined to 3.95 ± 3.95 % (**Table 3.5**).

| day of culture | % cholesterol | % plant sterols |
|---|---|---|
| DAY 0 | 60.12 ± 1.87 | 39.88 ± 1.87 |
| DAY 6 | 87.19 ± 7.99 | 12.81 ± 7.99 |
| DAY 10 | 96.05 ± 3.95 | 3.95 ± 3.95 |

**Table 3.5** Proportion of plant sterols in sitosterolemic lymphocytes before culture, and on day 6 and day 10 of culture (n=2; mean ± SD, duplicate samples except day 6 where one sample was available for patient #2).

## discussion

The levels of plant sterols measured in sitosterolemic peripheral mononuclear cells, were very high compared to levels typically reported for sitosterolemic tissues. Furthermore, plant sterol levels were higher in the mononuclear leukocytes (39.88%) than they were in the serum of the 2 patients

in the present study (26.79%).

Post-mortem studies of sitosterolemia have revealed that the proportion of plant sterols tends to be similar in all tissues, with the exception of brain, accounting for up to 25% of total sterols.[7, 21] The few studies which have examined plant sterol content in sitosterolemic blood cells however, have reported levels of plant sterols significantly higher than those in other tissues. Salen *et al.* noted that plant sterols accounted for approximately 20% of total sterols present in RBCs vs. approximately 15% in liver, plasma and lung tissue.[26] Jamal and Parsons similarly reported a greater proportion of plant sterols in sitosterolemic RBCs (26.85%) than in serum lipoproteins (18.20 - 21.87%) in a previous study of the same male patient who participated in the present study.[15] Nguyen *et al.* observed that mononuclear leukocytes obtained from 2 sitosterolemic patients contained 27.17% ß-sitosterol, while serum levels of ß-sitosterol in the same patients accounted for only 7.68 - 7.97% of total sterols.[79] He also noted that cholesterol content was not significantly different in control and sitosterolemic mononuclear cells (*ibid*), which is consistent with observations made in the present study.

Preliminary experiments revealed that very little (< 5%) of the sterol in patient lymphocytes was esterified. Nguyen *et al.* similarly reported that the percentage of sterols which were esterified in both control and sitosterolemic mononuclear leukocytes was very low (12%) when compared to that in other tissues (70 - 75%).[79] Ho *et al.* found that an even smaller proportion of the sterols in lymphocytes (<2%) was esterified.[166] Since lymphocytes do not function in sterol storage, the majority of sterols in these cells would likely be present as components of the plasma and ER membranes, and membrane

sterols are largely unesterified. As plant sterols are generally more difficult to esterify than cholesterol, [22] this structural characteristic might predispose plant sterols to accumulate in disproportionately high concentrations in cell membranes. This possibility would explain the high levels of plant sterols noted in sitosterolemic leukocytes.

The high concentrations of plant sterols in sitosterolemic lymphocytes decreased concomitantly with the duration of *In vitro* culture. After 10 days of rapid *In vitro* growth, plant sterol levels declined to 3.95% of total sterols. This observation confirmed the hypothesis that plant sterols could be 'cultured out' of sitosterolemic lymphocytes in a relatively short period of time, effectively permitting the study of these cells in the absence of abnormal sterols.

**conclusions**

Intracellular plant sterol concentrations (approximately 39%) were proportionally much higher than serum sterol levels of phytosterols (approximately 27%). While the GLC analyses of sitosterolemic cells revealed levels of cholesterol which were not significantly different from those in control mononuclear cells, plant sterols accounted for an additional 40% increase in sterol content in sitosterolemic cells. It was possible however, to reduce the intracellular plant sterol content by 98% after 10 days of *in vitro* culture. These observations suggested that plant sterols can accumulate in the membranes of sitosterolemic cells at very high concentrations. These results also indicated that it is possible to reduce intracellular plant sterol concentrations *in vitro* by culturing sitosterolemic cells under conditions stimulating rapid growth, thereby providing a system which facilitates the separation of effects due to plant sterols

from those due to inborn metabolic defects.

## 3.3 HMG-CoA REDUCTASE ACTIVITY

The rate of incorporation of [$^3$H]-sodium acetate into the cholesterol fraction of lymphocytes was used as a measure of HMG-CoA reductase activity in control and sitosterolemic cells. HMG-CoA reductase activity was measured in freshly isolated lymphocytes to determine if cholesterol synthesis was lower in sitosterolemic cells than in control cells. Cholesterol synthesis was also measured in lymphocytes cultured under IL-2 stimulation to determine what effect rapid growth, concurrent with the progressive depletion of plant sterols , had on the rate of cholesterol synthesis in sitosterolemic cells. HMG-CoA reductase activity was measured both in the presence and absence of exogenous cholesterol (supplied as LDL), to evaluate the effects of cholesterol loading and depletion, respectively. Furthermore, the effects of sterol loading, using sitosterolemic LDL vs. control LDL, was examined in both control and patient lymphocytes. Finally, the partitioning of [3H]-sodium acetate into the various lipid fractions under different culture conditions, was evaluated to compare partitioning in sitosterolemic cells with that in control cells.

### 3.3.1 HMG-CoA Reductase Activity in Freshly Isolated Lymphocytes

HMG-CoA reductase activity was initially measured in lymphocytes freshly

isolated from patient and control whole blood. Mean cholesterol synthesis in control cells was 1.59 ± 0.12 pmol/$10^6$ cells/hr, and mean cholesterol synthesis in sitosterolemic cells was 1.55 ± 0.14 pmol/$10^6$ cells/hr. The cholesterol synthetic rates in freshly isolated sitosterolemic lymphocytes were not significantly different from those in control cells.

Cells were then incubated for 24 hours in lipoprotein deficient medium (RPMI 1640 + 10% LPDS), and cholesterol synthesis was measured over the last 6 hours of the incubation period (Day 1, no LDL). Mean cholesterol synthesis in control cells incubated in lipoprotein deficient medium was 10.98 ± 0.79 pmol/$10^6$ cells/hr. Mean cholesterol synthesis in sitosterolemic cells was 3.94 ± 0.40 pmol/$10^6$ cells/hr, and was significantly lower ($p<0.001$) than that in control cells (**Figure 3.4**).

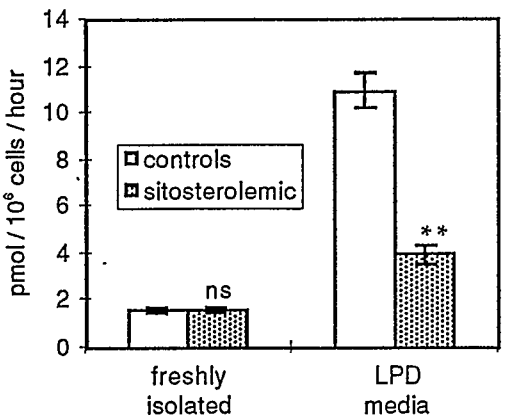


**Figure 3.4** [$^3$H]-acetate incorporation into the cholesterol fraction of freshly isolated control (n=10) and sitosterolemic (n=2) lymphocytes, and in lymphocytes incubated for 24 hours in lipoprotein deficient media (LPD media). Values represent mean ± SEM (** $p<0.001$).

## discussion

Reduced cholesterol synthesis has been reported previously in cases of

sitosterolemia. [66, 72, 74, 79] and Nguyen *et al.* have speculated that a deficiency in HMG-CoA reuctase may be the primary cause of this lipid disorder. [74, 178] In the present study however, cholesterol synthesis in freshly isolated sitosterolemic lymphocytes was not significantly different from that in control lymphocytes. Ho *et al.* noted that acetate incorporation into cholesterol was very low in freshly isolated lymphocytes. [166] As such, the apparent inability to detect any difference between freshly isolated control and sitosterolemic cells may be due to a lack of sensitivity in the HMG-CoA reductase assay. However, the concentration of acetate used in the assay was shown to be saturating, and isotope incorporation was shown to be linear over the incubation period (section 2.3.1), in this and other studies. [165, 166] Furthermore, the values obtained in the present study were within the range of values observed in other studies of cholesterol synthesis in leukocytes. [66, 72, 74, 165] Collectively, these observations suggest that assay sensitivity was not likely problematic.

Alternatively, the reduced cholesterol synthetic rates observed in previous studies of sitosterolemic cells may be attributable to methodological differences. Previous measurements of cholesterol synthesis in sitosterolemic leukocytes were obtained by incubating freshly isolated peripheral blood mononuclear cells in RPMI 1640 containing 50% autologous serum. While the use of monocytes (19%) in combination with lymphocytes (81%) would not likely affect any comparison between control and sitosterolemic cholesterol synthetic rates, [166] the use of autologous serum might. Studies of cholesterol synthesis in sitosterolemic cells incubated in autologous serum, cannot exclude differences due to the presence of plant sterols. Previously observed differences between control and sitosterolemic basal cholesterol synthetic rates in whole cell assays were

relatively small (4.2 - 5.2 vs. 1.5 - 3.5 pmol/$10^6$ cells/hr, respectively). These differences might be accounted for by the application of a methodology which does not control for either the quantity or type of sterol in the incubation medium.

Patient serum in previous studies not only contained high levels of plant sterols, but was also significantly enriched in cholesterol, relative to control serum. [72, 79] Sterol synthesis in freshly isolated lymphocytes has been shown to be directly subject to feedback inhibition mediated by exogenous cholesterol supplied as LDL. [166] Concentrations of LDL cholesterol as low as 2 μg/ml have been shown to decrease reductase activity by nearly 40%. [176] Furthermore, high circulating levels of LDL have been demonstrated to inhibit cholesterol synthesis in hypercholesterolemic subjects, [189] and sterol balance experiments have shown that chronic administration of a high cholesterol diet is frequently associated with suppression of whole body cholesterol synthesis. [192, 193, 194] The higher *in vitro* or *in vivo* levels of circulating sterols in untreated sitosterolemic patients might therefore, be expected to lead to reduced cholesterol synthetic rates in freshly isolated tissues, even in the absence of sterols in the incubation medium.

The normal basal cholesterol synthetic rates observed in sitosterolemic lymphocytes in the present study, could also be attributable to the effects of cholestyramine therapy. HMG-CoA reductase activity has not been previously measured in sitosterolemic patients following prolonged cholestyramine therapy (>5 yrs), and the subsequent complete normalization of serum lipid levels. Significant reductions in serum sterol levels, particularly of the magnitude seen in these 2 patients, might be expected to have some effect on basal levels of cholesterol synthesis. Previous studies of moderately hypercholesterolemic

subjects have demonstrated that decreases in serum LDL cholesterol as a result of reduced cholesterol absorption are compensated for by modest increases in cholesterol synthesis. [177] Cholestyramine however, has been shown to induce marked increases in the *in vitro* cholesterol synthetic rates of normal individuals. As such, basal levels of cholesterol synthesis may be elevated to normal levels in the 2 sitosterolemic patients in the present study, as a result of long-term bile acid sequestration. The administration of bile acid sequestering resins however, typically results in a 2-5 fold upregulation of cholesterol biosynthesis. [74, 72] This magnitude of upregulation was conspicuously absent in these 2 patients. This observation corroborates previous reports of defective cholesterol biosynthesis in cases of sitosterolemia, and confirms the abnormal response of sitosterolemics to hyperlipidemic drug therapy. [72]

Abnormal regulation of cholesterol synthesis was also suggested by the observation that sitosterolemic lymphocytes demonstrated a significantly lower rate of cholesterol synthesis than control cells, when they were preincubated in lipoprotein deficient medium ($p<0.001$). Ho *et al.* noted that normal lymphocytes incubated in lipoprotein deficient medium for 56 hours, increased acetate incorporation into cholesterol by 7 fold. [166] This is consistent with the 7 fold increase in cholesterol synthesis demonstrated by control cells in the present study. Sitosterolemic cells however, demonstrated an increase of less than 3 fold following incubation in lipoprotein deficient medium. This observation suggests that sitosterolemic cells not only respond abnormally to bile acid sequestering resins, but may also respond abnormally to other stimuli which induce the upregulation of HMG-CoA reductase activity.

Although sitosterolemic lymphocytes failed to increase HMG-CoA

reductase activity to control levels in the absence of exogenous cholesterol, these cells clearly demonstrated some capacity to upregulate cholesterol synthesis. This is a particularly significant observation, as there have been no previous reports of increased cholesterol synthesis in sitosterolemic cells. This observation suggests that the upregulation of cholesterol synthesis is not precluded in this lipid disorder, but is instead limited. The extent and cause of this limitation remains to be evaluated, and may facilitate elucidation of the primary defect in sitosterolemia.

**conclusions**

HMG-CoA reductase activity in freshly isolated sitosterolemic lymphocytes, isolated from patients undergoing long-term cholestryramine therapy, was not significantly different from that in control cells when measured using a assay medium which did not contain lipoproteins. However, reductase activity was approximately 60% lower in sitosterolemic cells than in control cells, following a 24 hour incubation period in lipoprotein deficient medium. Collectively, these observations suggests that the upregulation of cholesterol synthesis is defective in sitosterolemic lymphocytes.

### 3.3.2 HMG-CoA Reductase Activity in Cultured Cells

To further delineate the extent to which the upregulation of cholesterol synthesis was limited, sitosterolemic cells were subsequently cultured under conditions stimulating rapid growth. Sitosterolemic and control lymphocytes were

initially cultured in RPMI 1640 + PHA + 10% FBS to stimulate mitogenesis and to upregulate IL-2 receptor activity. During this time period, both sitosterolemic and control cells formed large cohesive aggregates visible to the naked eye. After 4 days of PHA stimulation, the cells were transferred to RPMI 1640 + 10% FBS + 10 U/ml IL-2. Cell aggregations gradually dissociated, and rapid growth was sustained until day 10 or 11 of culture.

HMG-CoA reductase activity was first assayed on day 6, following a 24 hour incubation period in RPMI 1640 + 10% LPDS. Mean cholesterol synthesis in control lymphocytes on day 6 of culture was 39.2 ± 4.39 pmol/$10^6$ cells/hour. Mean cholesterol synthesis in sitosterolemic lymphocytes was 34.91 ± 3.51 pmol/$10^6$ cells /hour, and was not significantly different from that of control cells.

Cholesterol synthesis was measured again on day 10 of culture, after 24 hours of incubation in lipoprotein deficient medium. On day 10, mean cholesterol synthesis in control lymphocytes was 50.49 ± 9.04 pmol/$10^6$ cells/hour, vs. 47.56 ± 4.41 pmol/$10^6$ cells/hour in sitosterolemic lymphocytes. This difference was not statistically significant. Furthermore, day 10 cholesterol synthetic rates were not statistically different from day 6 cholesterol synthetic rates, in either control or sitosterolemic cells. **(Table 3.6, Figure 3.5)**

| | HMG-CoA reductase activity (pmol acetate/$10^6$ cells/hr) | | | |
|---|---|---|---|---|
| | day 0 (freshly isolated) | day 1 (no LDL) | day 6 (no LDL) | day 10 (no LDL) |
| sitosterolemic | 1.55 ± 0.14 | 3.94 ± 0.40** | 34.91 ± 3.51 | 47.56 ± 4.41 |
| controls | 1.59 ± 0.12 | 10.98 ± 0.12 | 39.21 ± 4.39 | 50.49 ± 9.04 |

**Table 3.6** [$^3$H]-acetate incorporation into the cholesterol fraction of sitosterolemic (n=2) and control (n=10) lymphocytes before and during culture. Values represent mean ± SEM( ** $p<0.001$) Patient values were based on the mean of 3 separate experiments.

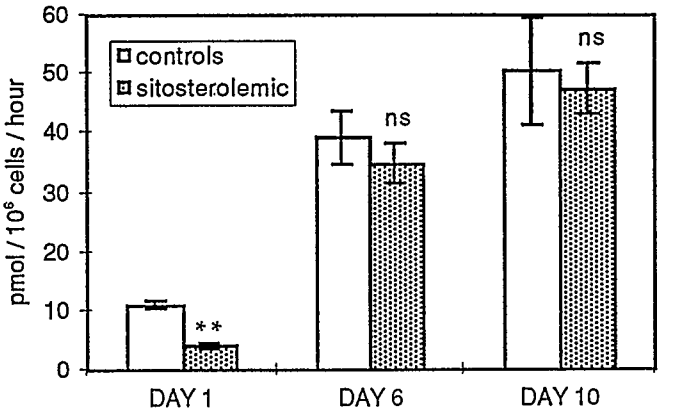


**Figure 3.5** [$^3$H]-acetate incorporation into the cholesterol fraction of control and sitosterolemic lymphocytes before and during culture Values represent mean ±SEM (** $p<0.001$). Patient values were based on the mean of 3 separate experiments.

## discussion

One of the earliest changes in lymphocyte metabolism following stimulation with mitogens is an increase in the rate of endogenous cholesterol biosynthesis. [179, 180, 181] The previous observation that cultured sitosterolemic lymphocytes demonstrated a growth rate which was not significantly different from that of control cells, strongly suggested that cholesterol synthesis was upregulated in these cells. This hypothesis was confirmed by the measurement of HMG-CoA reductase activity during IL-2 stimulated culture. Cultured sitosterolemic lymphocytes demonstrated full upregulation of cholesterol synthesis to control levels on both day 6 and day 10 of culture. This upregulation was approximately 10 fold above values obtained for freshly isolated sitosterolemic lymphocytes incubated for 24 hours in lipoprotein deficient medium, and approximately 30 fold above basal levels in sitosterolemic cells. These observations clearly indicated that sitosterolemic lymphocytes do not rely exclusively on exogenous cholesterol for growth. They also indicated that

sitosterolemic cells have the capacity to upregulate cholesterol synthesis to normal levels.

The upregulation of endogenous cholesterol synthesis occurs through a combination of transcriptional and post-transcriptional mechanisms. Upregulation in response to sterol depletion is thought to be regulated primarily through enhanced transcription, [97] and has been traced to the SRE in the promoter region of the reductase gene. [69, 105, 106, 113] The upregulation of HMG-CoA reductase activity in response to mitogenic stimulation is thought to occur primarily through post-transcriptional mechanisms, [97] including enhanced translation of the reductase gene, and reduced degradation of both reductase mRNA and protein. [123, 124, 126, 127] Post-transcriptional regulation of cholesterol synthesis has been shown to involve a non-sterol product of the mevalonate pathway, in addition to cholesterol . [97] The observation that rapidly growing sitosterolemic lymphocytes pre-incubated in lipoprotein deficient medium demonstrated cholesterol synthetic rates which were not significantly different from those of control cells, strongly suggests normal upregulation of HMG-CoA reductase activity by both transcriptional and post-transcriptional mechanisms in these cells.

The apparently normal response of cultured sitosterolemic cells to both sterol depletion and mitogenic stimulation is at odds with the reduced cholesterol synthesis reported to characterize this lipid disorder. Nguyen *et al.* demonstrated deficiencies in both reductase mRNA and reductase protein in the hepatic tissue of 2 sitosterolemic subjects, [74] and attributed reduced cholesterol synthetic rates in this disorder to an inherited defect at the level of HMG-CoA reductase. [74, 183] The sub-normal response of freshly isolated sitosterolemic lymphocytes to

exogenous cholesterol depletion, which was noted in the present study, would appear to be consistent with this hypothesis. The latter observations however, were all based on measurements of cholesterol synthesis in freshly isolated tissues. As such, the contradictory response observed in freshly isolated cells may be attributable to *in vivo* limitations which are absent *in vitro.*

One possible explanation for the normalization of cholesterol synthesis *in vitro* is the progressive reduction in cellular plant sterol levels which is concurrent with long-term culture. A gas chromatographic analysis of sitosterolemic lymphocytes revealed that the plant sterol content in freshly isolated cells was reduced by approximately 77% after 6 days of culture, and by 96% after 10 days. In this regard, it is interesting to note that cholesterol synthetic rates in sitosterolemic cells were still somewhat less than those in control cells on day 6 of culture. Although this difference was not statistically significant, it was much less evident on day 10 of culture. The high concentrations of plant sterols in sitosterolemic cell membranes could exert regulatory effects on HMG-CoA reductase activity at both transcriptional and post-transcriptional levels. Recently, Wang *et al.* described a novel mechanism by which membrane lipids might directly regulate transcription. [184] The transcription factor SREBP-1, has been shown to be cleaved in sterol depleted cells generating a fragment that translocates to the nucleus where it interacts with the SRE to activate the transcription of sterol regulated genes. Membrane sterols have been demonstrated to inhibit the cleavage of this protein, thereby reducing HMG- CoA reductase, LDL receptor, and HMG-CoA synthase mRNA levels. [184, 185] Post-transcriptionally, the degradation of reductase protein in has been shown to be accelerated by sterols, [97, 182] and this degradation is exclusively mediated by

those domains of the enzyme which traverse the ER membrane. [124, 125] Moreover, the modulation of reductase activity by membrane microviscosity has been postulated. The enrichment of membrane sterol content has been shown to decrease membrane microviscosity , and decreases in membrane microviscosity have been correlated with reduced reductase activity. [141, 142] Clearly, membrane sterols have the capacity to regulate HMG-CoA reductase activity at a number of different levels. The hydrophobicity and lower esterification rate of plant sterols would tend to favor the deposition of these sterols in cell membranes. As such, it is not unlikely that the high levels of plant sterols in sitosterolemic lymphocyte membranes might exert regulatory effects similar to cholesterol, and that culture-induced reductions in plant sterol levels might release this sterol mediated suppression of reductase activity.

**conclusions**

Cholesterol synthesis on day 6 and day 10 in cultured sitosterolemic lymphocytes was not significantly different from that in control cells, suggesting that the primary defect in this disorder may not be reduced cholesterol synthesis. Rather, reduced cholesterol synthesis may be secondary to the abnormally high concentrations of plant sterols in sitosterolemic cells.

### 3.3.3 Effect of LDL on HMG-CoA Reductase Activity

Cholesterol synthesis was also measured on day 10 of culture, after a 24 hour incubation period in RPMI 1640 + 50 μg/ml LDL (Sigma) to evaluate the

response of sitosterolemic lymphocytes to LDL cholesterol. Following incubation in LDL, cholesterol synthesis decreased significantly ($p<0.001$) in both control and sitosterolemic cells, measuring $21.79 \pm 3.15$ and $14.49 \pm 4.79$ pmol/$10^6$ cells /hour, respectively. This represents a decrease of approximately 56% in control cells vs. approximately 70% in sitosterolemic cells. Although the downregulation of cholesterol synthesis following sterol loading was more pronounced in the sitosterolemic cells, this difference was not statistically significant ($p<0.07$) **(Figure 3.6)**

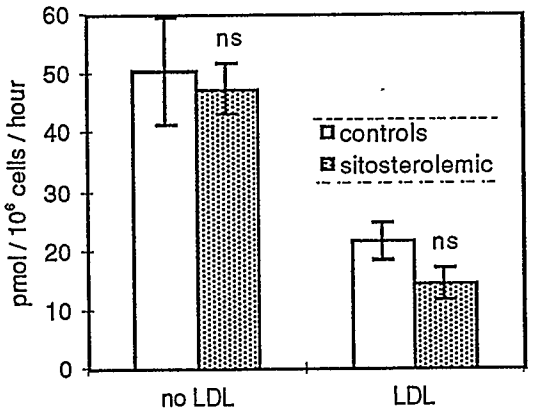


**Figure 3.6** [$^3$H]-acetate incorporation into the cholesterol fraction of control (n=10) and sitosterolemic (n=2) lymphocytes on day 10 of culture, following a 24 hr incubation in lipoprotein deficient medium (no LDL) or in medium containing 50 µg/ml of commercially prepared (Sigma) LDL. Values represent mean ± SEM; patient values were based on the mean of 3 separate experiments.

Cholesterol synthesis was also measured on day 10 of culture, after a 24 hour incubation period in RPMI 1640 + 50 ug/ml control LDL (containing no detectable plant sterols), and in RPMI 1640 + 50 µg/ml sitosterolemic LDL to evaluate the short-term regulatory effects of sitosterolemic LDL (containing approximately 26% plant sterols). There was no significant difference between cholesterol synthetic rates in either control or sitosterolemic cells incubated in

control LDL vs. cells incubated in sitosterolemic LDL. Overall however, sitosterolemic cells evidenced significantly lower cholesterol synthetic rates than control cells, when incubated in LDL ($p<0.02$). The mean reduction in HMG-CoA reductase activity in control cells was 52.3%, whereas enzyme activity declined by 76.5% in the sitosterolemic lymphocytes during the same incubation period (**Table 3.7, Figure 3.7**).

| | HMG-CoA red. (pmol acetate/$10^6$ cells/hr) | | |
|---|---|---|---|
| | no LDL | control LDL | sitosterol. LDL |
| controls | 68.79 ± 18.99 | 34.97 ± 8.21 | 30.55 ± 4.57 |
| sitosterolemic | 77.13 ± 18.82 | 18.38 ± 8.66 | 17.80 ± 5.60 |

**Table 3.7** [$^3$H]-acetate incorporation into the cholesterol fraction of control (n=4) and sitosterolemic (n=2) cultured lymphocytes (day 10) incubated for 24 hours in lipoprotein deficient medium (no LDL), or in medium containing 50 µg/ml of control LDL or sitosterolemic LDL. Values represent mean ± SEM; experiment was repeated twice for each control (with LDL from patient #1 and with LDL from patient #2), and twice for each sitosterolemic patient.

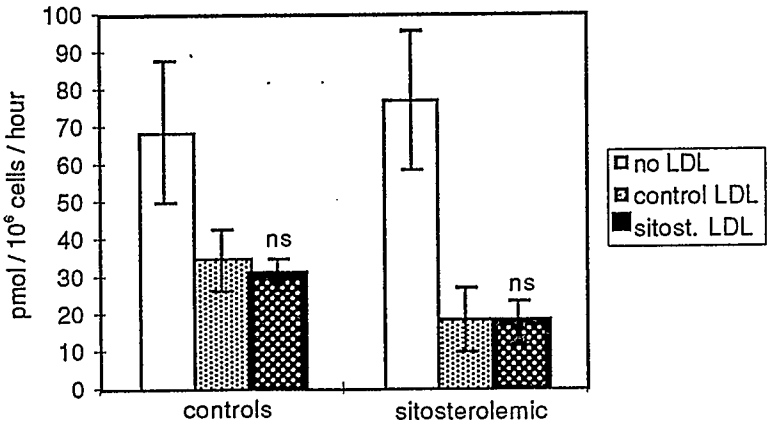


**Figure 3.7** [$^3$H]-acetate incorporation into the cholesterol fraction of control (n=4) and sitosterolemic (n=2) cultured lymphocytes (day 10) incubated for 24 hours in lipoprotein deficient medium (no LDL), or in medium containing 50 µg/ml of control LDL or sitosterolemic LDL. Values represent mean ± SEM; experiment was repeated twice for each control (with LDL from patient #1 and with LDL from patient #2), and twice for each sitosterolemic patient.

**discussion**

Sitosterolemic LDL had no apparent short-term regulatory effects on HMG-CoA reductase activity in either control or sitosterolemic cells. The observation that patient LDL exerted effects very similar to control LDL, suggested that this LDL was assimilated normally by both patient and control lymphocytes. This observation also suggested that the quantities of plant sterol potentially introduced over a 24 hour incubation period did not alter cholesterol synthesis to any significant degree. While these results do not preclude the possibility that plant sterols adversely affect cholesterol synthesis, they do demonstrate that lymphocytes do not respond to small quantities of plant sterols any differently than they do to cholesterol.

If plant sterols exert their effects by accumulating in cell membranes, a 24 hour incubation period in LDL containing 26% plant sterols may be insufficient to demonstrate any effect. Cholesterol synthetic rates in sitosterolemic cells on day 6 of culture were not significantly different from rates in normal cells, despite relatively high cellular levels of plant sterols. Regulation of reductase activity by membrane sterols presumably operates at some threshold level. [195] Wang *et al.* noted that the logical place for a cell to sense cholesterol or related sterol content is the site of cholesterol biogenesis, the ER membrane. Wang *et al.* and Gasic noted that cholesterol and related sterols occur in the plasma membranes of eukaryotic cells in concentrations equimolar to that of all the other membrane lipids combined, whereas the ER is relatively poor in cholesterol. [195, 184] As such, large changes in plasma membrane sterol content likely reflect much smaller changes in ER membrane sterol content. Gasic speculated that increases in the sterol content of the ER membrane may cause the bilayer to expand thereby

shielding a proteolytic site on SREBP-1 and preventing cleavage of the SRE transcription factor. Similarly Chun *et al.* noted that degradation of HMG-CoA reductase protein in the ER membrane was accelerated by sterols, and speculated that changes in ER sterol content might increase susceptibility to proteolysis. [126] Both mechanisms of regulating HMG-CoA reductase activity presume that some threshold level of membrane sterols must be exceeded before reductase activity is reduced. As such, small quantities of plant sterols would not be expected to exert short-term regulatory effects which were any different from those of cholesterol.

Curiously, sitosterolemic lymphocytes demonstrated significantly lower rates of cholesterol synthesis than controls, following incubation in control and sitosterolemic LDL. One possible explanation for this observation is that sitosterolemic cells may have a lower threshold for exogenously supplied sterols, possibly due to the presence of increased levels of membrane sterols. This hypothesis is consistent with the observation that subnormal cholesterol synthetic rates have invariably been observed in sitosterolemic cells in assays where the incubation medium has contained serum. [66, 72, 74, 79] The failure to detect a significant difference between control and sitosterolemic cholesterol synthetic rates when commercially prepared LDL was used in the incubation medium might similarly be attributed to differences in threshold levels of cellular sterols. The sterol content of the commercially prepared LDL was higher than that of both the control and sitosterolemic LDL prepared in the laboratory. The ratio of sterol to protein (as determined by colourimetric assay) was 3.10 for the control LDL, 2.65 for the LDL prepared from patient #1, and 3.0 for the LDL prepared from patient #2. The control used in this study was selected on the basis of lower than

normal serum sterol levels, similar to those of the 2 sitosterolemic patients. Sigma-LDL was prepared commercially from pooled serum, and evidenced a higher cholesterol-protein ratio (3.6-3.8) which is more reflective of the general population. Over a 24 hour incubation period, the sterol threshold of the controls might be expected to more closely approach that of the sitosterolemic patients following incubation in LDL which was high in cholesterol content. It is tempting to suggest that sitosterolemic cells have a lower threshold level for exogenous sterols due to the presence of plant sterols, although the observation that phytosterols accounted for less than 4% of total cellular sterols on day 10 of culture suggests this is not a likely explanation.

Alternatively, the reduced cholesterol synthetic rates demonstrated by sitosterolemic cells incubated in LDL may be attributable to enhanced LDL receptor activity. Nguyen *et al.* revealed that sitosterolemic mononuclear cells expressed high numbers of LDL receptors than normal cells, [27] and that sitosterolemic monocytes took up and degraded LDL more actively than control monocytes. [187] He speculated that enhanced receptor uptake of sterols was a potential means of compensating for reduced cholesterol synthesis. On day 10 of culture however, cholesterol synthesis in sitosterolemic cells was not significantly different from that in control cells. This observation contradicts Nguyen's hypothesis, but raises the possibility LDL receptor mediated uptake may be abnormally high in sitosterolemia, irrespective of cholesterol synthetic rates.

Another possible explanation for the apparent hyper-responsiveness of sitosterolemic cells to LDL sterol may be increased uptake of LDL sterol by a receptor independent pathway. Goldstein and Brown proposed that the LDL

receptor independent catabolism of LDL was mediated *in vivo* by uptake and degradation via a pathway involving pinocytosis. [196] They further noted that degradation of LDL by this route may be unregulated in the sense that it would continue irrespective of cholesterol content in the cell, and could result in the massive accumulation of cholesterol which characterizes certain dyslipidemias. Spady *et al.* in a study of receptor-independent LDL catabolism in normal rats, concluded that this catabolic pathway functions in the parenchymal cells of most tissues, including the liver and the small intestine. Sterols from LDL particles may also enter cells by a sterol exchange mechanism operating at the level of special plasma membrane domains. [198] Although numerous researchers have suggested that cholesterol entering cells by this pathway does not enter the regulatory pool, cholesterol exchange has been shown to be capable of inhibiting HMG-CoA reductase activity. Uptake of chylomicron remnants by the liver has been shown to lead to rapid suppression of HMG-CoA reductase activity. [199] Field *et al.* demonstrated a dose-dependent decrease in HMG-CoA reductase activity in CaCo-2 intestinal cells incubated in taurocholate micells containing cholesterol. [190] In view of these observations, increased unregulated uptake of sterols by sitosterolemic cells could explain both the hyper-responsiveness of freshly isolated and cultured sitosterolemic lymphocytes to serum LDL, and the abnormal absorption of plant sterols by intestinal cells.

**conclusions**

Cultured sitosterolemic lymphocytes demonstrated normal downregulation of cholesterol synthesis, by approximately 70%, in response to exogenous LDL. Sitosterolemic LDL did not affect this process any differently than control LDL,

suggesting that short-term incubation in small quantities of plant sterols has no apparent effect on HMG-CoA reductase activity. Sitosterolemic cells however, demonstrated conspicuously lower reductase activity than control cells when lymphocytes were preincubated in LDL. This observation indicates that reduced cholestrol synthesis in these cells may be related to an abnormal response to exogenous sterol.

### 3.3.4 Partitioning of [$^3$H]-sodium acetate into Lipid Fractions

The partitioning of isotope into the various lipid fractions was determined to compare the distribution of isotope in sitosterolemic lymphocytes with that in control lymphocytes, under different culture conditions. Five different lipid fractions were separated by TLC: phospholipids, free fatty acids, cholesterol, triglycerides, and cholesterol esters. Isotope incorporation into each lipid fraction was analyzed, and data was expressed as a percent of total incorporation into all fractions. Isotope incorporation into lipid fractions other than cholesterol is not however, a valid measure of lipid synthesis, and changes in percent incorporation may not reflect absolute changes. [221]

In freshly isolated control and sitosterolemic cells [$^3$H]-sodium acetate was incorporated primarily into the cholesterol, phospholipid and triglyceride fractions, and this pattern of incorporation in sitosterolemic lymphocytes was not significantly different from that in control cells . In freshly isolated cells, the greatest proportion of isotope was incorporated into the phospholipid and triglyceride fractions. When freshly isolated cells were incubated in lipoprotein

deficient medium, proportionally more isotope was incorporated into the cholesterol fraction, and incorporation into phospholipids and triglycerides was reduced. In liporotein deficient medium however, significantly less ($p<0.02$) [$^3$H]-sodium acetate was incorporated into the cholesterol fraction of sitosterolemic cells, and significantly more was incorporated into the phospholipid fraction ($p<0.05$), when compared to controls. This difference was not evident on day 10 of culture.

| [$^3$H] acetate % incorporation | lipid fraction | controls (mean ± SEM) | sitosterolemic (mean ± SEM) |
|---|---|---|---|
| freshly isolated | phospholipid | 62.65 ± 3.51 | 63.15 ± 1.40 |
| | cholesterol | 11.36 ± 0.78 | 16.4 ± 2.80 |
| | fatty acids | 6.61 2.74 | 1.38 ± 0.23 |
| | triglycerides | 15.38 ± 3.15 | 14.48 ± 1.08 |
| | cholesterol esters | 3.98 ± 0.40 | 4.30 ± 0.01 |
| DAY 1 (no LDL) | phospholipid | 23.60 ± 1.50 | 37.00 ± 3.85 * |
| | cholesterol | 69.35 ± 0.65 | 45.30 ± 0.55 ** |
| | fatty acids | 0.65 ± 0.35 | 5.73 ± 2.98 |
| | triglycerides | 5.35 ± 1.55 | 7.23 ± 0.43 |
| | cholesterol esters | 1.05 ± 0.25 | 4.73 ± 0.98 |
| DAY 10 (no LDL) | phospholipid | 28.26 ± 0.98 | 28.18 ± 0.73 |
| | cholesterol | 60.13 ± 1.11 | 58.65 ± 0.60 |
| | fatty acids | 1.78 ± 0.19 | 4.23 ± 2.53 |
| | triglycerides | 5.67 ± 0.37 | 6.58 ± 0.08 |
| | cholesterol esters | 3.95 ± 0.43 | 5.38 ± 0.58 |
| DAY 10 (LDL) | phospholipid | 37.39 ± 1.51 | 59.48 ± 2.93 *** |
| | cholesterol | 38.75 ± 2.28 | 19.6 ± 3.85 ** |
| | fatty acids | 8.92 ± 3.62 | 0.53 ± 0.52 |
| | triglycerides | 10.67 ± 1.10 | 11.35 ± 3.25 |
| | cholesterol esters | 4.33 ± .66 | 4.53 ± 0.18 |

**Table 3.8** Percent [3H]-sodium acetate incorporation into the various lipid fractions of control (n=4) and sitosterolemic (n=2) lymphocytes, under different experimental conditions. Values represent mean ± SEM (*significantly greater than control value, $p<0.05$; **significantly less than control value, $p<0.02$; ***significantly greater than control value, $p<0.01$).

When day 10 lymphocytes were incubated for 24 hours in medium containing LDL, incorporation into the cholesterol fraction declined, while

incorporation into triglycerides and phospholipids increased. In sitosterolemic cells however, the proportion of isotope incorporated into the cholesterol fraction was significantly lower ($p<0.02$) than in control cells. (**Table 3.8**).

**discussion**

The partitioning data confirmed the observations of the previous experiments. The cholesterol synthetic rate in freshly isolated sitosterolemic lymphocytes was not significantly different from that in controls. This conclusion was supported by the observation that isotope partitioning into the 5 lipid fractions was similar for both control and sitosterolemic cells before culture. The increase in cholesterol synthesis following 24 hr incubation in lipoprotein deficient medium, was significantly less in sitosterolemic cells than in control cells. Similarly, a significantly smaller proportion of isotope appeared in the cholesterol fraction of sitosterolemic cells. These observations suggest defective upregulation of HMG-CoA reductase activity in freshly isolated sitosterolemic lymphocytes. After 10 days of culture, following a similar 24 hr incubation period in lipoprotein deficient medium, these differences were ameliorated. This observation suggests that normal upregulation of cholesterol synthesis occurs in sitosterolemic lymphocytes, after a prolonged period of IL-stimulated growth. When day 10 cells were incubated for 24 hr in 50 µg/ml LDL however, cholesterol synthetic rates were reduced to significantly lower levels in sitosterolemic cells than in control cells, and this was reflected by the incorporation of a significantly lower proportion of [3H] sodium acetate into the cholesterol fraction of sitosterolemic lymphocytes. Collectively, these observations suggest that the reduced cholesterol synthesis which has been

reported to characterize sitosterolemia may be related to an abnormal response to exogenously supplied sterols.

**conclusions**

A significantly smaller proportion of acetate was incorporated into the cholesterol fraction of sitosterolemic lymphocytes and a significantly greater proportion of isotope was incorporated into phospholipids, when freshly isolated cells were incubated in lipoprotein deficient medium for 24 hours, or day 10 cells were incubated in LDL. These observations confirm reduced cholesterol synthesis in sitosterolemic cells under these culture conditions. Otherwise, the pattern of isotope partitioning into the various lipid fractions was similar, suggesting that acetate is metabolized normally in sitosterolemic cells

## 3.4 ACAT Activity

The rate of incorporation of [$^3$H]-oleate into the cholesteol ester fraction of lymphocytes was used as a measure of acyl-CoA cholesterol acyltransferase (ACAT) activity in control and sitosterolemic cells. ACAT activity was measured in freshly isolated lymphocytes to determine if sterol esterification was higher in sitosterolemic cells. ACAT activity was also measured in lymphocytes cultured under IL-2 stimulation to determine what effect rapid growth, concurrent with the progressive depletion of plant sterols, had on the rate of sterol esterification. ACAT activity was measured both in the presence and absence of exogenous cholestrol (supplied as LDL) to evaluate the effects of cholesterol loading and

depletion, respectively. Furthermore, the effects of sterol loading, using sitosterolemic LDL vs. control LDL, was examined in both control and patient lymphocytes. Finally, partitioning of [$^3$H]-oleate into the various lipid fractions under different culture conditions was evaluated, to compare the partitioning of isotope in sitosterolemic cells with that in control cells.

### 3.4.1 ACAT Activity in Freshly Isolated Cells

ACAT activity was initially measured in lymphocytes freshly isolated from patient and control whole blood. Mean sterol esterification in control cells was 5.89 ± 0.50 pmol/$10^6$ cells/hr, and mean esterification in sitosterolemic cells was 5.01 ± 0.89 pmol/$10^6$ cells/hr. ACAT activity in freshly isolated sitosterolemic lymphocytes was not significantly different from that in control cells.

Cells were then incubated for 24 hours in RPMI 1640 containing 50 µg/ml LDL and 10% LPDS, and ACAT activity was measured over the last 6 hours of the incubation period (day 1, LDL). ACAT activity was upregulated significantly ($p<0.001$) in both control and sitosterolemic lymphocytes, increasing to 21.59 ± 1.05 pmol/$10^6$ cells/hr in sitosterolemic cells, and to 17.49 ± 1.74 pmol/$10^6$ cells/hour in control cells. The sterol esterification rate in sitosterolemic lymphocytes was significantly higher than that in control cells in the presence of exogenous LDL ($p<0.05$) (**Figure 3.8**).

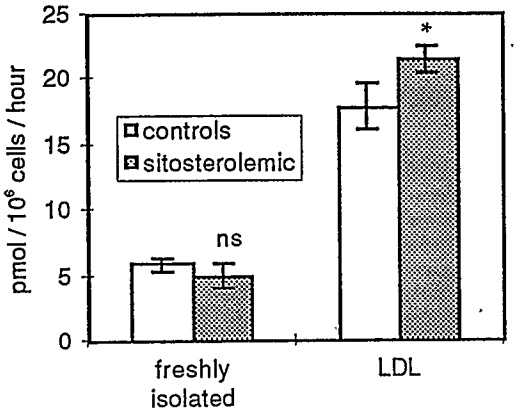


**Figure 3.8** [$^3$H]-oleate incorporation into the ester fraction of freshly isolated lymphocytes and lymphocytes incubated for 24 hr in medium containing 50 µg/ml LDL. Values represent mean ± SEM (controls, n=10; sitosterolemic, n=2; patient values were based on the mean of 3 separate experiments; * $p<0.05$)

**discussion**

ACAT activity has not been measured previously in sitosterolemic tissues. This enzyme however, has been demonstrated to play a role in both atherosclerosis and sterol absorption (Sections 1.3.2 and 1.3.3). The massive accumulation of sterol esters and the abnormal absorption of dietary sterols, which characterize sitosterolemia, suggest that ACAT activity might be either indiscriminate or excessive in this lipid disorder. ACAT activity in freshly isolated lymphocytes however, was not significantly different from that in control cells.

In the absence of exogenous cholesterol, the rate of cholesterol ester formation has been shown to be dependent on intracellular cholesterol content. [48] The cholesterol content of sitosterolemic lymphocytes was not significantly different from that of control cells (0.351 vs 0.383 µg/106 cells). In view of the direct regulation of ACAT activity by cellular cholesterol, the normal levels of cholesterol in sitosterolemic cells would tend to support the observation that ACAT activity was similar to that in control cells. Overall sterol content in

sitosterolemic lymphocytes however, was shown to be significantly higher than that in normal cells, with plant sterols accounting for an additional 0.235 µg/106 cells. ACAT activity in freshly isolated sitosterolemic lymphocytes then, would appear to be unaffected by the high levels of plant sterols present in these cells.

These data suggest that the plant sterols in sitosterolemic cells may be poor substrates for ACAT. Previous reports have corroborated this observation. The percent esterification of campesterol by ACAT has been reported to be 23%, and to be less than 5% for both sitosterol and stigmasterol. [144] Moreover, this substrate specificity is reportedly common to most eukaryotic cells. [48] As such, the normal ACAT activity demonstrated by freshly isolated lymphocytes, despite high plant sterol levels, suggests that sterol esterification by this enzyme, is not likely indiscriminate in this disorder.

The sterol esterification rate in both sitosterolemic and control lymphocytes increased significantly following a 24 hour incubation period in medium containing LDL. This observation is consistent with previous reports that sterol esterification in fibroblasts [146] and macrophages [156] has been shown to increase by as much as 10 fold following the supply of exogenous cholesterol as LDL.. The magnitude of this increase was significantly greater in sitosterolemic lymphocytes than in control cells. Sitosterolemic lymphocytes evidenced a 4.3 fold increase in sterol esterification following incubation in LDL, versus a 2.9 fold increase in control cells. This observation suggests that ACAT activity may be higher than normal in sitosterolemia.

The high ACAT activity in sitosterolemic lymphocytes following incubation in LDL, is not likely attributable to cholestyramine therapy. Cholestyramine has been shown to have no effect on hepatic or intestinal ACAT activity in rats [209, 210]

or hamsters. [52] Moreover, cholestyramine has been shown to reduce rather than increase ACAT activity in rabbit intestinal preparations. [211] Burrier *et al.* noted that ACAT activity was generally similar in cholestyramine-treated animals at saturating levels of exogenous cholesterol. [174]

The high ACAT activity in sitosterolemic cells may reflect the increase in the size of the intracellular sterol pool, due to the presence of plant sterols. Studies in a variety of tissues have repeatedly demonstrated that endogenous microsomal cholesterol is not sufficient to saturate this enzyme. [IN 48] Billheimer and Gillies noted that assays of ACAT activity in the absence of cholesterol illustrate the effect of various environmental conditions on cholesterol esterification, whereas assays in the presence of exogenous cholesterol reveal the extent to which observed changes in ACAT activity are due to increases (or decreases) in the amount of sterol in the substrate pool. Although plant sterols may not be suitable substrates for ACAT, the presence of phytosterols in the membranes of sitosterolemic cells may reduce the capacity of these cells to retain free cholesterol. The poor esterification of plant sterols by ACAT would tend to favour their retention in the ACAT substrate pool. As such, the liklihood that any surplus cholesterol entering the cell would be esterified rather than retained in the cells regulatory pool as free sterol, would be greater than in normal cells. Okwu *et al.* noted that macrophage ACAT activity was stimulated by lipoproteins only after a 'threshold' amount of sterol had accumulated in the cells. [172] Accordingly, plant sterols may reduce the threshold at which LDL supplied sterol stimulates ACAT activity. This possibility could explain both the increased ACAT activity in sitosterolemic cells when LDL is present, and the accumulation of sterol esters which occurs in sitosterolemic tissues.

**conclusions**

Baseline ACAT activity in freshly isolated sitosterolemic lymphocytes was not significantly different from that in control cells, suggesting that the high levels of plant sterols in these cells do not directly affect sterol esterification and that plant sterols may be a poor substrate for ACAT. However, ACAT activity was significantly higher in sitosterolemic lymphocytes when the cells were preincubated in medium containing LDL. This observation suggests that in the presence of LDL, ACAT activity in sitosterolemia is high, possibly due to an abnormal responsiveness of these cells to exogenous sterols.

## 3.4.2 ACAT Activity in Cultured Lymphocytes

Sterol esterification was measured again, both in the presence and absence of LDL, after 10 days of rapid IL-2 stimulated growth. Following 24 hours of incubation in lipoprotein deficient medium, ACAT activity in day 10 sitosterolemic lymphocytes was 6.38 ± 0.73 pmol/$10^6$ cells/hr vs. 5.02 ± 0.66 pmol/$10^6$ cells/hr in control cells. Control and sitosterolemic values were not significantly different. Moreover, day 10 values for both control and sitosterolemic cells were not significantly different from values obtained before culture, in the absence of exogenous LDL.

When day 10 lymphocytes were incubated for 24 hours in medium containing 50 μg/ml LDL however, sterol esterification was significantly higher in sitosterolemic cells than in control cells (18.26 ± 0.96 vs. 11.90 ± 1.15 pmol/$10^6$

cells/hr, respectively). There was no significant difference between LDL-stimulated ACAT activity on day 10 and LDL-stimulated activity before culture, in sitosterolemic cells. In control lymphocytes however, day 10 values were significantly lower than values obtained for freshly isolated cells **(Table 3.9, Figure 3.9)**.

| | ACAT activity (pmol oleate/$10^6$ cells/hr) | | | |
|---|---|---|---|---|
| | day 0 (freshly isolated) | day 1 (LDL) | day 10 (no LDL) | day 10 (LDL) |
| sitosterolemic | 5.01 ± 0.89 | 21.59 ± 1.05* | 6.38 ± 0.73 | 18.26 ± 0.96** |
| control | 5.89 ± 0.50 | 17.49 ± 1.74 | 5.02 ± 0.66 | 11.90 ± 1.15 |

**Table 3.9** [$^3$H]-oleate incorporation into the cholesterol ester fraction of cultured lymphocytes in the presence or absence of 50 µg/ml LDL. Values represent mean ± SEM (controls, n=10; sitosterolemic, n=2; patient values represent the mean of 3 separate experiments; *p<0.05; **p<0.01)

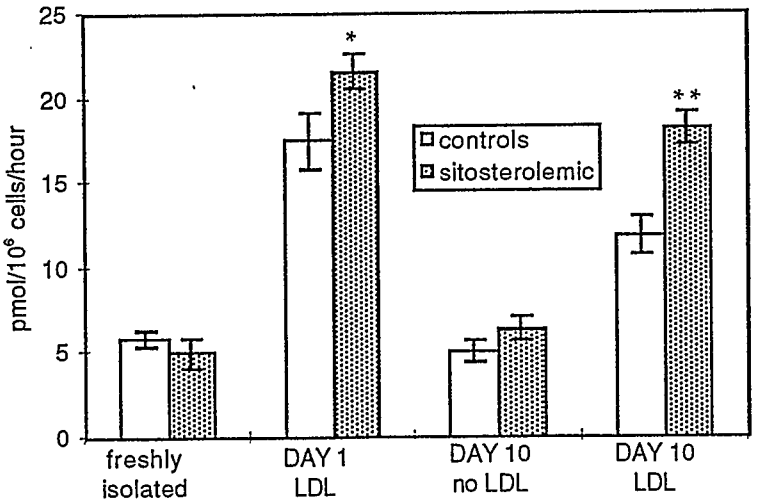


**Figure 3.9** [$^3$H]-oleate incorporation into the cholesterol ester fraction of cultured lymphocytes in the presence or absence of 50 µg/ml LDL. Values represent mean ± SEM (controls, n=10; sitosterolemic, n=2; patient values represent the mean of 3 separate experiments; *p<0.05; ** p<0.01)

**discussion**

Rapid IL-2 stimulated growth had no apparent effect on baseline ACAT activity in either control or sitosterolemic cells. This observation is consistent with the modulation of ACAT activity primarily by substrate supply. Bilheimer and Gillies noted that, unlike HMG-CoA reductase, there is little evidence to suggest that ACAT is regulated through modulation of the amount of enzyme synthesized. [95] ACAT is widely believed to function in the buffering of changes in membrane cholesterol content, effectively monitoring the intracellular concentration of free cholesterol. In conjunction with HMG-CoA reductase and the LDL receptor system, ACAT acts to minimize fluctuations in free cholesterol. As such, ACAT activity increases primarily in response to increased levels of intracellular cholesterol. Goldstein and Brown noted that in lipoprotein deficient medium, the rate of incorporation of [$^{14}$C]-oleate into intracellular esterified cholesterol remained low in cultured fibroblasts. [197] In rapidly growing cells, the demand for cholesterol would be similarly high, and cholesterol esterification should likewise remain low.

When ACAT was measured in the presence of LDL however, sterol esterification in day 10 sitosterolemic cells was significantly higher than esterification in day 10 control cells. This observation is consistent with data obtained using freshly isolated cells. Moreover, this observation is also consistent with the HMG-CoA reductase data, which suggested that sitosterolemic cells may be more responsive than normal cells to LDL cholesterol. As noted previously, this increased responsiveness may be attributable to the increased LDL receptor mediated uptake which has been

reported to characterize sitosterolemic mononuclear cells. [79] The regulation of ACAT activity in cultured cells has been shown to be tightly linked to the rate at which cells take up lipoprotein cholesterol. [52] LDL uptake however, is yet to be measure in cultured sitosterolemic cells. Alternatively, increased ACAT activity may be attributable to increased uptake by non-receptor mediated pathways. Although Attie *et al.* showed that LDL taken up and catabolized by non-receptor mediated pathways in rat hepatocytes did not stimulate cholesterol ester synthesis, [212] Brown *et al.* have demonstrated stimulation of macrophage ACAT activity in response to sterols taken up by non-receptor mediated pathways. [208] Moreover, the addition of free cholesterol to the incubation medium of normal and FH cultured fibroblasts has been demonstrated to stimulate the synthesis of cholesterol esters.[146] There is also evidence to suggest that plasma membrane cholesterol is accessible to ACAT. Slotte and Bierman were able to decrease the avidity of the plasma membrane for cholesterol by treating fibroblasts with sphingomyelinase. The subsequent flux of cholesterol back into the cell resulted in a 15 fold increase in oleate incorporation into cholesterol esters. The synthesis of cholesterol was also inhibited by 60%, effectively demonstrating that plasma membrane cholesterol was entering the regulatory pool. [213] Increased or unregulated non-receptor mediated uptake of sterols could explain both the increased ACAT and HMG-CoA reductase activity, as well as the absorption of abnormal sterols, which characterize sitosterolemia.

The possibility also remains that abnormally high ACAT activity in the presence of LDL may be due to an expanded intracellular pool of sterols. Hashimoto noted that very small increases in microsomal free cholesterol stimulated very large increases in ACAT activity in rabbit aortic microsomes. [214]

However, ACAT activity in day 10 sitosterolemic cells was not significantly different from that in freshly isolated cells, either in the presence or absence of LDL, despite a 96% reduction in the intracellular plant sterol content. This observation suggests that plant sterol levels do not directly affect ACAT activity, and favours the hypothesis that the primary defect in sitosterolemia is at the level of sterol absorption.

**conclusions**

Baseline ACAT activity in control and sitosterolemic lymphocytes was not significantly different in rapidly growing cells than in freshly isolated cells, suggesting that the activity of this enzyme is not altered by mitogenic stimulii or the depletion of plant sterols. When exogenous sterol was supplied as LDL however, ACAT activity increased significantly. Moreover, this increase was significantly greater in sitosterolemic lymphocytes than control lymphocytes. This corroborates observations made in freshly isolated cells, suggesting that sitosterolemia may be characterized by high ACAT activity, possibly due to an abnormal responsiveness to exogenous sterols.

### 3.4.3 Effect of Sitosterolemic LDL on ACAT activity

Sterol esterification was also measured on day 10 of culture after a 24 hr incubation period in RPMI 1640 containing 50 μg/ml of control LDL and in RPMI containing 50 μg/ml of sitosterolemic LDL, to evaluate the regulatory effects of patient LDL. ACAT activity increased significantly in cells incubated in both types

of LDL ($p<0.01$). There was however, no significant difference between sterol esterification rates in control or sitosterolemic cells incubated in sitosterolemic LDL vs. Cells incubated in control LDL. Although mean ACAT activity was uniformly higher in sitosterolemic cells than in control cells, this difference was not statistically significant. **(Table 3.10)**

| | ACAT (pmol oleate/$10^6$ cells/hr) | | |
|---|---|---|---|
| | no LDL | control LDL | sitosterol. LDL |
| controls | 6.22 ± 1.17 | 10.72 ± 2.98 | 9.21 ± 1.35 |
| sitosterolemic | 5.76 ± 1.92 | 12.90 ± 0.65 | 10.71 ± 1.69 |

**Table 3.10** [$^3$H]-oleate incorporation into the cholesterol ester fraction of cultured lymphocytes (day 10) incubated for 24 hours in lipoprotein deficient medium (no LDL), or in medium containing 50 µg/ml of control LDL or 50 µg/ml of sitosterolemic LDL. Values represent mean ± SEM; experiment was repeated twice for each control (with LDL from patient #1 and LDL from patient #2), and twice for each sitosterolemic patient.

## discussion

LDL obtained from sitosterolemic patients did not affect ACAT activity any differently than LDL obtained from controls. Both types of LDL upregulated sterol esterification to a similar extent. This observation is consistent with previous data (Sections 3.4.1 and 3.4.2), indicating that the presence or absence of plant sterols has no apparent effect on ACAT activity. Although sterol esterification in sitosterolemic lymphocytes in both types of LDL was consistently higher than esterification in control cells, this difference was not statistically significant. This observation is at odds with the significantly higher esterification noted previously in sitosterolemic cells incubated in LDL (Sections 3.4.1 and 3.4.2). In this context, it is relevant to note that LDL prepared from control and sitosterolemic

serum did not elevate ACAT activity to the same extent as commercially prepared (Sigma) LDL. Sigma LDL increased [$^3$H]-oleate incorporation into the ester fraction by 2.3 to 2.9 fold, whereas sitosterolemic/control LDL increased incorporation by only 1.7 to 2.0 fold. The lower cholesterol content of the latter may explain the disparity in statistical significance (as discussed previously in section 3.3.3).

**conclusions**

Sitosterolemic LDL did not exert regulatory effects on ACAT activity in control and sitosterolemic lymphocytes, which were any different from effects exerted by control LDL. This observation suggests that short-term incubation in small quantities of plant sterols does not affect ACAT activity.

### 3.4.4 Partitioning of [$^3$H]-Oleate into Lipid Fractions

The partitioning of isotope into the various lipid fractions was determined to compare the distribution of isotope in sitosterolemic lymphocytes with that in control lymphocytes, under different culture conditions. Five different fractions were separated by TLC: phospholipids, free fatty acids, cholesterol, triglycerides, and cholesterol esters. Isotope incorporated into each lipid fraction was analyzed by ß-scintillation counting, data was expressed as a percent of total incorporation into all fractions. Isotope incorporation into fractions other than cholesterol ester is not however a measure of lipid synthesis, and changes in percent incorporation may not reflect changes in the absolute amount of lipid.[221]

Under all culture conditions, isotope was incorporated primarily into the phospholipid and triglyceride fractions. A much smaller proportion of [$^3$H]-oleate was incorporated into cholesterol, free fatty acids, and cholesterol esters. Following incubation in LDL, isotope incorporation into the triglyceride fraction increased, while incorporation into phospholipids decreased, in freshly isolated control and sitosterolemic cells. Preincubation of cells in LDL also significantly increased the proportion of oleate incorporated into the cholesterol ester fraction of both control and sitosterolemic lymphocytes. This increase, was significantly greater in day 1 and day 10 cultured sitosterolemic cells than in control cells ($p<0.02$). Incorporation of oleate into the triglyceride and cholesterol fractions of cultured sitosterolemic cells tended to be lower than incorporation into these fractions in control cells, but these differences were not statistically significant. Overall, the pattern of oleate incorporation into the various lipid fractions was similar before and after 10 days of IL-2 stimulated culture, in both control and sitosterolemic cells. **(Table 3.11)**

| [$^3$H] oleate % incorporation | lipid fraction | controls (mean ± SEM) | sitosterolemic (mean ± SEM) |
|---|---|---|---|
| freshly isolated | phospholipid | 31.47 ± 0.65 | 40.73 ± 2.17 |
| | cholesterol | 3.30 ± 2.00 | 0.77 ± 0.07 |
| | fatty acids | 1.07 ± 0.62 | 0.62 ± 0.62 |
| | triglycerides | 65.69 ± 1.29 | 57.40 ± 1.50 ** |
| | cholesterol esters | 0.57 ± 0.38 | 0.48 ± 0.03 |
| DAY 1 (LDL) | phospholipid | 18.18 ± 1.48 | 23.38 ± 0.53 ** |
| | cholesterol | 1.33 ± 0.09 | 1.33 ± 0.03 |
| | fatty acids | 2.87 ± 1.80 | 2.38 ± 2.38 |
| | triglycerides | 76.74 ± 1.22 | 68.05 ± 4.21 |
| | cholesterol esters | 1.14 ± 0.19 | 1.88 ± 0.08 ** |
| DAY 10 (LDL) | phospholipid | 41.4 ± 1.16 | 48.37 ± 0.89 ** |
| | cholesterol | 1.95 ± 0.18 | 1.00 ± 0.25 |
| | fatty acids | 4.09 ± 1.08 | 2.79 ± 2.34 |
| | triglycerides | 51.39 ± 0.78 | 46.04 ± 3.67 |
| | cholesterol esters | 1.43 ± 0.12 | 1.95 ± 0.08 ** |
| DAY 10 (no LDL) | phospholipid | 39.49 ± 1.25 | 44.35 ± 0.35 ** |
| | cholesterol | 1.86 ± 0.12 | 1.35 ± 0.02 |
| | fatty acids | 9.56 ± 2.92 | 1.95 ± 1.95 |
| | triglycerides | 48.36 ± 3.40 | 51.65 ± 2.20 |
| | cholesterol esters | 0.72 ± 0.06 | 0.63 ± 0.08 |

**Table 3.11** Percent [$^3$H]-oleate incorporation into the various lipid fractions of control (n=4) and sitosterolemic (n=2) lymphocytes under different culture condidtions. Values represent mean ± SEM (**p,0.02)

## discussion

The partitioning of [$^3$H]-oleate into the lipid fractions of freshly isolated sitosterolemic cells was similar to that in control cells. Notably, the proportion of isotope incorporated into the cholesterol ester fraction was not significantly different. This is consistent with the previous observation that baseline ACAT activity in sitosterolemic lymphocytes was normal. In the presence of LDL however, the proportion of oleate incorporated into cholesterol esters was higher in sitosterolemic lymphocytes than in control lymphocytes. These data are consistent with the higher esterification rate in sitosterolemic cells, which was

noted previously in freshly isolated cells and day 10 cells cultured in the presence of LDL.

Overall, these data support the conclusion that sterol esterification in sitosterolemic cells is higher than that in control cells, and that the esterification process is not significantly affected by long-term culture.

**conclusions**

The partitioning of oleate into the various lipid fractions of sitosterolemic lymphocytes confirmed the observations made in previous experiments. The proportion of isotope incorporated into sterol esters was higher than normal, when sitosterolemic cells were preincubated in LDL. Overall, the pattern of isotope partitioning in sitosterolemic cells was similar to that in control cells, suggesting normal uptake and metabolism of fatty acids in this disorder.

# CHAPTER 4
# CONCLUSIONS

## 4.1 GENERAL CONCLUSIONS

Lymphocytes from two sitosterolemic subjects demonstrated significantly lower upregulation of cholesterol synthesis following 24 hours of incubation in lipoprotein deficient medium than control cells. However, sitosterolemic lymphocytes, demonstrated normal growth rates in medium containing FBS and in lipoprotein deficient medium. After 6 and 10 days of IL-2 stimulated rapid *in vitro* growth, these cells demonstrated cholesterol synthetic rates which were not significantly different from those of control cells. Before culture, plant sterols were shown to account for 38% of intracellular sterols, while the intracellular cholesterol concentration was similar to that in control cells. Plant sterol concentration however, was reduced to approximately 4% over the 10 culture period. The incubation of control and sitosterolemic cells for 24 hours in medium supplemented with sitosterolemic LDL (containing 26% plant sterols) did not affect cholesterol synthesis any differently than control LDL. However, the incubation of sitosterolemic cells in sitosterolemic and control LDL reduced cholesterol synthesis significantly more in sitosterolemic cells than in control cells. A similar enhanced responsiveness to LDL cholesterol was demonstrated when ACAT activity was measured in sitosterolemic lymphocytes. ACAT activity before and after 10 days of IL-2 stimulated culture, was not significantly different in sitosterolemic cells. When sitosterolemic lymphocytes were preincubated in LDL (Sigma) however, ACAT activity was significantly higher in sitosterolemic

cells than in control cells, irrespective of culture duration.

## 4.2 SUMMARY

In summary, the results of this study indicated that sitosterolemic lymphocytes have the capacity to upregulate cholesterol synthesis to normal levels in culture. While this upregulation was concurrent with the depletion of plant sterols, direct regulation of reductase activity by phytosterols remains to be conclusively demonstrated. ACAT activity was similarly shown to be normal, and the depletion of intracellular plant sterols did not alter the rate of sterol esterification. However, both HMG-CoA reductase activity and ACAT activity in sitosterolemic lymphocytes were shown to be significantly different from activity in control cells when the cells were preincubated in LDL (Sigma). These observations occurred irrespective of changes in intracellular plant sterol concentration. Collectively, these results strongly suggest that the primary defect in sitosterolemia is at the level of sterol absorption, and that the reduced cholesterol synthesis and the accumulation of sterol esters which characterize this lipid disorder, may be secondary to abnormal sterol uptake.

## 4.3 FUTURE RESEARCH

While the present study strongly suggests that the primary defect in this disorder does not reside in the cholesterol synthetic pathway, the role of plant

sterols in the downregulation of cholesterol biosynthesis remains to be clarified. To conclusively demonstrate that plant sterols affect HMG-CoA reductase activity, concentrations of plant sterols similar to those found in sitosterolemic lymphocytes, must be introduced into normal cells. This could be accomplished by the long-term incubation of normal cells in sitosterolemic LDL, or more practically, in LDL which has been reconstituted with plant sterols. The tissue culture system used in these experiments should prove very useful in this regard, and should circumvent many of the problems encountered in previous attempts to establish animal models suitable for the study of this disorder.

The observation that two of the enzymes involved in intracellular cholesterol homeostasis demonstrated abnormal activity when sitosterolemic cells were incubated in LDL, suggests that the factors governing the responsiveness of these cells to exogenous sources of sterol are abnormal. As such, LDL receptor mediated uptake, as well as non-receptor mediated uptake, should be evaluated in cultured sitosterolemic lymphocytes. Moreover, it is important that ACAT activity and HMG-CoA reductase activity be measured in cultured lymphocytes using microsomal membrane preparations. If the defect in this disorder is at the level of the plasma membrane, microsomal assays of cultured cells should yield normal enzyme activity in the absence of plant sterols.

Recent research has provided compelling evidence that microsomal membrane sterols may exert direct regulatory effects on HMG-CoA reductase and ACAT activity. It would therefore, be worthwhile to measure plant sterol levels in microsomal membranes, and determine to what extent intracellular membranes are enriched with plant sterols. Plant sterols were not completely cultured out of sitosterolemic cells in this culture system. The possibility remains

that plant sterols in the microsomal membranes of sitosterolemic cells may be acting to reduce the threshold level at which exogenous cholesterol stimulates reductase downregulation and ACAT upregulation. Longer-term culture of sitosterolemic lymphocytes should be undertaken to achieve more complete removal of phytosterols from these cells. To this end, transformed cell lines have been established in Dr. Parson's lab. These cell lines will facilitate further study of this lipid disorder by providing a continuous supply of plant-sterol free cells.

Finally, cholesterol synthesis and sterol esterification should be evaluated in primary cultures of cells obtained from other sitosterolemic patients. ACAT activity has not been measured previously in sitosterolemic tissues, and the observations made in the present study remain to be verified. Ideally, the present experiments should also be repeated in the absence of patient cholestyramine therapy, and further studies of sitosterolemic patients who have been treated with bile acid sequestering resins for more than 5 years are required. The effects of prolonged cholestyramine therapy on HMG-CoA reductase activity and intracellular levels of plant sterols in sitosterolemic patients have not been previously evaluated, and the role of cholestyramine needs to be clarified.

Further development of alternate tissue culture systems appropriate for the study of sitosterolemia, such as hepatocyte cell lines or intestinal cell lines, should be undertaken, and will undoubtedly assist in the resolution of the primary defect in this disorder.

## 4.4 CONTRIBUTION TO SCIENTIFIC RESEARCH

This study provided evidence to suggest that the primary defect in sitosterolemia is at the level of sterol absorption rather than cholesterol biosynthesis, as has been suggested previously. Moreover, it comprises the first report of ACAT activity in sitosterolemic cells, and the first evaluation of reductase activity and intracellular sterol levels in sitosterolemic patients following prolonged cholestyramine therapy. More importantly, this thesis has advanced a novel approach to the study of the etiology of sitosterolemia. Long-term *in vitro* culture of sitosterolemic cells has not been previously attempted. It was shown to be a viable approach for differentiating effects due to plant sterols from those due to inborn metabolic errors. Although sitosterolemia is a rare hereditary disorder, study of this defect may further the understanding of the mechanisms which regulate cholesterol homeostasis and cholesterol absorption in human cells generally. Specifically, the study of sitosterolemia may provide valuable information regarding the location of the regulatory pool of sterols in cells and further clarify the role of microsomal sterols in the regulatory process, in additon to providing information concerning the mechanism by which cell membranes selectively absorb cholesterol. These processes are currently poorly understood, and are the subject of considerable ongoing research.

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