Mechanism of cholesterol reduction to coprostanol by cholesterol reductase

by

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Signatures have been redacted for privacy

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GENERAL INTRODUCTION

Heart disease is the leading cause of human death in the United States, accounting for nearly 40 percent of the annual deaths (National Research Council, 1989; U.S. Department of Health and Human Services, 1988). An increasing amount of the medical literature has documented the relationship between concentrations of serum cholesterol and risk of heart disease in men and to a smaller extent in women. Populations from developed countries, which consume diets high in meat and low in fiber, a much greater risk for coronary artery disease than are more rural populations. It has been proven that dietary cholesterol, fat, protein, carbohydrate, fibers, minerals, and vitamins can affect the concentration of plasma cholesterol.

Diet plays an important role in the control of hypercholesterolemia. Amount of fat ingested can affect concentration of plasma cholesterol; high fat diet increase the incidence of hypercholesterolemia, whereas low fat diets decrease the incidence (Groot and Scheek, 1984). Decrease in dietary lipids containing saturated fatty acids and cholesterol is a basic precaution that is recommended for the prevention, arrest, and reversal of atherosclerosis.

Several processes to decrease the cholesterol concentration in various food products have been proposed. Cholesterol reductase, an enzyme that converts cholesterol to

poorly absorbed coprostanol, shows promise for "decholesterolizing" meat, egg, and dairy products.

A primary reaction of intestinal bacteria on neutral steroids is the conversion of cholesterol to coprostanol. This reaction results in reduction of the 5-6 double bond of cholesterol to form the saturated derivative coprostanol that is absorbed poorly in humans (Bhattacharyya, 1986). Eyssen et al. (1973) identified Eubacterium ATCC 21,408 as an intestinal gram-positive anaerobe that was capable of reducing cholesterol to coprostanol. This organism now has been isolated from the feces of humans, baboons, and rats (Macdonald et al., 1983).

The conversion of cholesterol into coprostanol by intestinal microorganisms has been reported to occur by means of two different pathways. One pathway involves a direct reduction of the double bound at C-5 (Rosenfeld et al., 1954; Rosenfeld and Gallagher, 1964); the other involves the intermediate formation of 4-cholesten-3-one and coprostanone (Bjorkhem and Gustafsson, 1971). Coprostanol produced by incubating Eubacterium ATCC 21,408 with [4β-^{3}H, 4-^{14}C]-cholesterol had retained 81% of the tritium originally present in cholesterol, most of which had been transfered to the C-6 position in coprostanol, indicating that the conversion of cholesterol in coprostanol involved isomerization of a 5-6 double bond to a 4-5 double bond and an intramolecular shift

of the major amount of tritium from C-4 to C-6 (Parmentier and Eyssen, 1973).

Recently, my colleagues isolated a pure culture of a cholesterol-reducing microorganism from a swine sewage lagoon. This bacterium classified as Eubacterium sp. strain HL converts cholesterol into coprostanol in the incubation media to greater than 90%. The present investigation was designed to elucidate the pathways of biohydrogenation of cholesterol by Eubacterium sp. strain HL.

EXPLANATION OF THESIS FORMAT

This thesis has been prepared according to the alternate thesis format as described in the Iowa State University Graduate College Thesis Manual. Results of research completed to partially complete requirements for the Master of Science degree are contained within the thesis. The thesis contains a review of the literature and an introduction to the problem, as well as materials and methods, results and discussion sections, and is in a form suitable for publication in a scientific journal.

LITERATURE REVIEW

Atherosclerosis

Atherosclerosis and its complications are the main cause of death in civilized countries. Elements of atherosclerosis are present in all arteries, young or old, animal or human.

It is well established that atherosclerosis is a multifactorial disease and that both genes and environment contribute to its development (Davignon et al., 1977). However, many complex and numerous cellular factors are involved in atherogenesis. The end product remains primarily the accumulation of cholesterol and cholesterol esters in the intima of the arterial wall at vulnerable sites. Fatty deposits in the interior of arteries contain 70% cholesterol (Linder, 1985). Many studies have shown that in groups of humans whose concentrations of blood cholesterol are high the incidence of atherosclerosis also is high (Hulley, 1988). Dietary studies in man have produced much evidence to support this relationship. Numerous studies have shown that excess dietary cholesterol has an undeniable hypercholesterolemic effect and induces atherosclerosis (Page, 1954). Furthermore, it was initially thought that man's maximal daily absorption of dietary cholesterol, despite variable dietary intake, could not exceed 300-500 mg per day (Kaplan et al., 1963; Wilson et

al., 1965). It is now clear that the amount of cholesterol absorbed varies directly with amounts of cholesterol intake (Borgström, 1969; Quintao et al., 1971). A drastic lowering of cholesterol intake (from 600 mg/day to less than 50 mg/day) will decrease plasma cholesterol from 15% to 20% in human. (Connor, 1980).

Because cholesterol of an atherosclerotic lesion originates in large part from blood plasma, much attention has been focused on the interaction between lipoproteins and arterial epithelial cells. Research on the etiology of atherosclerosis received a new impetus when it was suggested that low-density lipoproteins (LDL) and high-density lipoproteins (HDL) play opposite roles in cholesterol deposition during lesion development (Kannel at al., 1979). Studies suggested that LDL are associated positively with atherosclerosis and HDL do not seem to be associated with atherosclerosis (Small, 1977). The concentration of circulating LDL is related directly to the consumption of cholesterol and saturated fats (American Heart Association Nutrition Committee, 1982). Furthermore, when either monounsaturated or polyunsaturated dietary fats are substituted for saturated dietary fats, plasma cholesterol concentration decreases (American Heart Association Nutrition Committee, 1982).

Even though numerous studies have been done, the

mechanism of atherogenesis still remains unclear. At least 37 different variables have been correlated with atherosclerosis (Strasser, 1972). Hypercholesterolemia, hypertension, and cigarette smoking have the strongest positive correlation to premature coronary heart disease (Gordon et al., 1971).

Cholesterol Metabolism

For many years, the metabolism of cholesterol has been a subject of great interest. Cholesterol is a necessary body substance that is found in all animal tissues. It is the precursor of steroid hormones and bile acids, and a major constituent of atherosclerotic plaques and most gallstones. Much has been learned about cholesterol metabolism in both animals and man during the last 20 years. Interestingly, the adrenal gland contains a relatively high concentration of cholesterol that is depleted rapidly under stress when cortical activity is high (Pike, 1975). Adipose tissue is a major cholesterol storage organ (Farkas et al., 1973). Cholesterol in the blood stream comes from two sources: firstly, cholesterol is produced naturally by the body's cells at about 700-900 mg per day. Secondly, cholesterol in blood is derived from the intake of cholesterol-containing foods at about 300 to 500 mg per day in man (Turley and Dietschy, 1982).

Absorption of cholesterol

The average American diet supplies approximately 400 to 700 mg of cholesterol per day. Intestinal cholesterol, however, is derived not only from the diet but also from intestinal secretions and bile. Bile usually transports from 750 to 1250 mg cholesterol per day (Bennion and Grundy, 1975); the contribution from the sloughed mucosal cells is significantly less. Most of the cholesterol in bile is unesterified, but a portion of dietary cholesterol may be esterified with fatty acids (Grundy, 1983). Any cholesterol ester entering the intestinal lumen is de-esterified rapidly by pancreatic cholesterol esterase (Sabine, 1977). Free cholesterol must be solubilized before absorption. This cholesterol solubilization is achieved by its incorporation into mixed micelles containing conjugated bile acids, fatty acids, monoacyglycerols, and lysolecithin (Hofmann and Borgstrom, 1964). Efficiency of cholesterol absorption is limited; only from 30% to 60% of dietary and biliary cholesterol is absorbed (Grundy and Mok, 1977). Increasing the cholesterolintake results in increased total absorption, although the percentage absorbed is usually lower. Passage through the lipid of the mucosal cell membrane occurs by a passive diffusion. In the mucosal cell, cholesterol is re-esterified largely with unsaturated fatty acids, incorporated into chylomicrons, and transferred into intestinal lymph.

Dietary cholesterol absorption may be affected by the amount of biliary cholesterol released into the intestine. Evidence shows that biliary cholesterol is absorbed similarly to that of dietary cholesterol but is absorbed more efficiently than the dietary cholesterol as a result of a difference in the physical state of the cholesterol (Grundy, 1983). Biliary cholesterol enters the intestine in micellar solution, whereas dietary cholesterol must be digested and transformed into a micellar state before absorption. This transformation of dietary cholesterol usually is not 100% efficient. Absorption of endogenous cholesterol ranges between 60 and 80% (Grundy and Mok, 1977), but absorption of dietary cholesterol usually is lower and varies from 20 to 80% (Grundy, 1979).

Degradation of cholesterol

The regulation of blood cholesterol concentration depends not only on the rate of de novo synthesis but also on the rate of degradation and excretion of cholesterol. Some cholesterol is excreted into the bile, a portion of which may be reabsorbed from the intestine. Some biliary cholesterol is reduced by bacteria in the digestive tract to coprostanol and coprostanone, which are excreted in the feces along with unabsorbed cholesterol (Dietschy and Wilson, 1970).

The main disposal mechanism for excess body cholesterol

is by way of metabolism to bile acids. Conversion of cholesterol to bile acids occurs primarily in the liver (Salen and Shefer, 1983). Bile acids, which are synthesized de novo in the liver, are called primary bile acids, whereas secondary bile acids are formed from the metabolism of the primary bile acids by intestinal microorganisms. The two most abundant primary bile acids in humans, cholic acid and chenodeoxycholic acid, are synthesized in the liver from cholesterol (Bergstrom et al., 1960). These acids are conjugated with taurine or glycine and appear in the bile as the sodium or potassium salts (Sabine, 1977). The conjugated bile acids excreted with bile enter into the gastrointestinal tract and are transformed by microorganisms into secondary bile acids, such as deoxycholic and lithocholic acids. Most bile acids are reabsorbed rapidly and return to the liver via the portal circulation where they are further metabolized and are resecreted into bile. Only a small daily turnover of bile acids through the intestine and into the feces occurs (Dietschy and Wilson, 1979). In humans, approximately 20-30 g of bile salts are secreted by the liver but only about 0.5 g appears in the feces each day (Zubay, 1988).

Reduction of cholesterol to coprostanol

Unabsorbed cholesterol is eliminated ultimately from the body in the feces as fecal neutral steroids. The fecal neutral

sterols represent a mixture of derivatives of endogenous cholesterol and a varying amount of unabsorbed dietary sterols of animal and vegetable origin (Gould and Cook, 1958). The fecal neutral steroids of rats originate primarily from three sources: 1. de novo cholesterol synthesis by the intestinal mucosa and liver, 2. dietary and mucosal sterols, and 3. a rapidly exchangeable cholesterol pool excreted through bile, the intestinal wall, or both (Miettinen et al., 1981). It is possible that fecal neutral sterols are derived predominantly from dietary and intestinal origin rather than from homogeneous lumenal pool derived from the diet, the bile, and the intestinal wall (Wilson and Reinke, 1968).

During intestinal transit, cholesterol is converted mainly into two bacterial conversion products, coprostanol and coprostanone (McNamara et al., 1981). Cholesterol, coprostanol, and coprostanone accounts for more than 95% of the neutral steroid in human feces; the remainder is predominantly cholestanol. Depending upon the diet, 50% or more of the total fecal sterol can be present in the form of coprostanol (Heftman, 1970). In addition, evidence was obtained for the presence in human feces of trace amounts of epicoprostanol and cholestanone (McNamara et al., 1981). The early observations that mixed fecal cultures of human or rat readily transform cholesterol to coprostanol led to later attempts to isolate the microorganisms responsible for this

transformation (Snog-kjaer et al., 1956). The microorganisms responsible for these transformations were identified as Eubacterium species (Eyssen, 1973; Sadzikowski et al., 1977).

Eyssen et al. (1973) reported that the steroid-reducing microorganism isolated from the intestine of the rat is a small gram-positive, strictly anaerobic bacterium. On the basis of its morphological and physiological characteristics, it has been classified as a Eubacterium and was referred to as Eubacterium 21,408. A pure culture of Eubacterium 21,408 reduced cholesterol in the growth media to coprostanol with an efficiency exceeding 90%. This bacterium required at least 1 mg per ml of a 5-6 double bond 3β-hydroxy steroid and bovine brain tissue for growth. Growth was very sparse on media containing 0.125 or 0.25 mg cholesterol per ml, and optimal growth was not obtained unless the medium contained 1.5-2 mg cholesterol per ml. The large amount of steroid required for maximal growth would indicate that cholesterol is not acting as a growth factor. It seems logical to assume that the 5-6 double bond of cholesterol and plant sterols is acting as a hydrogen acceptor in the metabolism of the microorganism. In 1982, Brinkley et al. (1982) isolated nine strains of cholesterol-reducing bacteria from intestinal contents of baboons. Most of these isolates required cholesterol or a plasmalogen found in brain for growth.

Isolation and characterization of the cholesterol-

reducing strains of Eubacterium have been difficult because these organisms failed to grow on agar media. Freier and Hartman at Iowa State University (1991) reported growth of a pure culture of a cholesterol-reducing bacterium in medium that did not contain plasmalogen. This isolate is a small, anaerobic, nonsporing, Gram-positive rod. The isolate does not require cholesterol for growth, but it requires lecithin and seems to possess phospholipase activity. Two pure cultures were isolated, one from a hog sewage lagoon (HL) and one from stream sediment (SS). Isolate HL has been classified tentatively as a Eubacterium sp.

Coprostanol formation may occur by more than one pathway (Rosenfeld et al., 1967). Two major pathways have been postulated for formation of coprostanol from cholesterol (Macdonald et al., 1983). One pathway involves the intermediate formation of 4-5 double bond in cholestenone and coprostanone (Bjorkhem and Gustafsson, 1971). In the other pathway, cholesterol is transformed into coprostanol by the direct reduction of the 5-6 double bond (Rosenfeld and Gallagher, 1964). Rosenfeld and Gallagher (1964) incubated [3α-^{3}H]-cholesterol with human feces and observed that the resulting coprostanol retained most of the label of C-3, indicating a direct reduction of the double bond. However, Bjorkhem and Gustafsson (1971) demonstrated that during the microbial conversion of [3α-^{3}H]-cholesterol to coprostanol 3α-

tritium can be removed and then reinserted into the same position. Thus, it is not possible to distinguish between the two reduction pathways by using [3α-^{3}H]-cholesterol as a substrate. Evidence for the multistep pathway has been presented by Bjorkhem and Gustafsson (1971) with cecal contents from rats and similarly by Parmentier and Eyssen (1974) with Eubacterium ATCC 21,408. By using [4β-^{3}H, 4-^{14}C]-cholesterol as a substrate, Parmentier and Eyssen (1974) found that conversion to coprostanol occurred with a loss of about 20% of the tritium; more than 70% of the tritium in cholesterol had been transferred to the C-6 position. The results of the experiments with [4β-^{3}H]-cholesterol leave no doubt that the multistep pathway is of major importance for coprostanol formation.

Past Approaches for Decreasing Cholesterol in Food Products

Much research had been conducted for several years to develop technologies to decrease fat and cholesterol in human diets. The food industry has responded with many new products. The technologies used can be classified as chemical, physical, microbiological, enzymatic, and genetic.

The use of organic solvents to remove cholesterol and lipids from eggs has shown some success. Supercritical fluid extraction, however, has shown excellent potential. Research

at the University of Nebraska (Froning, 1991) has indicated that cholesterol and fat can be removed from dried egg yolk without impairing its functionality. Cholesterol content in dried egg yolk was decreased substantially (up to a two-thirds removal) by supercritical carbon dioxide extraction. Novak et al. (1991) are conducting research on the removal of cholesterol from liquid egg yolk by using supercritical extraction with carbon dioxide. They reported cholesterol removal of up to 34 percent in liquid and up to 76 percent in dried egg yolk. These results indicate that supercritical carbon dioxide extraction may have commercial feasibility in the future.

A physical process for simultaneous deodorization and cholesterol removal from fats and oils was reported by Marschuer and Fine (1989). A stream-stripping method was used by the Omega Source Corp., Burnsville, Minnesota to remove 93% of the cholesterol from milk fat. This process was thought to be less expensive than supercritical fluid extraction; even so, it is still an expensive and complicated procedure.

Enzymes offer another alternative for decreasing the cholesterol content of dairy and egg products. A number of microorganisms have been shown to degrade cholesterol; however, only a few do so without accumulating any steroid intermediates (Arima, 1969; Masheck, 1972). Johnson and Somkuti (1989) reported the loss of 40% of the cholesterol

found in egg yolk preparations following treatment with sonicated extracts of Rhodococcus equi. Extracts of R. equi 21107 and R. equi 33706 removed 3.3% and 3.1% of the cholesterol from egg yolk per min per mg of crude enzyme protein, respectively. Incubation of fresh cream with R. equi 33706 extracts resulted in only a 2.4% reduction in cholesterol content. Cholesterol degradation by R. equi 33706 had an optimal temperature of 40°C and an optimal pH of 8.0. There was no apparent requirement for divalent metal ions. Approximately 44% of enzyme activity was lost after a 60 min exposure at 60°C. Thin layer chromatographic analysis of cholesterol degradation products revealed only a few steroid-like compounds, primarily 4-cholesten-3-one and 1,4-cholestadiene-3-one. The results indicated that the Rhodococcus cholesterol degrading system is highly effective with free cholesterol in micellar solution but much less so with cholesterol in complex food systems, such as egg yolk and milk cream.

Work is being conducted at Iowa State University to develop an enzymatic process to convert cholesterol to the coprostanol (Dehal et al., 1991). They demonstrated that cholesterol reductase is present in cucumber, alfalfa, and pea leaves. The cholesterol reductases of these plants are cytosolic and have an optimal pH of about 6.5. NADPH and NADH are the effective reducing cofactors. Preparations of

cholesterol reductase from alfalfa and cucumber leaves and cholesterol-reducing bacteria converted some cholesterol in homogenized milk, homogenized cream, ground beef and pork, and fresh and dried egg yolk to coprostanol. The conversion of skeletal muscle cholesterol to coprostanol also was studied after intravenous injections of an alfalfa cholesterol reductase into rats. However, at the present time, the use of cholesterol degrading enzymes in food remains unrealistic for lack of toxicological data on both enzyme extracts and intermediate products of cholesterol degradation.

SECTION 1. MECHANISM OF CHOLESTEROL REDUCTION TO COPROSTANOL BY CHOLESTEROL REDUCTASE

ABSTRACT

The mechanism of biohydrogenation of cholesterol to coprostanol was studied by incubating Eubacterium sp. strain HL with [4-^{3}H, 4-^{14}C]-cholesterol as a mixture of α and β isomers. In a growth medium under anaerobic conditions, coprostanol isolated after incubation of [4-^{3}H, 4-^{14}C]-cholesterol retained 97% of the tritium originally present in cholesterol. The main part of this tritium (64%) is in the C-6 position in coprostanol, showing that the conversion of cholesterol by Eubacterium HL into coprostanol involves the intermediate formation of 4-cholesten-3-one followed by reduction of the latter to coprostanol. Furthermore, [4-^{3}H, 4-^{14}C]-cholesterol and coprostanone were converted efficiently to coprostanol. Thus, these results support the hypothesis that the major pathway for biohydrogenation of cholesterol by Eubacterium HL involves the intermediate formation of 4-cholesten-3-one followed by reduction of the latter to coprostanol.

INTRODUCTION

The conversion of cholesterol into coprostanol by intestinal microorganisms has been reported to occur by two different pathways (Figure 1). One involves the intermediary formation of 4-cholesten-3-one and coprostanone (Eyssen and Parmentier, 1977; Parmentier and Eyssen, 1974; Bjorkhem and Gustafsson, 1971). In the other pathway, cholesterol is transformed into coprostanol by the direct reduction of the 5-6 double bond (Rosenfeld and Gallagher, 1964).

In the previous work with cecal contents from rats, coprostanol isolated after incubation of [4β-^{3}H, 4-^{14}C] cholesterol retained 60% of the tritium (Bjorkhem and Gustafsson, 1971). Most of this tritium had been transferred to the C-6 position, showing that the conversion of cholesterol into coprostanol involves isomerization of a 5-6 double bond to a 4-5 double bond. Rosenfeld and Gallagher (1964) incubated [3α-^{3}H]-cholesterol with human feces and observed that the resulting coprostanol retained most of the label of C-3, indicating a direct reduction of the double bond. However, Bjorkhem and Gustafsson (1971) demonstrated that during the microbial conversion of [3α-^{3}H]-cholesterol to coprostanol 3α-tritium can be removed and then reinserted with high efficiency into same position. Thus, it is not possible

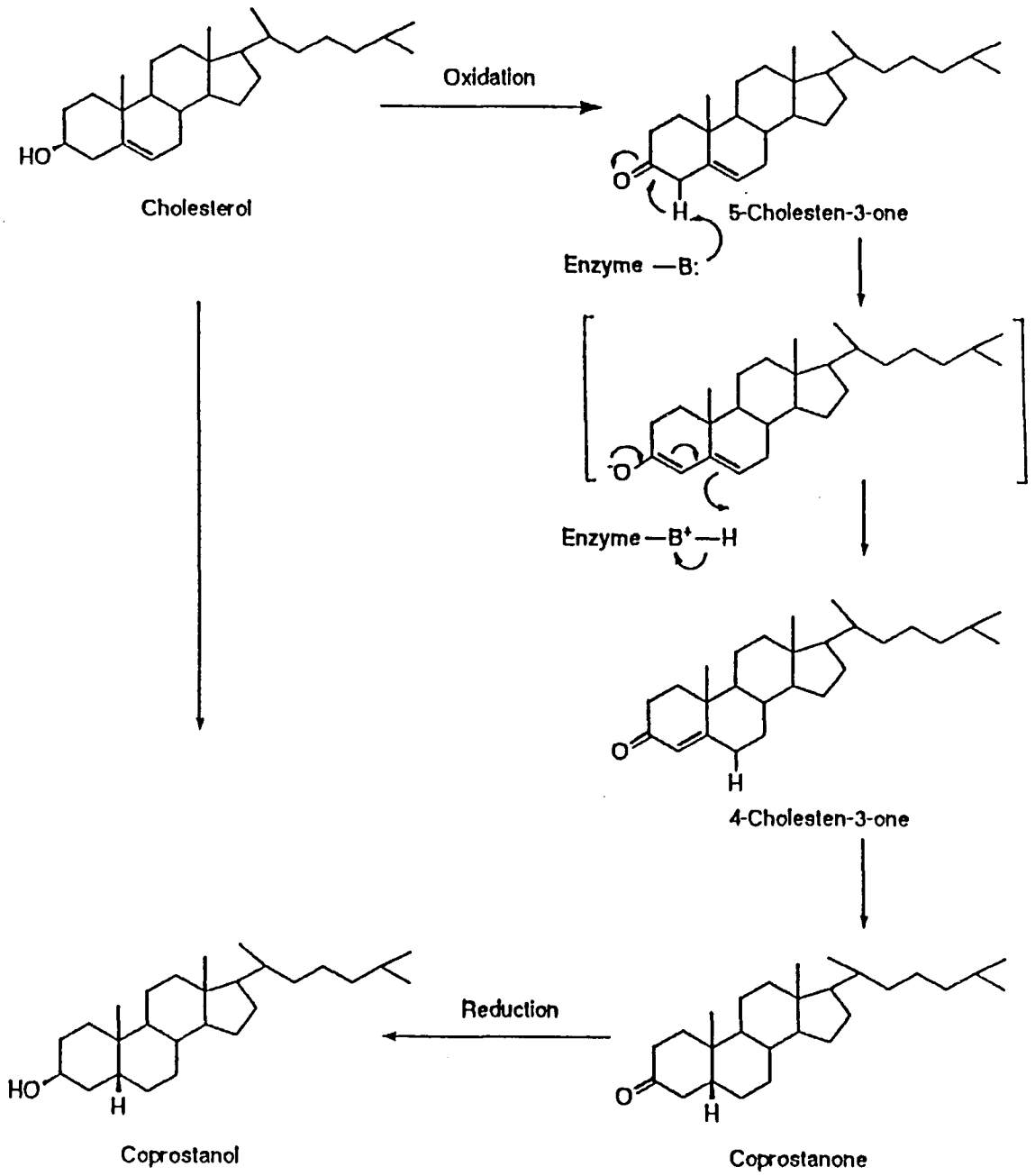

Figure 1. Proposed multistep reaction sequence for enzymatic cholesterol conversion to coprostanol

to distinguish between the two reduction pathways when using [3α-^{3}H]-cholesterol as a substrate. The study of Bjorkhem and Gustafsson invalidates the conclosion of Resenfeld and Gallagher. But they concluded that both pathways were of equal importance, whereas Parmentier and Eyssen (1974) determined that the indirect pathway predominated.

Recently, Freier and Hartman at Iowa State University isolated a pure culture of cholesterol-reducing bacterium from a swine sewage lagoon sample (1991). The isolate has been classified tentatively as a Eubacterium sp. strain HL. This isolate is a small, anaerobic, non sporing, Gram-positive rod that reduced almost 90% of the cholesterol in growth medium to coprostanol.

In the present investigation, an attempt has been made to elucidate the pathways of biohydrogenation of cholesterol by Eubacterium HL. The reaction has been carried out with [4-^{3}H, 4-^{14}C]-cholesterol as substrates in a growth medium under anaerobic conditions.

EXPERIMENTAL DESIGN

[4-^{14}C]-Cholesterol was purchased from Amersham Inc. (Arlington Heights, IL), and [4-^{3}H]-cholesterol was prepared by using modifications of published procedures for preparation of unlabeled cholesterol. Cholesterol derivatives were prepared initially with deuterium label and then spectroscopically characterized. Tritiated materials were prepared by using the optimized procedures. Stereochemistry of introduction of label was determined by nuclear magnetic resonance (NMR).

[4-^{3}H, 4-^{14}C]-Cholesterol was incorporated into a liposome preparation (Freier and Hartman, 1991) and incubated for five days with a recently isolated monoculture of coprostanol-producing bacteria (Eubacterium sp. strain HL). The coprostanol was isolated by thin-layer chromatography. The amount of ^{3}H and ^{14}C in the cholesterol substrate and coprostanol product was determined by dual-label counting techniques on the liquid scintillation counter. The results will be interpreted to determine the specific metabolic pathway for coprostanol synthesis in this bacterium.

EXPERIMENTAL PROCEDURE

Materials and Methods

Synthesis of [4-^{2}H]-cholesterol

The scheme for this synthesis is shown in Figure 2. This sequence provides label in the 4 position as a mixture of α and β isomers, but is much more concise than the procedures of Lockley et al. (1978) to the individual stereoisomers.

Cholesta-3,5-dienyl-3-acetate synthesis

Cholesta-3,5-dienyl-3-acetate was prepared by the following procedure using a modification of the method of Dauben and Eastham (1950). 4-Cholesten-3-one (0.5 g, 1.30 mmole) was dissolved in (2.5 ml, 22.7 mmole) isopropenyl acetate with (0.002 ml, 0.038 mmole) H_2SO_4. The solution was refluxed for 2 hours under N_2 balloon attaching it. At one hour of reflux, the acetone formed was removed via a needle through the septum with an aspirator vacuum protected by a $CaSO_4$ drying tube. At the end of the second hour, (0.02 g, 0.24 mmole) anhydrous sodium acetate was added to quench the reaction. The mixture was concentrated at reduced pressure. The residual bluegreen fluorescent oil was diluted with a few ml of chloroform and then decanted from the sodium acetate into about 6 ml of methanol. Additional chloroform was added

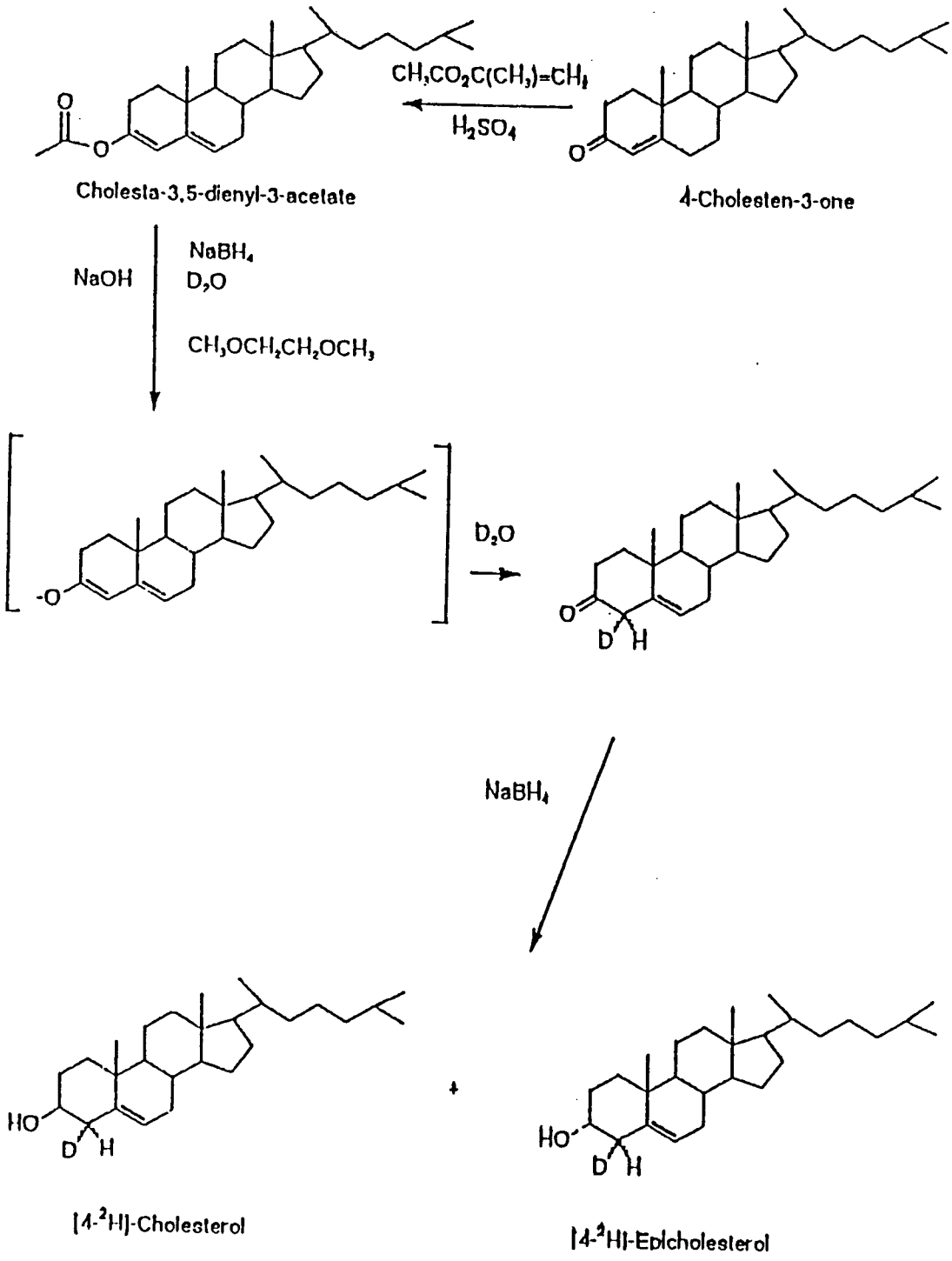

Figure 2. Synthesis of [4-^{2}H]-cholesterol

to bring the oil into solution in the refluxing methanol. Seeding and slow cooling of this solution yielded 0.32 g (58%) cholesta-3,5-dienyl-3-acetate with mp 75.5-77°C.

[4-^{2}H]-cholesterol synthesis

$NaBH_4$ (30 mg, 0.79 mmole) and NaOH (8.5 mg, 0.21 mmole) were added to 1 ml 1,2-dimethoxyethane, then (0.03 ml, 1.66 mmole) of D_2O was added to the mixture. Cholesta-3,5-dienyl-acetate (45 mg, 0.11 mmole) was added to the solution. The mixture was stirred for 96 hours at room temperature. The mixture was extracted with hexanes and washed with water four times. After drying with $MgSO_4$, the hexanes was evaporated under reduced pressure. The residue was separated by flash chromatography with 1:13 (v/v) CH_3CN/CH_2Cl_2 (Still et al., 1978). The yield is 22 mg (59%).

Synthesis of [4-^{2}H]-5-Cholesten-3-one

[4-^{2}H]-Cholesterol (0.0064 g, 0.017 mmole) was dissolved in 0.56 ml CH_2Cl_2. Anhydrous $CaCO_3$ powder (0.007 g, 0.070 mmole) was added to the solution. Pyridininum chlorochromate (0.012 g, 0.571 mmole) was added, and the mixture stirred for 30 min under nitrogen at 25°C. A saturated NaCl solution (5 ml) then was added. The suspension was filtered and then thoroughly extracted with diethyl ether (10 ml) three times. The ether layer was filtered through anhydrous $MgSO_4$ and

evaporated to dryness under reduced pressure to yield [4-^{2}H]-5-cholesten-3-one (0.006 g; 93% yield). TLC analysis showed a single spot at Rf 0.55 in 4:6 (v/v) ethyl acetate : hexanes (Parish and Chitrokorn, 1983).

Synthesis of [4-^{3}H, 4-^{14}C]-cholesterol

[4-^{3}H]-Cholesterol was prepared in the same manner as [4-^{2}H]-cholesterol, with the substitution of 0.04 ml of 50 mCi/mmole tritiated water (Amersham Inc., Arlington Heights, IL) for D_2O.

The product obtained after purification by column chromatography on silica gel (0.040-0.063 mm) had specific radioactivity 8.7×10^{-4} mCi/μl [4-^{14}C]-Cholesterol was purchased from Amersham Inc. (Arlington Heights, IL) and had a specific radioactivity of 70 mCi/mmol. The mixture of [4-^{3}H]-cholesterol with [4-^{14}C]-cholesterol is referred to as [4-^{3}H, 4-^{14}C]-cholesterol.

Proof for location of isotope in [4-^{2}H]-cholesterol

Mass spectrometry was used to identify [4-^{2}H]-cholesterol by using Finingan 4000 GC-MS. Deuterium content was calculated from M, M+1, M+2, M+3, and M+4 peaks in the mass spectra of the steroids.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian 500 spectrometer (operating at 500 MHz

for proton nuclei) by using $CDCl_3$ or pyridine-d_5 as the solvent and tetramethylsilane (Me_4Si) as the internal standard.

^{13}C NMR spectra were recorded on a VXR-300 spectrometer (operating at 75 MHz for carbon nuclei) by using $CDCl_3$ as both the solvent and internal standard.

Deuterium NMR spectrum was recorded on a VXR-300 spectrometer (operating at 46 MHz) using chloroform as solvent.

Incubation and purification methods

The basic cholesterol medium used throughout the study contained the follwing components per liter: casitone 10 g; yeast extract 10 g; lecithin 1 g; cholesterol 2 g; resazurin 1 mg; sodium thioglycolate 0.5 g; and dihydrate $CaCl_2$ 1 g (Freier and Hartman, 1991).

[4-^{3}H]-Cholesterol (in 95% ethanol 0.3 μl, 2.6 μCi) and [4-^{14}C]-cholesterol (in 95% ethanol 0.3 μl, 2.5 μCi) were added to 1 ml of basic cholesterol medium that contains (9 μl) inoculum. After incubation of the labeled sterols with Eubacterium HL for 5 days at 37°C under strictly anaerobic conditions, the sterols were extracted twice with 2 ml chloroform-methanol (2:1, v/v). The nonpolar phase was concentrated to about 100 μl. Chromatography was performed on silica gel-H thin-layer plate (0.75 mm thickness) with hexanes:ethyl acetate (75:25, v/v) as solvent. When stained

with a 0.2% ethanolic solution of 2,7-dichlorofluorescein, two bands were observed. Bands corresponding to cholesterol and coprostanol were scraped into scintillation vials for isotopic measurements.

Determination of isotope in different positions in coprostanol

Determination of the position of tritium in the isolated coprostanol was carried out by oxidation of the coprostanol to coprostanone and base-catalyzed exchange of the hydrogens at the 2 and 4 positions. This procedure was developed by using deuterium and subsequently applied to the tritiated compound. The following procedure was adapted from the method of Parish and Chitrokorn (1983). Coprostanol (24 mg, 0.062 mmole) was dissolved in 2 ml CH_2Cl_2. Anhydrous $CaCO_3$ powder (25 mg, 0.25 mmole) was added to the solution. Pyridinium chlorochromate (45 mg, 0.209 mmole) was added. The product was purified by flash chromatography with hexanes:ethyl acetate (8:2, v/v) as solvent. The identity and purity of the compound was further checked by NMR.

Coprostanone (4 mg, 0.010 mmole) was heated at 95°C in a solution of sodium methoxide (15 mg, 0.28 mmole) in dioxane (100 µl) and deuterated water (50 µl, 2.8 mmole, 99.9 atom %D) for 24 hours. The reaction mixture was cooled and acidified with HCl and extracted with diethyl ether. The ether extract was washed with water until neutral; then the solvent was

dried over $MgSO_4$ and evaporated. The residue was checked with NMR. These conditions completely exchanged all hydrogens at C-2 and C-4. This treatment is expected to remove all isotope in the C-4 position after incubation with the cholesterol-reducing bacteria.

The procedure was carried out on radioactive coprostanol in exactly the same manner, but without the NMR spectra. To confirm that exchange was complete, the sample was resubmitted to the exchange conditions. No further loss of tritium was observed.

Radioactivity assay

Tritium and ^{14}C in the double-labeled sterols were determined by the two-channel ratio method in a Beckman L5-8000 liquid scintillation spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA)

RESULTS and DISCUSSION

Proof for Location of Isotope in [4-^{2}H]-Cholesterol

Determination of structure by the mass spectrum

Mass spectra were used to help establish the structure of synthesized compound. Two samples of undeuterated cholesterol were run by solid probe at 70 eV; two samples of deuterated cholesterol were run in the same manner. Figure 3 shows the mass spectrum of cholesterol, and Figure 4 shows the mass spectrum of [4-^{2}H]-cholesterol.

The averaged results for the molecular ion region were as shown in Table 1.

To determine the amounts of ^{2}H present in the

Table 1. Mass data for cholesterol and [4-^{2}H]-cholesterol

M/Z	Normalized peak height	
	Cholesterol	[4-^{2}H]-Cholesterol
384	2.20	0.74
385	1.01	1.69
386	100.00	46.52
387	29.08	100.00
388	4.62	47.01
389	0.48	11.02
390	0.05	2.39

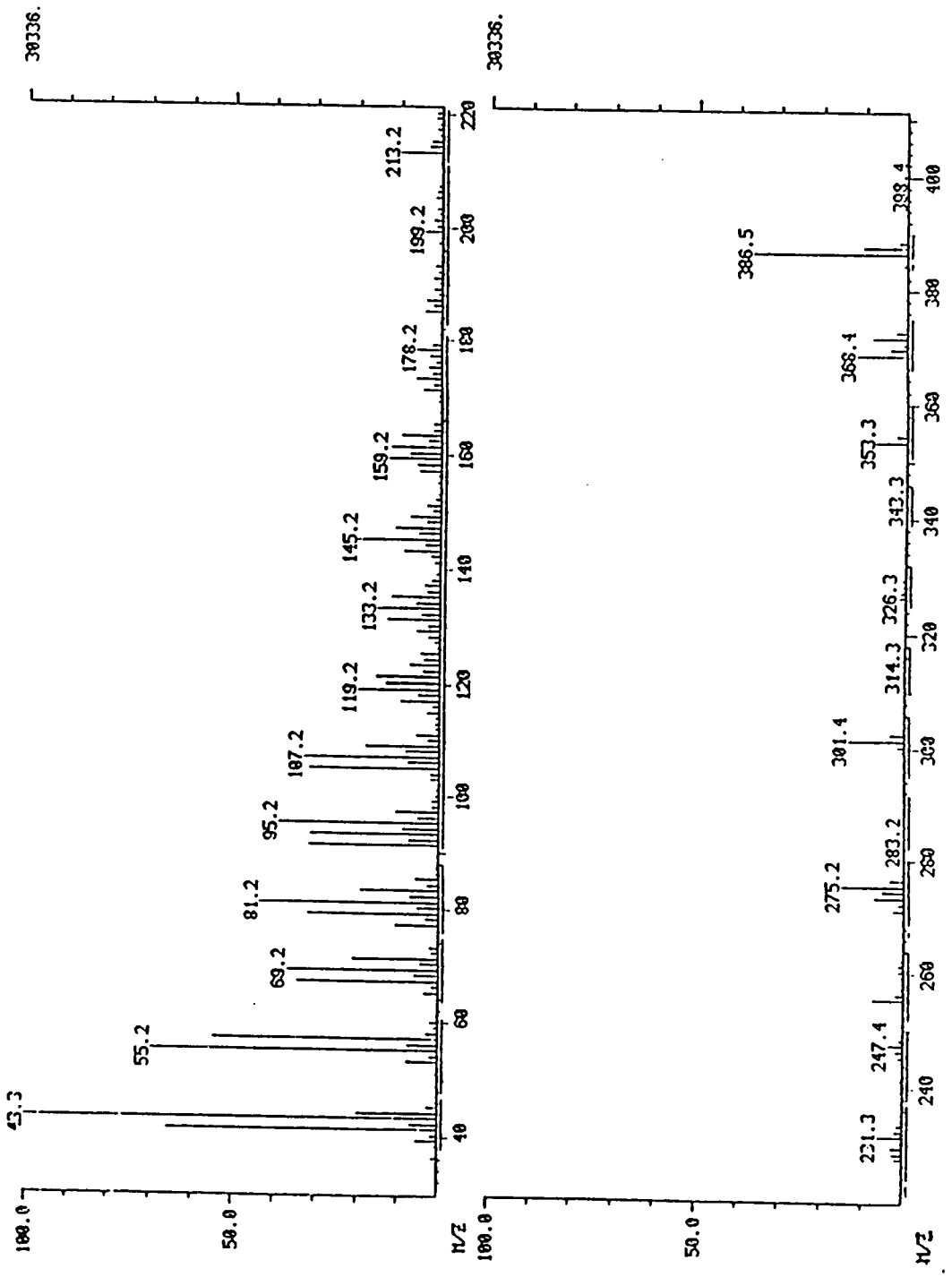

Figure 3. Mass spectrum of cholesterol

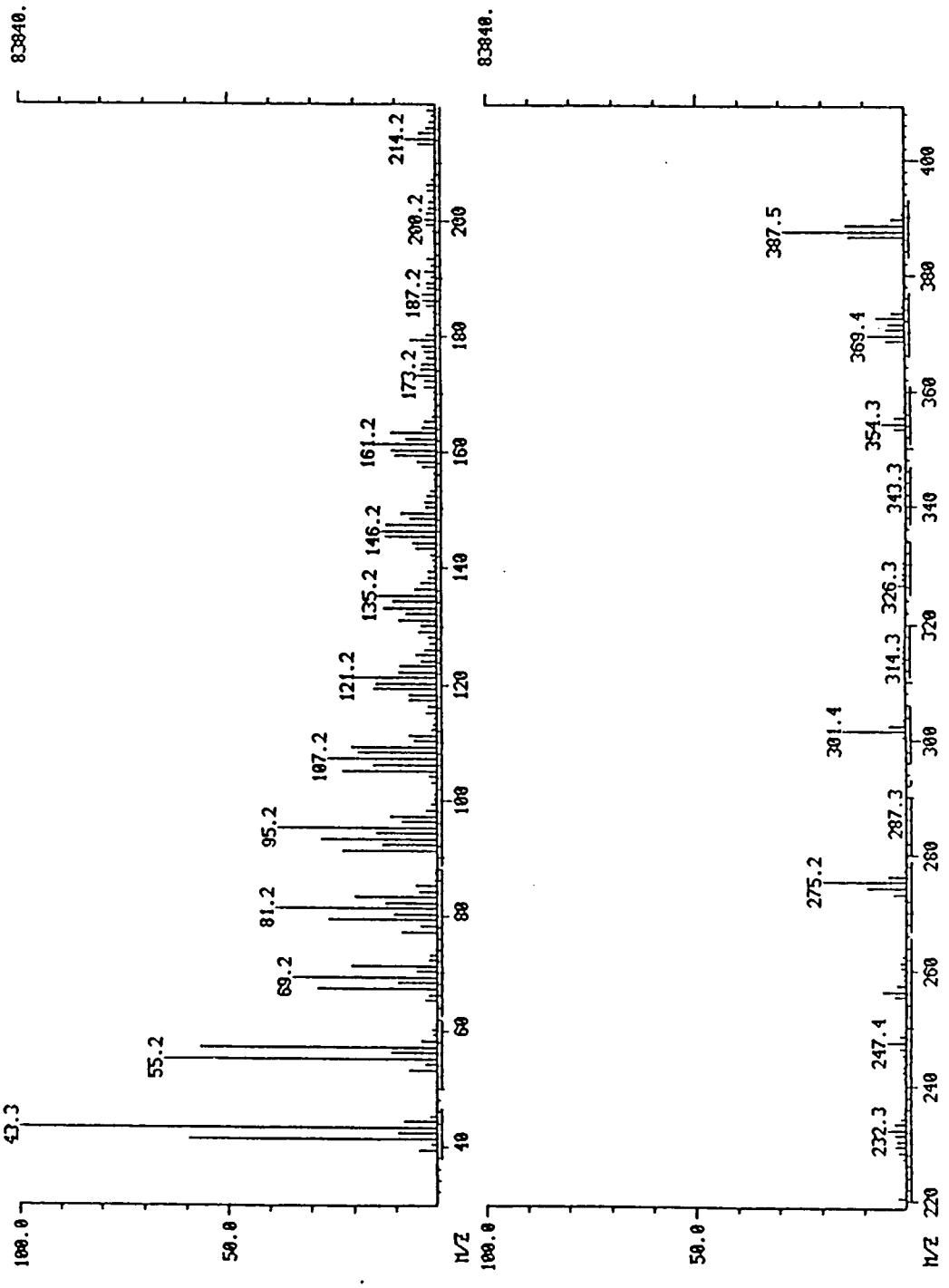

Figure 4. Mass spectrum of [4-^{2}H]-cholesterol

isotopically labeled cholesterol mixture, it is necessary to correct the [4-^{2}H]-cholesterol values for contributions from carbon-13. The measured values for unlabeled cholesterol are very close to the theoretical values calculated using natural abundance tables; we used the measured values for the following analysis. As can be seen in Table 1, the M-1(1.01) and M-2(2.20) peaks are small, so these fragmentation pathways were ignored.

The following solution was used:

386			D0
387		(D0+1)	D1
388	(D0+2)	(D1+1)	D2
389	(D1+2)	(D2+1)	D3
390	(D2+2)	(D3+1)	D4

The solution is now complete:

386	46.52	D0=46.52
387	(46.52x.2908)+D1= 100	D1=86.47
388	(46.52x.0462)+(86.47x.2908)+D2=47.01	D2=19.72
389	(86.47x.0462)+(19.72x.2908)+D3=11.02	D3= 1.29
390	(19.72x.0462)+(1.29x.2908)+D4= 2.39	D4= 1.10

Expressed as fractional abundances, the results are: 30% undeuterated cholesterol, 56%-D1 and 13%-D2. Further examination was carried out to obtain evidence of where cholesterol was labeled.

Proton NMR assignments for cholesterol and deuterated cholesterol

The 500-MHz proton magnetic resonance spectra of

cholesterol and deuterated cholesterol have been recorded in both pyridine and chloroform.

Figures 5 and 6 show the 500-MHz ^1H NMR spectra of cholesterol and deuterated cholesterol in chloroform. Figures 7 and 8 show the 500-MHz ^1H NMR spectra of cholesterol and deuterated cholesterol in pyridine. Table 2 shows chemical shift assignments and integration for cholesterol and deuterated cholesterol in $CDCl_3$. Table 3 shows chemical shift assignments and integration for cholesterol and deuterated cholesterol in pyridine-d_5. Integration of the resonance at 2.68-2.55 ppm in pyridine solvent and 2.33-2.18 ppm in chloroform solvent showed one proton each as compared with two protons each in the undeuterated compound. From these spectra, it can be concluded that one H-4 proton contributes to the resonance at 2.68-2.55 ppm in pyridine solvent and at 2.33-2.18 ppm in chloroform solvent. These spectra show that deuterium is at C-4. But, because the C-4α and C-4β resonances can not be resolved, the spectra provide no evidence of the stereochemistry of deuteration.

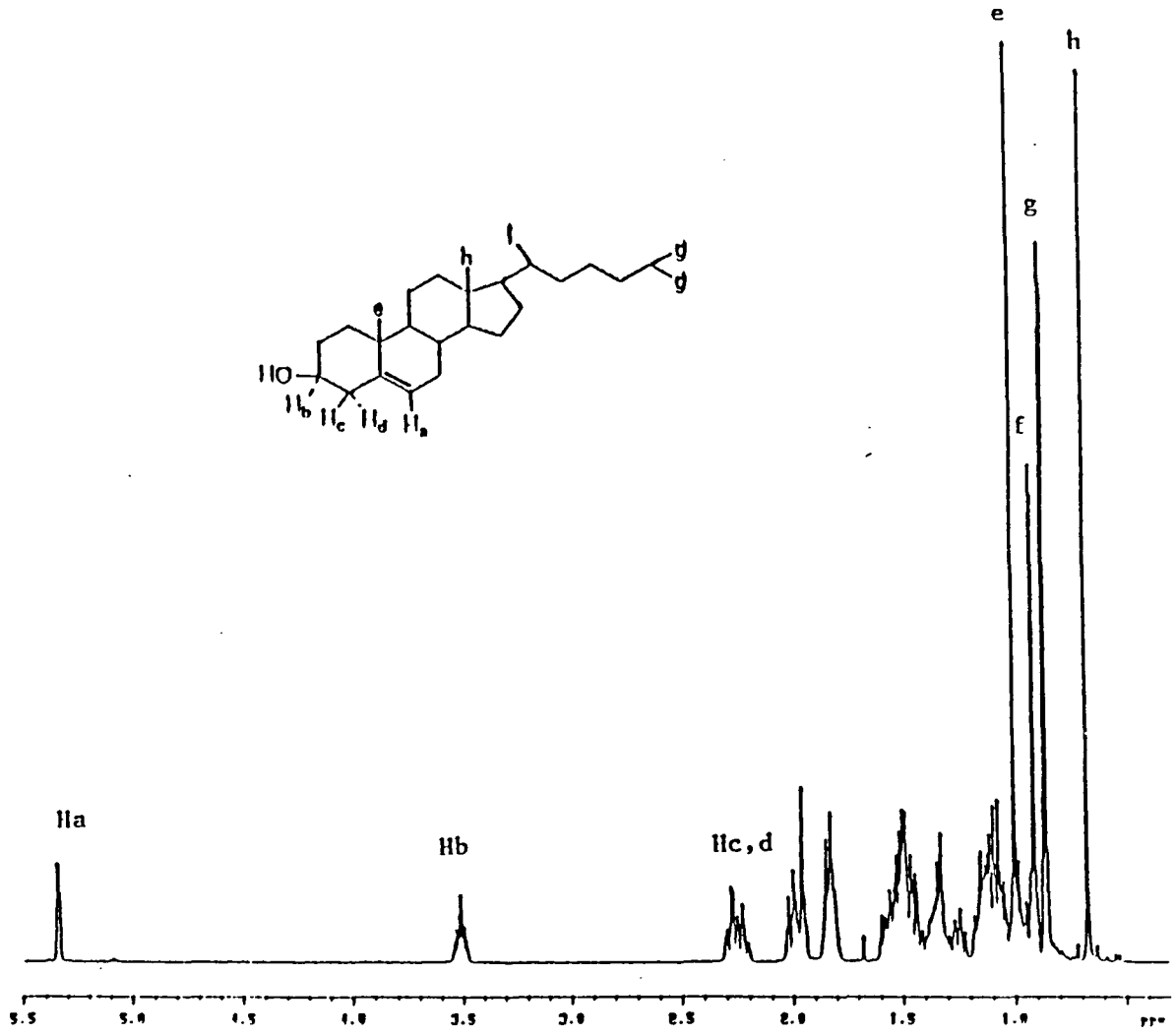

Figure 5. 500-MHz ^{1}H NMR spectrum of cholesterol in chloroform.

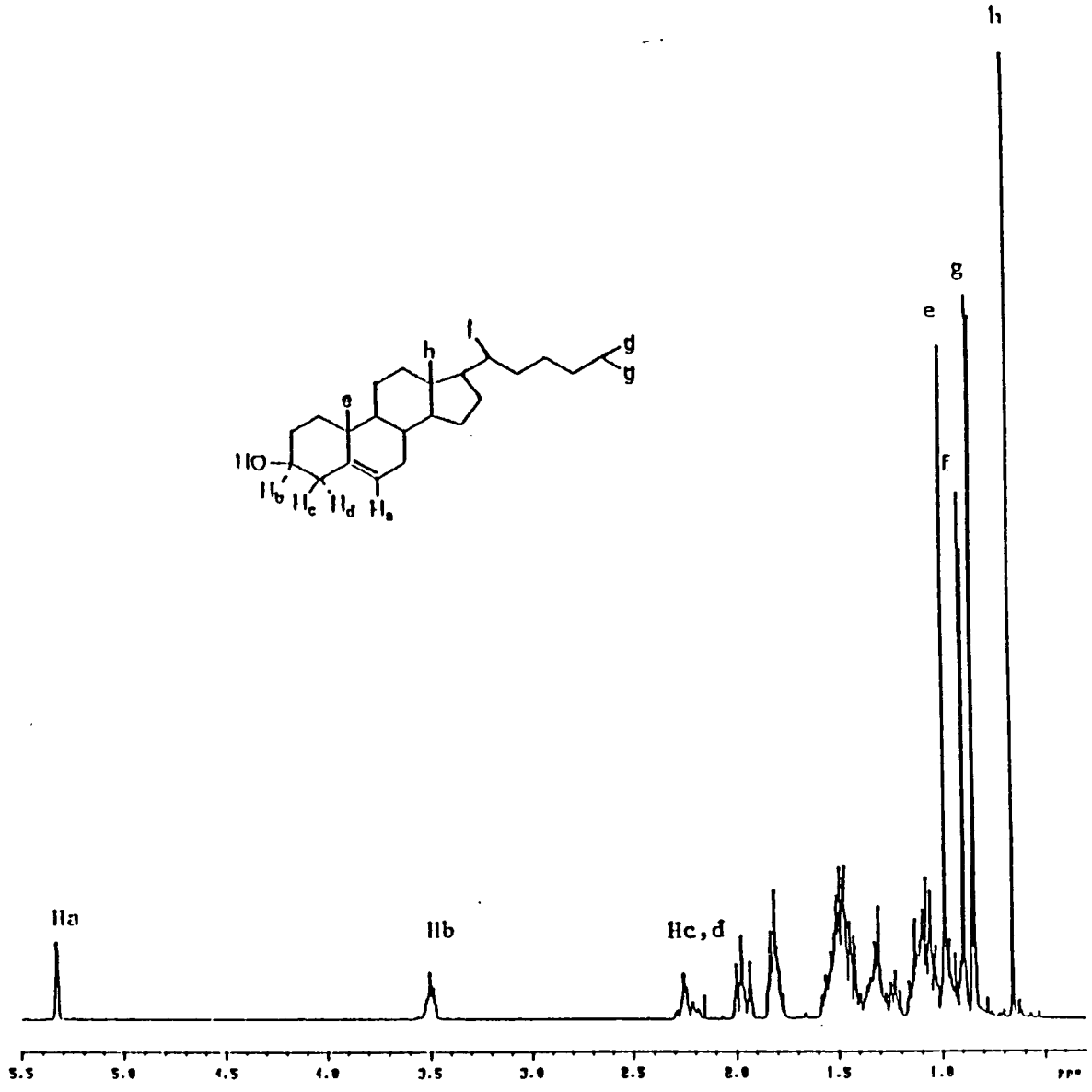

Figure 6. 500-MHz ^{1}H NMR spectrum of deuterated cholesterol in chloroform

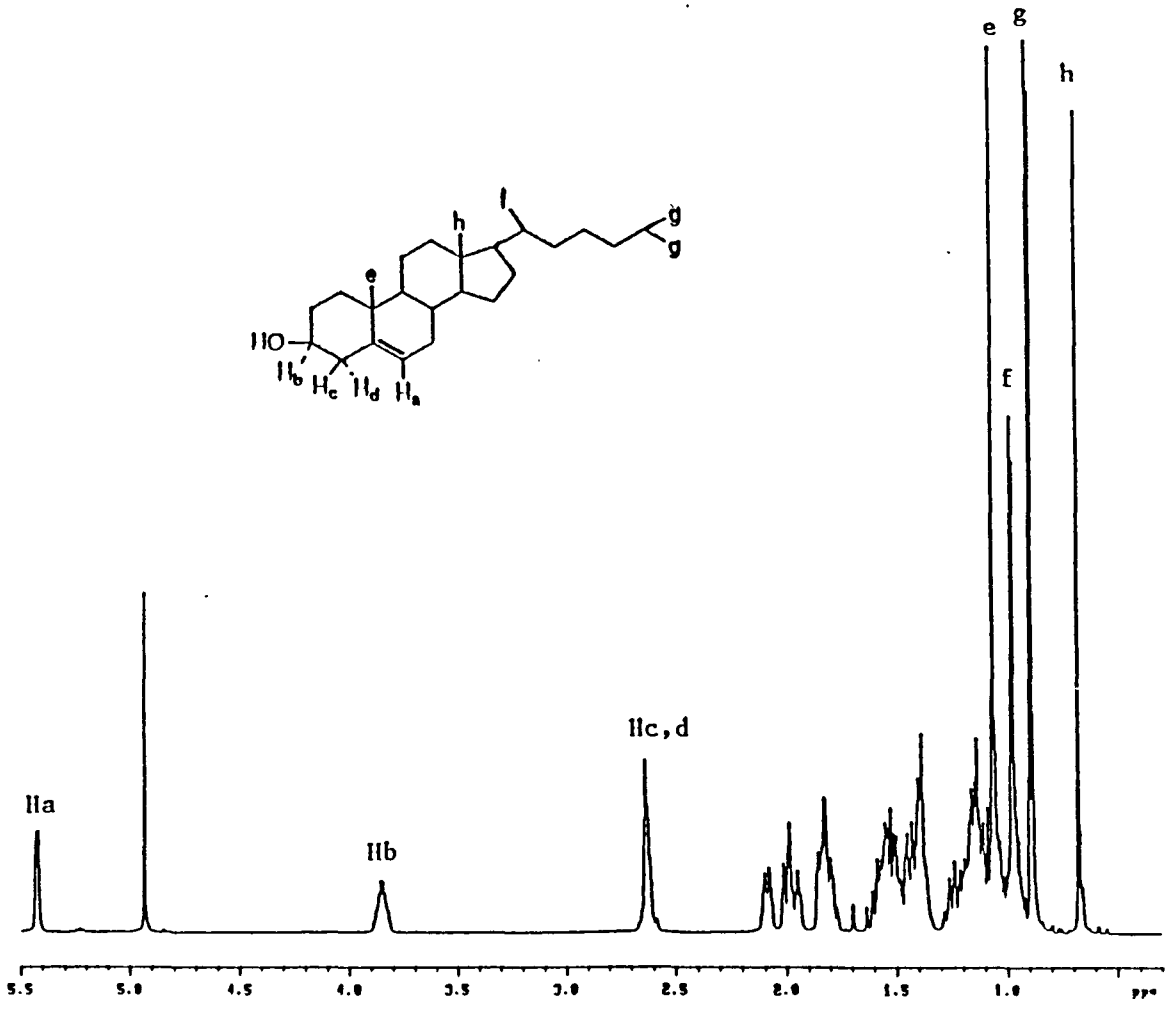

Figure 7. 500-MHz ^{1}H NMR spectrum of cholesterol in pyridine-d_5

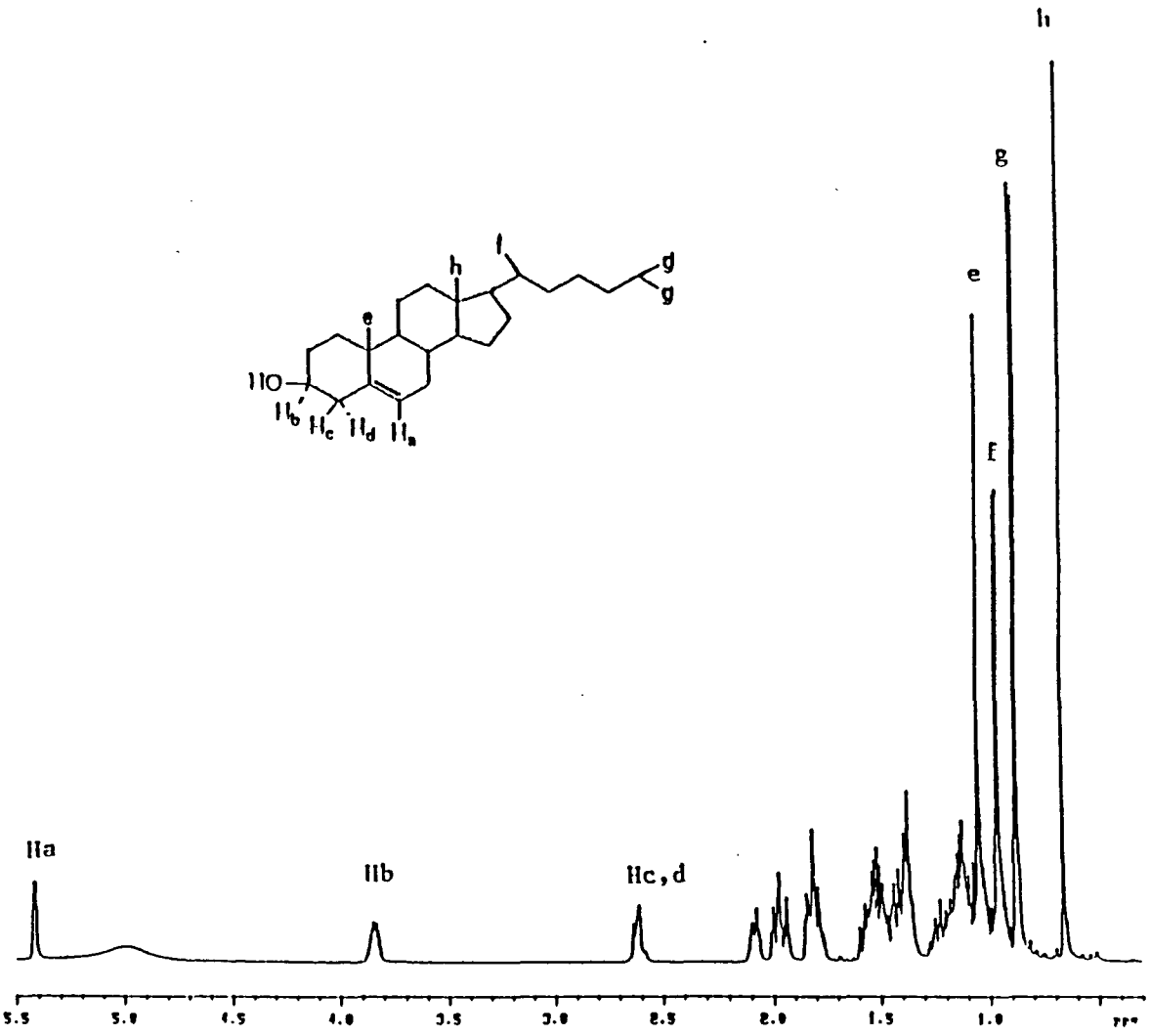

Figure 8. 500-MHz ^{1}H NMR spectrum of deuterated cholesterol in pyridine-d_5

Table 2. Proton chemical shift assignments and integral for cholesterol and deuterated cholesterol in $CDCl_3$

Proton location[a]	Chemical shift(ppm)[b]	Cholesterol integral	^{2}H-Cholesterol integral
6	5.34	1.0	1.0
3	3.51	1.0	1.0
4a ,4e	2.26	2.1	1.2

[a] Number refers to carbon number of cholesterol, a to axial, and e to equatorial.

[b] Assignments from the reference of Barry et al (1973).

Table 3. Proton chemical shift assignments and integral for cholesterol and deuterated cholesterol in pyridine-d_5

Proton location[a]	Chemical shift(ppm)[b]	Cholesterol integral	^{2}H-cholesterol integral
6	5.43	1.0	1.0
3	3.85	1.0	0.9
4a, 4e	2.62	2.1	1.0
2e	2.11	1.0	0.9
7a, 7e	2.03	2.0	2.0
1a, 1e, 2a	1.80	2.9	2.9

[a] Number refers to carbon number of cholesterol, a to axial, and e to equatorial.

[b] Assignments from the reference of Sawan et al (1979).

^{13}C Nuclear magnetic resonance spectra of cholesterol and deuterated cholesterol

Although proton NMR gave us much information in structural and conformational studies of cholesterol and deuterated cholesterol, the overlap of many lines with closely similar chemical shifts causes great difficulty in explaining the spectra. This is one thing, the other thing is that so far I did not show strong evidence for the deuterium position.

^{13}C NMR spectra are in general much more informative than ^{1}H NMR spectra for structural analyses of complex molecules.

^{13}C NMR spectra were recorded on a VXR-300 spectrometer (operating at 75 MHz for carbon nuclei) using $CDCl_3$ as both the solvent and internal standard (77.06 ppm) with proton decoupling. The chemical shifts are shown in Table 4. They are also displayed in Figure 9 and Figure 10.

When hydrogen is replaced by deuterium the carbon-4 undergoes an appreciable upfield shift of about 0.2 ppm per D atom (Silverstein et al., 1981). In this experiment, isotope effect resulted in a slight upfield shift of C-4 absorption (0.25 ppm). In addition, the carbon-4 resonance appears as 1:1:1 triplet due to the spin coupling of deuterium (I=1) with carbon. The resonances of the carbons-3, 5, and 6 are broadened somewhat due to unresolved carbon-deuterium coupling (data not shown). Figure 11 shows an expansion of the carbon-4 region. The coupling constant is 18 Hz. A normal coupling

constant Table 4. ^{13}C Chemical shifts for cholesterol and deuterated cholesterol in $CDCl_3$ solvent in ppm relative to $CDCl_3$ at 77.06 ppm

Carbon	Deuterated cholesterol	Cholesterol[a]	Cholesterol[b]
1	37.3	37.3	37.5
2	31.7	31.6	31.6
3	71.8	71.6	71.3
4	42.2, 42.0, 41.7	42.3	42.4
5	140.8	140.8	141.2
6	121.8	121.6	121.3
7	32.0	31.9	32.0
8	32.0	31.6	32.0
9	50.2	50.1	50.5
10	36.6	36.5	36.5
11	21.1	21.1	21.2
12	28.3	28.2	28.3
13	42.4	42.2	42.4
14	56.8	56.8	56.9
15	24.3	24.3	24.3
16	39.8	39.8	40.0
17	56.2	56.2	56.5
18	11.9	11.9	12.0
19	19.4	19.4	19.4
20	35.8	35.8	35.8
21	18.8	18.8	18.8
22	36.2	36.2	36.4
23	23.9	23.9	24.1
24	39.6	39.5	39.6
25	28.1	28.0	28.0
26	22.6	22.6	22.5
27	22.9	22.8	22.8

[a] Assignments made in this work.

[b] Assignment from Breitmaier and Voelter (1987).

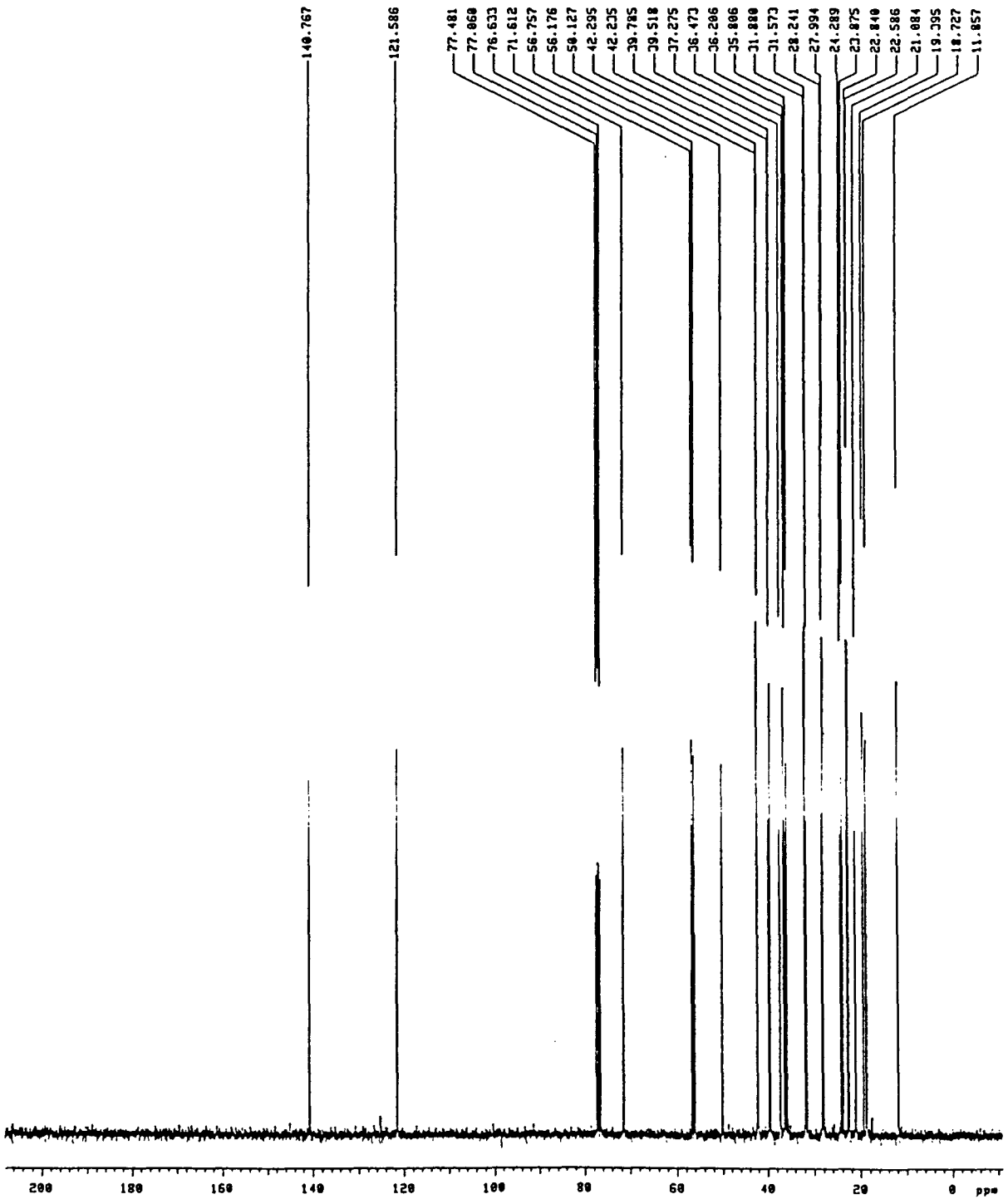

Figure 9. ^{13}C NMR spectrum of cholesterol

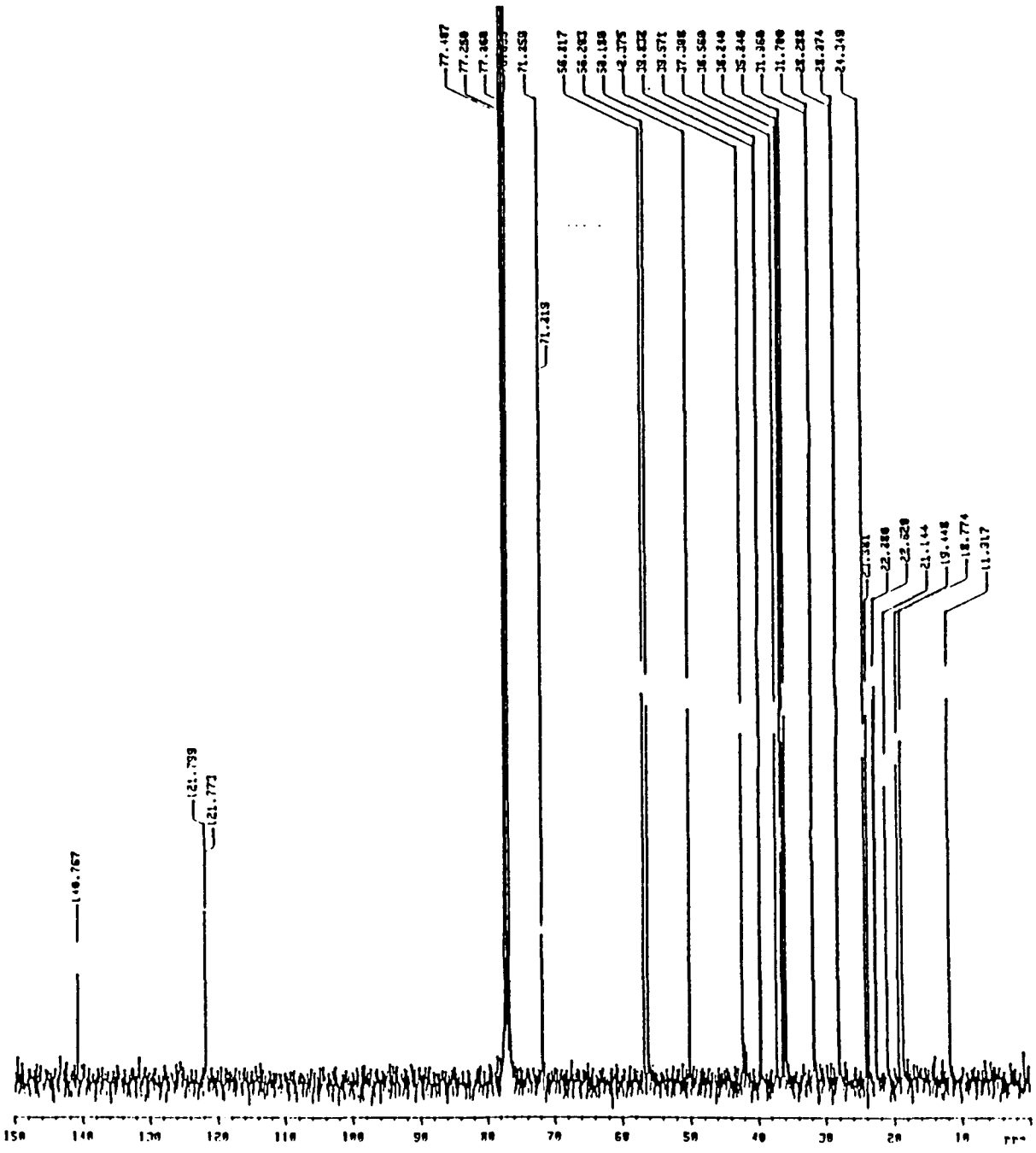

Figure 10. ^{13}C NMR spectrum of deuterated cholesterol

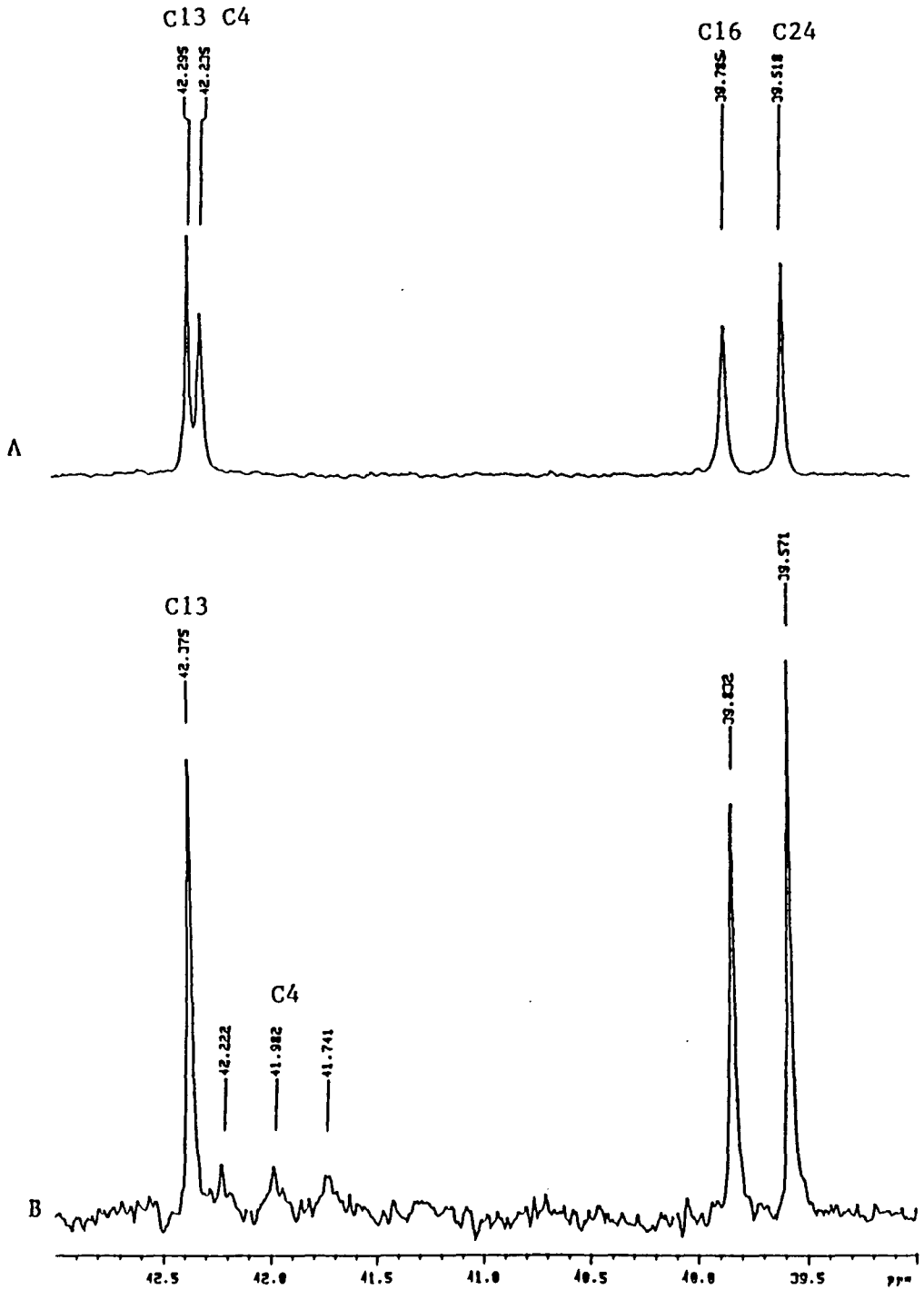

Figure 11. The expansion of the carbon-4 region of ^{13}C spectra of cholesterol (A) and deuterated cholesterol (B) in $CDCl_3$. Note that the C-4 carbon has been broadened, shifted, and split by the deuterium.

deuterium coupled to ^{13}C is 18.5 Hz (Silverstein et al., 1981).

The introduction of a second deuterium on the carbon-4 usually results in a disappearance of the carbon-4 signal, or 5 multiplicities. However, appreciable upfield shifts and line broadening are observed at the carbons-3, 5, maybe carbon-6 (Leyden and Cox, 1977). In this experiment, line broadening was observed at C-3, 5 and 6. Interestingly, only a very small (about 10%) residual D0 signal remains for C-4. This is in contrast to the 30% D0 seen in the mass spectrum.

Deuterium NMR spectrum

To confirm the above evidence and to find whether deuterium was present at a position other than C-4, Deuterium NMR spectrum of deuterated cholesterol was recorded at 46 MHz in chloroform (Figure 12). The chemical shifts (2.24 ppm) observed for deuterium are the same as seen for the C-4 proton (2.26 ppm) in Figure 5 and 6.

From these spectra, it can be concluded that only one proton is attached to C-4 position. In other words, one deuterium was labeled on H-4. However, there is no strong evidence to show the deuterium is axial or equatorial. This question remained to be answered.

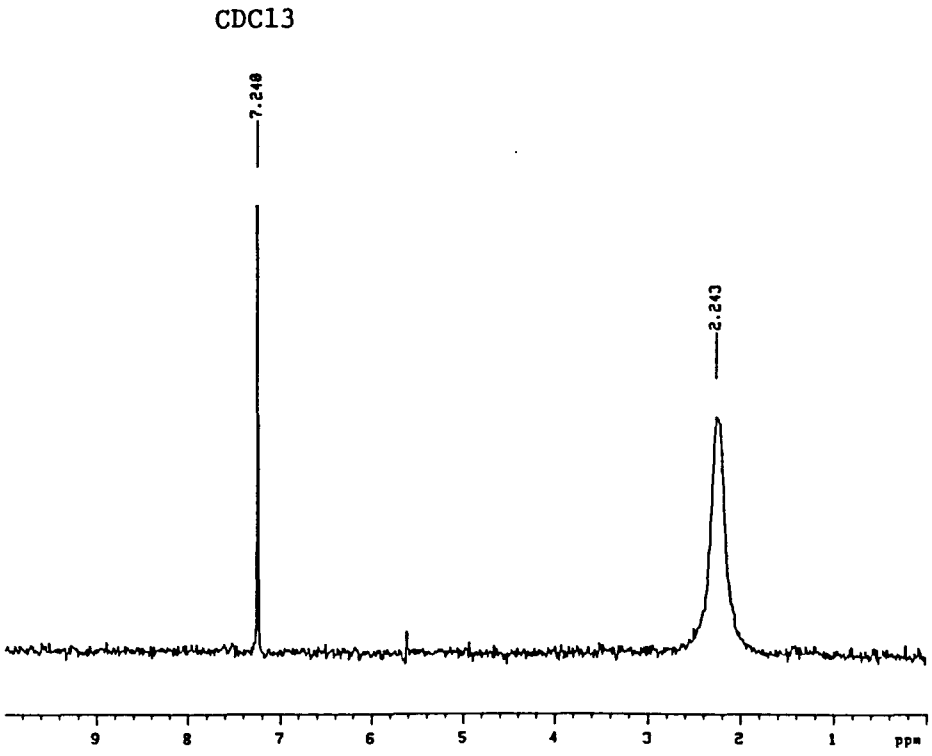

Figure 12. Deuterium NMR spectrum of deuterated cholesterol

The nuclear overhauser effect (NOE) in structural analysis of deuterated cholesterol

NOE experiments are used to provide information about molecular geometries. Experimentally, the method involves the saturation of one signal in the spectrum and observation of changes in the intensities in the other signals. The magnitude of the intensity changes depend upon internuclear distances between the nuclei concerned. These intensity changes arise from perturbations of the the relaxation processes, which lead to thermal equilibrium between the spin states. The relaxation of a given nucleus is affected by all surrounding nuclei and the relaxation from other nuclei is dominated by short range interactions and is proportional to r^{-6} where r is the distance separating the nuclei.

Two dimensional NMR experiment (NOESY) Two dimensional NOE spectra allow the measurement of fairly weak NOE effects. The 2D NOE experimental data are presented in Table 5. Figure 13 represents 2D NOE spectrum of deuterated cholesterol. Some of the relevant assignments are presented on the structure. From the spectrum and data, I note that H-4 has a weak NOE with C-19, a modetate NOE with H-3 and the strongest NOE with H-6. That means that some deuterium is in the axial position (β). Deuterium in the equatorial position H-4 should have the strong NOE with C-19 and a weaker NOE with

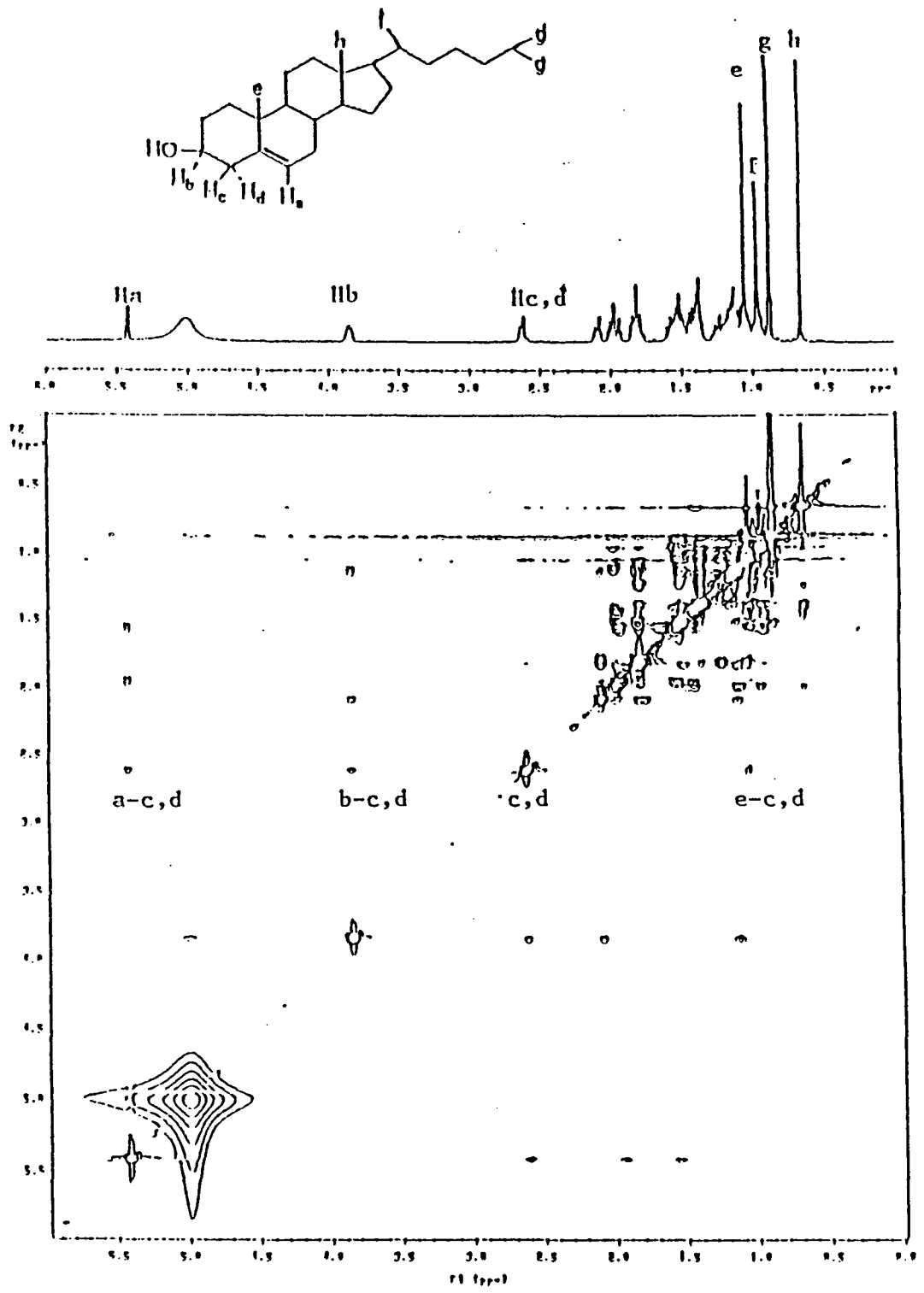

Figure 13. 2D NOE spectrum of deuterated cholesterol

Table 5. Two dimensional NOE spectroscopy (NOESY), cross peak to C-4 (2.6 ppm)

Signal	Shift (ppm)	Relative intensity	Distance[a] (Å)
C-6	5.4	1	3.30
C-3	3.8	0.3	2.49
C-19	1.0	0.2	2.24

[a] Distance is calculated by PCMODEL using the mmx force field.

H-3, H-6. To determine the ratio of axial to equatorial deuterium, a quantitative measure of the NOE values is needed.

One dimensional NMR experiment for deuterated cholesterol

Because NOESY allowed us to establish that both stereochemical dispositions of deuterated cholesterol were present, I hoped that one dimensional (1D) NOE spectroscopy could establish the ratio of stereochemical dispositions of deuterated cholesterol with more confidence. The 1D NOE results are presented in Table 6.

Irradiation at C-4 of deuterated cholesterol led to enhancement at the C-19 methyl group in an amount only 38% of that seen with unlabeled cholesterol. This observation suggests that 62% of the 4β position may bear deuterium. Mass spectral data show 0.82 deuterium in the cholesterol molecules; in other words, 76% of deuterium is in the axial position (β). However, irradiation at C-19, the inverse

experiment, led to 76% as much NOE to C-4 in the deuterated compound as compared with the unlabeled cholesterol. This observation corresponds to 65% of the deuterium in the β position, assuming the mass spectrum data are correct. Thus, I estimate about 65%-76% of the deuterium is in the β position (or about β:α=2:1). The problem is that the H-4α and H-4β peaks are not separate in ^{1}H NMR spectrum of cholesterol. If H-4α and H-4β can be resolved, I could determine directly how much deuterium is in an axial and how much in an equatorial position.

Table 6. One dimensional NOE spectroscopy

Irradiated at	Shift (ppm)	Signal	Shift (ppm)	^{2}H-Cholesterol enhancement(%)	Cholesterol enhancement(%)	^{2}H/H	Distance[a] (Å)
C-4	2.6	C-6	5.4	4.58	6.69	0.68	3.30
C-4	2.6	C-19	1.0	0.94	2.49	0.38	2.24
C-4	2.6	C-3	3.8	1.76	2.83	0.62	2.49
C-19	1.0	C-3	3.8	0.29	0.23	1.62	
C-19	1.0	C-4	2.6	2.80	3.70	0.76	2.24
C-19	1.0	C-6	5.4	0.13	0.03	4.30	

[a] Distance is calculated by PCMODEL using the mmx force field.

One dimensional NOE experiment for [4-^{2}H]-5-cholesten-3-one Figure 14 shows the 300-MHz ^{1}H NMR spectrum of 5-cholesten-3-one in $CDCl_3$. Examination of this spectrum yielded the complete assignments for the protons on carbon atom number

4 (referred to as Hb and Hc). Doublets are seen at 2.79 ppm and 3.25 ppm with a geminal coupling constant 16.5 Hz. These peaks were assigned to 4α and 4β by NOE spectroscopy. Figure 15 shows the 1D NOE spectrum. On irradiation at C-19 (referred to as d, 1.18 ppm), a strong NOE was seen to Hb (3.25 ppm). On the other hand, no NOE effects were observed at Hc (2.79 ppm). Consequently, Hb is assigned to the axial H-4β and Hc is assigned to the equatorial H-4α. Thus, the amount of deuterium in an axial and equatorial position can be determined in this compound simply by integration.

Table 7 shows chemical shift assignments and integration for 5-cholesten-3-one and [4-^{2}H]-5-cholesten-3-one. Integration of the 3.25 ppm doublet shows a 58% decrease because of deuterium being located at the axial position of C-4, and integration of the 2.79 ppm doublet shows a 28% decrease because of deuterium being located at the equatorial position of C-4. Therefore, 67% {0.58/(0.58+0.28)} of the deuterium is in the β position. This value compares favorably with the two previous estimates of 65% and 76% which were based on NOE on the cholesterol itself. This third estimate, thus provides more evidence for stereochemical location of deuterium and the deuterium also provides an independent check of the value of 17% derived from mass spectral data (30%D0-13%D2).

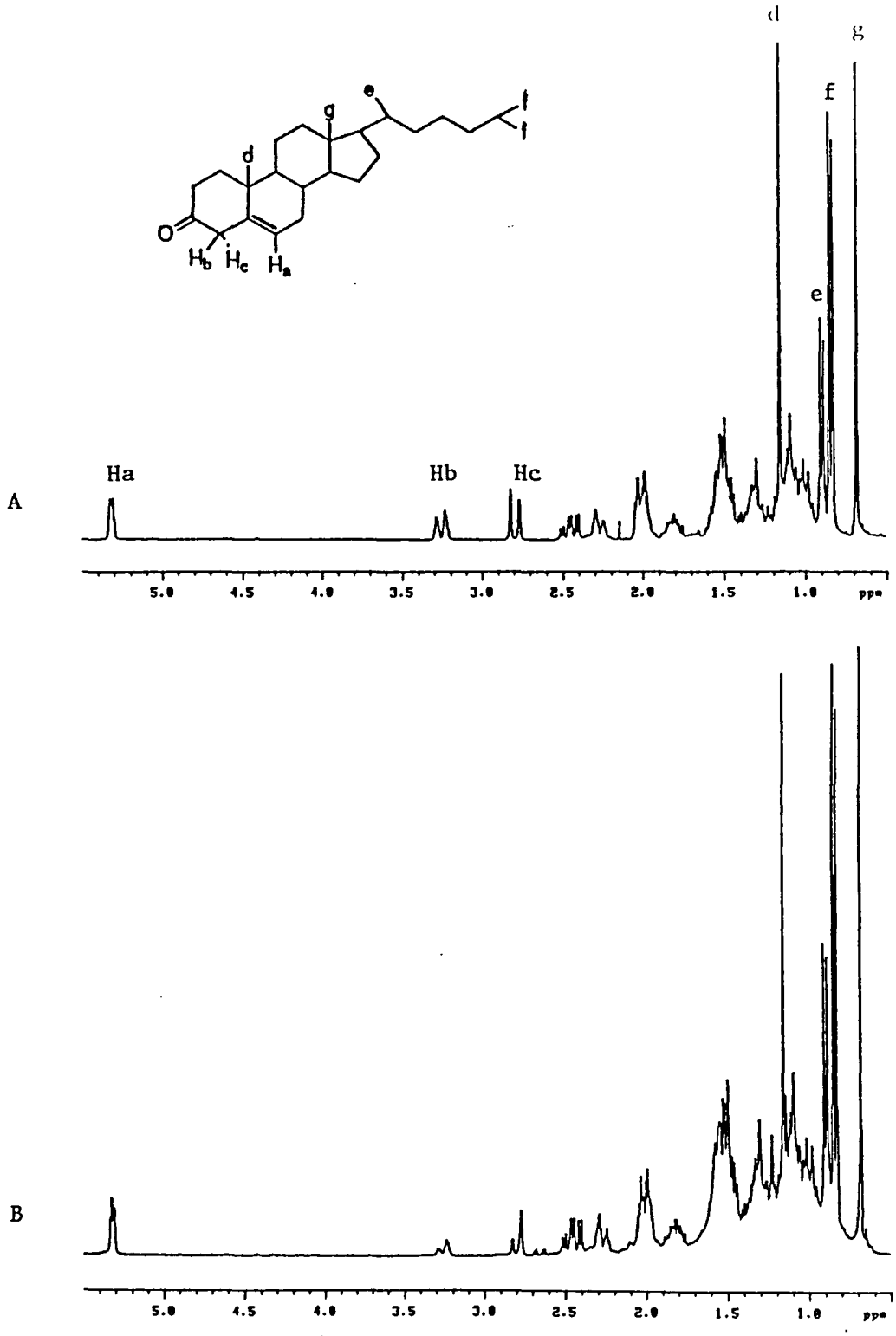

Figure 14. 300-MHz ^{1}H NMR spectra of 5-cholesten-3-one (A) and [4-^{2}H]-5-cholesten-3-one (B) in $CDCl_3$

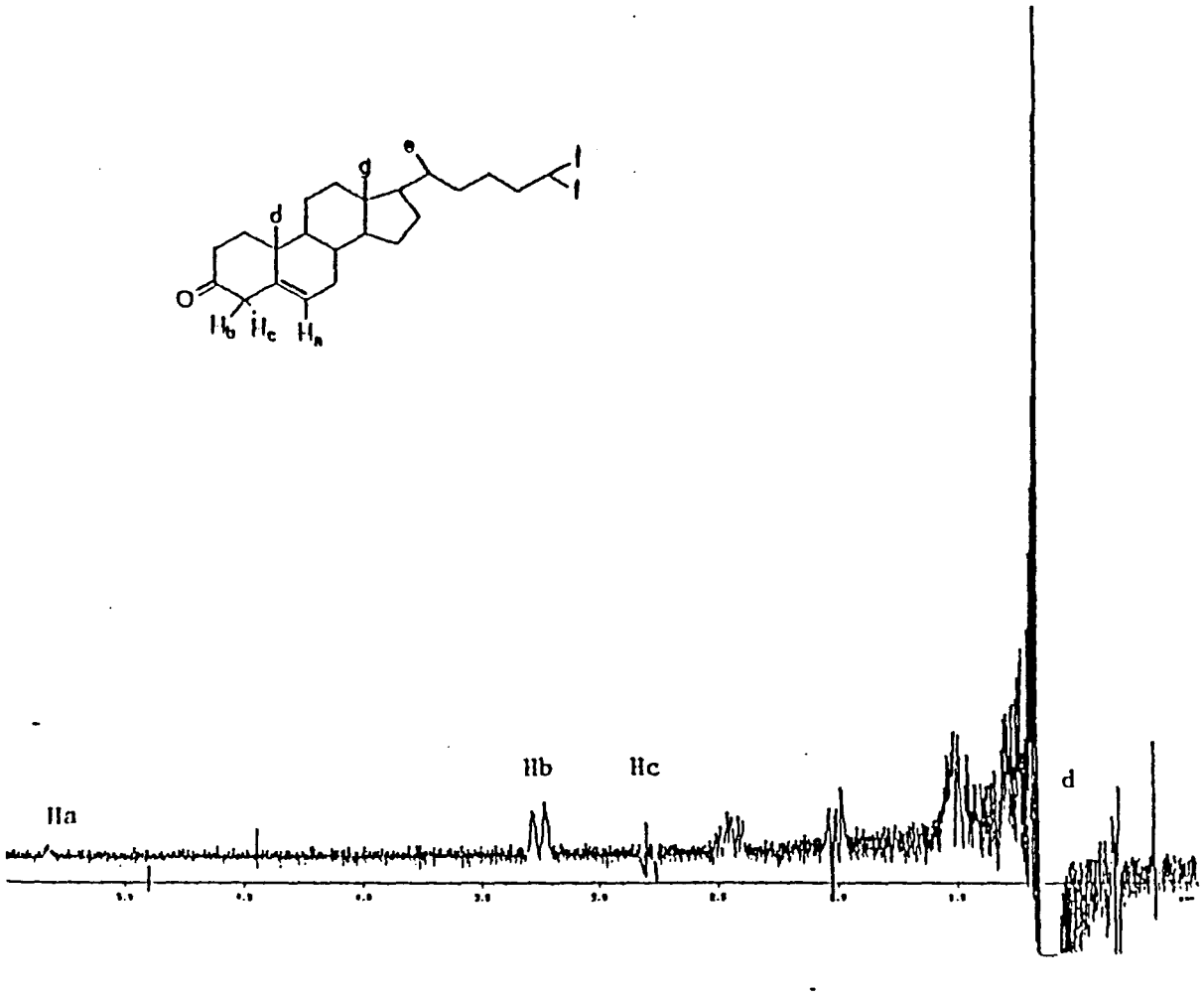

Figure 15. 1D NOE spectrum of 5-cholesten-3-one

Table 7. Chemical shift assignments and integration for 5-cholesten-3-one and [4-^{2}H]-5-cholesten-3-one

Proton location[a]		Chemical shift(ppm)[b]	Integral[c]	Integral[d]
6	(6a)	5.36	1.0	1.0
4β	(4b)	3.25	1.0	0.42
4α	(4c)	2.79	1.0	0.72

[a] Number refers to carbon number in cholesterol

[b] With respect to internal TMS.

[c] Relative area under peak for 5-cholesten-3-one.

[d] Relative area under peak for [4-^{2}H]-5-cholesten-one.

Incubation Studies

Determination of time required for maximal reduction of cholesterol by Eubacterium HL

Table 8 summarizes the changes in [4-^{3}H, 4-^{14}C]-cholesterol as the fermentation of Eubacterium HL proceeded. The maximal yield of reduced sterol (coprostanol) was obtained in 5 days when almost 90% of cholesterol was converted into coprostanol (Figure 16).

As shown in Table 8, the isolated coprostanol had retained 97% ± 2.5% of the tritium originally present in cholesterol.

Table 8. Conversion of [4-^{3}H, 4-^{14}C]-cholesterol to coprostanol by Eubacterium HL[a]

Measurement	Day of incubation	^{3}H (dpm)	^{14}C (dpm)	Yield[b] (%)	^{3}H/^{14}C
Cholesterol	0[c]	10743	9735	100	1.10
disappearance	1	10400	9511	98	1.09
	2	10596	9282	95	1.14
	3	7380	6432	66	1.14
	4	1922	1794	18	1.07
	5	1716	1511	16	1.13
	6	1332	1142	12	1.16
	7	1519	1323	13	1.15
Coprostanol	0[c]	50	22	0	
appearance	1	202	176	1.8	1.15
	2	475	444	4.5	1.07
	3	4676	4433	46	1.05
	4	8770	8170	84	1.07
	5	9108	8561	89	1.06
	6	8613	8058	83	1.06
	7	8187	7932	82	1.03
Background[d]		49	19		

[a] Each value is an average of two measurements.

[b] The first set of numbers (100-13) refers to percentage of ^{14}C labeled cholesterol that remains in the media; the second set of numbers (0-82) refers to percentage of ^{14}C label found in coprostanol.

[c] Time 0 indicates [4-^{3}H, 4-^{14}C]-cholesterol isolated from an uninoculated blank.

[d] Background refers to the background of a Beckman L5-8000 liquid scintillation spectrophotometer. Background was not subtracted from other values in the table.

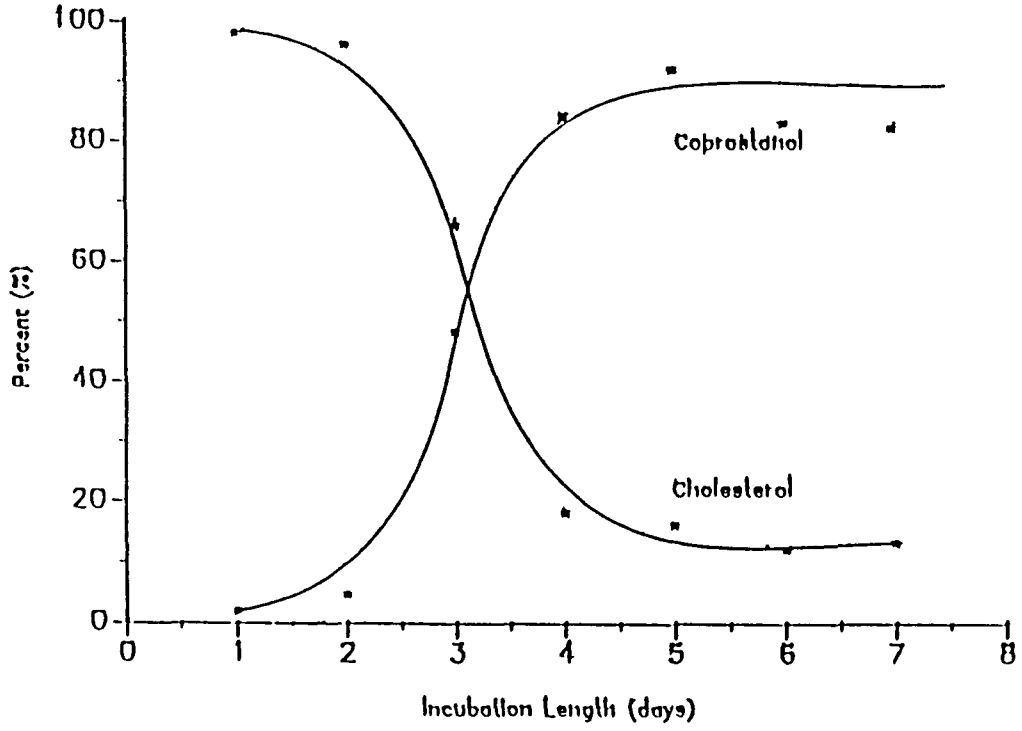

Figure 16. Percentage change in cholesterol and coprostanol concentration as the fermentation proceeded.

Determination of the products of Eubacterium HL other than coprostanol

According to Bjorkhem and Gustafsson (1971), 4-cholesten-3-one and coprostanone might be intermediates in the conversion of cholesterol into coprostanol. Therefore, I investigated whether Eubacterium HL was able to convert cholesterol into 4-cholesten-3-one and coprostanone. The procedures used in this experiment were same as before. The only difference was that to the organic extract was added

Table 9. Products of cholesterol produced by Eubacterium HL

Compound	^{3}H (dpm)[a]	^{14}C (dpm)[a]	$^{3}H/^{14}C$	^{3}H retained (%)
Blank[b]	4375	4009	1.09	100
Coprostanol	3935	3676	1.07	98
4-Cholesten-3-one	55	30		
Coprostanone	52	24		
Background[c]	49	19		

[a] each value of an average of two measurements.

[b] Blank indicates [4-^{3}H, 4-^{14}C]-cholesterol isolated from the uninoculated blank after 5 days of incubation.

[c] Background refers to the background of a Beckman L5-8000 liquid scintillation spectrophotometer. Backgound was not subtracted from other values in the table.

authentic 4-cholesten-3-one (Kodak Inc., Rochester, NY) and coprostanone (Steraloids Inc., Wilton, NH) (each 1 mg/ml). The TLC plates of the extracts of incubations described in the previous section only showed two bands--one for cholesterol and one for coprostanol. The data are presented in Table 9.

Eyssen et al. (1973) reported that production of coprostanol on a preparative scale by Eubacterium 21,408 always yielded small amounts of 4-cholesten-3-one and coprostanone. Their data support the indirect pathway for formation of coprostanol. In my experiment, I demonstrated, however, that coprostanone and 4-cholesten-3-one, if formed, are not released, or are further transformed more rapidly than is cholesterol to coprostanol. Thus, this observation suggests

that cholesterol is converted to coprostanol as a direct reduction or more likely as a multistep reduction without release of intermediates from the enzyme.

Determination of isotope in different position in coprostanol

To determine whether the label had been retained at C-4 or transferred to C-6, I oxidized coprostanol to coprostanone and exchanged the C-4 and C-2 hydrogens with base. I worked out conditions, as before, by using deuterated materials.

In the deuterium exchange experiment, the ^{13}C NMR spectra confirmed complete deuteration at the expected position(s), C-4 (non radioactive coprostanone was used rather than radioactive coprostanone). Figure 17 shows chemical shifts for coprostanone and deuterated coprostanone in $CDCl_3$ solvent. Figure 18 shows expansion of chemical shifts for coprostanone and deuterated coprostanone in $CDCl_3$ solvent. The peaks corresponding to C-2 and C-4 were deuterated completely.

Table 10 summarizes the results of localization of ^3H in coprostanol isolated from incubations with Eubacterium HL. Treatment with pyridinium chlorochromate did not remove any of the ^3H, whereas base-catalyzed exchange of coprostanone into deuterated coprostanone with sodium methoxide and deuterated water removed about 36% of the ^3H. Therefore, it was concluded that the main part of ^3H (64%) in coprostanone was situated in the C-6 position.

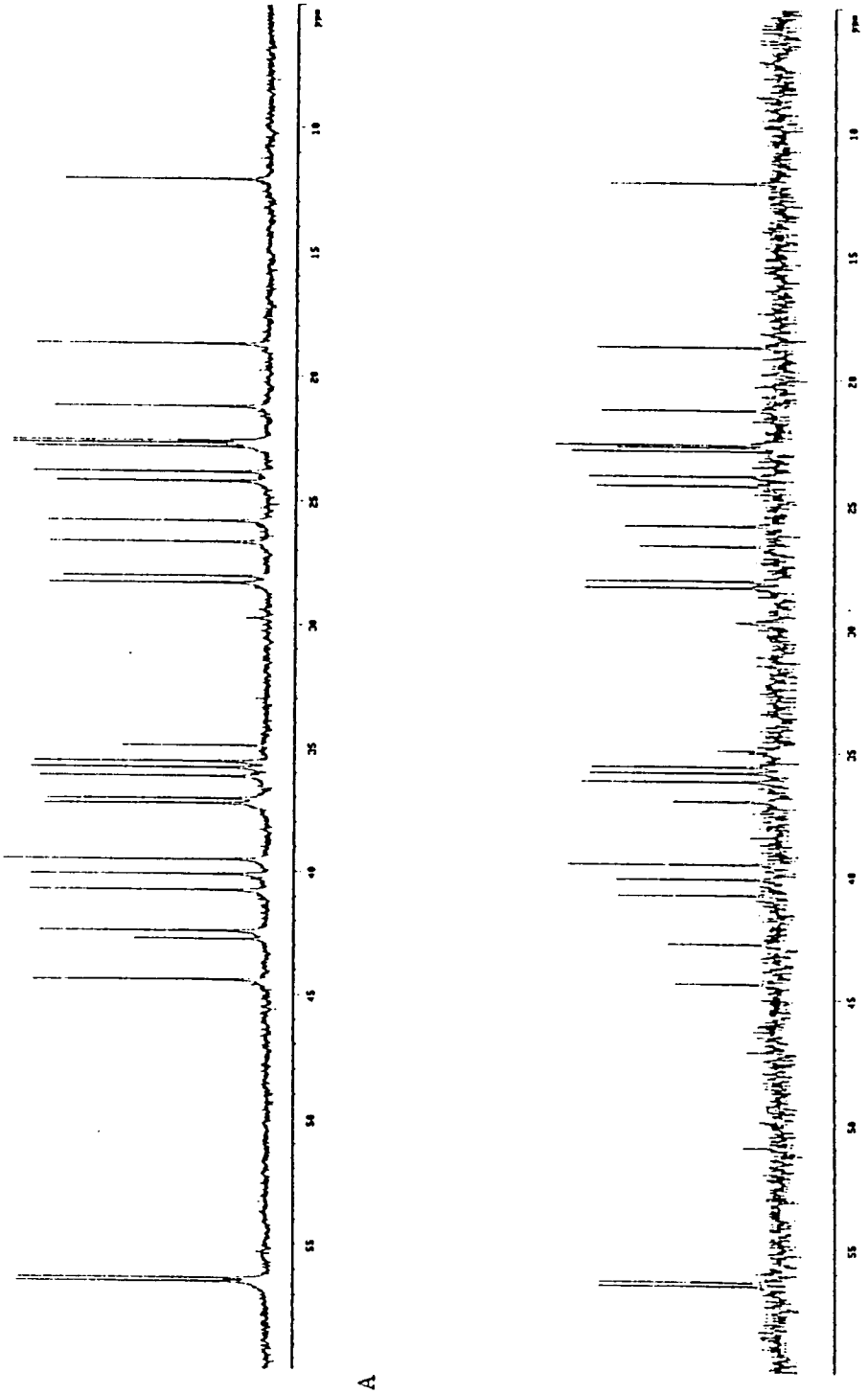

Figure 17. Chemical shifts for coprostanone (A) and deuterated coprostanone (B) in $CDCl_3$ solvent

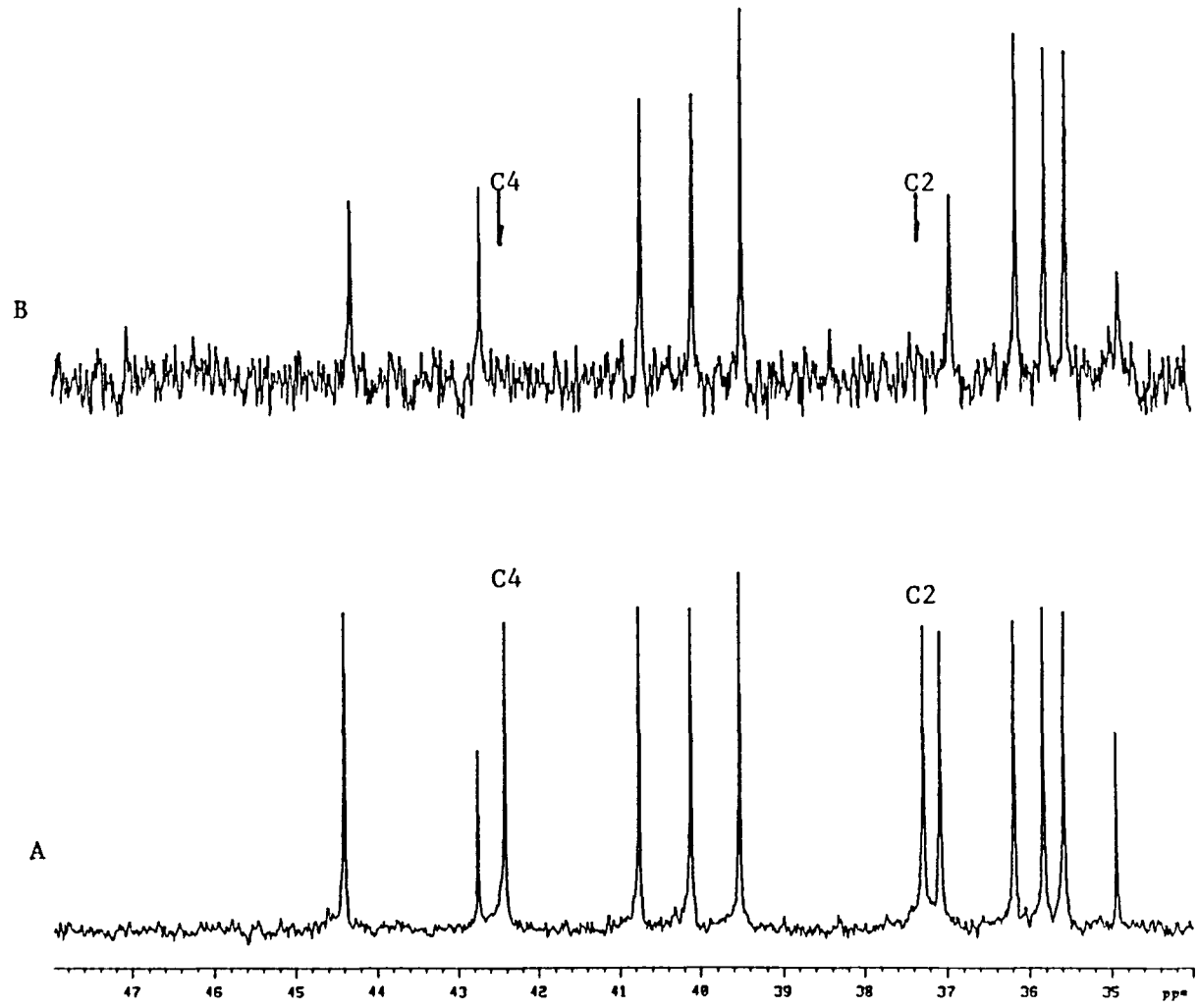

Figure 18. The expansion of chemical shifts for coprostanone (A) and deuterated coprostanone (B) showing that C-2 and C-4 disappeared because of deuterium substitution

Table 10. Localization of ^{3}H in coprostanol isolated from incubations with Eubacterium HL

Compound	^{3}H (dpm)[a]	^{14}C (dpm)[a]	$^{3}H/^{14}C$	^{3}H retained (%)
Coprostanol	2810	2620	1.07	100
Coprostanone	2086	1963	1.06	99
Deuterated-coprostanone	688	1031	0.67	63
Re-deuterated coprostanone	373	535	0.69	65

[a] Each value is an average of two measurements

Resubmission of the exchanged coprostanone to the sodium methoxide for a further 96 hours caused no more change in $^{3}H/^{14}C$ ratio, indicating that exchange was completed and not just slowed by a tritium isotope effect.

This ratio of about 2:1 for C-6:C-4 matches the 2:1 ratio of β:α seen for the deuterium at C-4. If tritium goes on in the same ratio, that would seem to be the maximal C-6:C-4 ratio possible. This data indicates that no solvent exchange occurs during proton transfer from C-4 to C-6 by the enzyme. Whereas an incomplete exchange of coprostanone also would explain such a high retention of label, I believe the re-deuteration of coprostanone has ruled out that possibility.

The results of the present work are consistent with the presence of a pathway from cholesterol to coprostanol involving 4-cholesten-3-one and coprostanone as intermediates.

From studies on biohydrogenation of [4β-^{3}H, 4-^{14}C]-cholesterol by Eubacterium ATCC 21,408, Parmentier and Eyssen (1973) concluded that significant amounts of tritium could be removed from the C-4 position and that more than 70% of this tritium was transferred to the C-6 position of coprostanol. This observation suggested that the major pathway for production of coprostanol by Eubacterium 21,408 involves the intermediate formation of 4-cholesten-3-one. The present investigations with a pure culture of Eubacterium HL extend these observations. Isomerization of a 5-6 double bond to a 4-5 double bond by a mechanism involving transfer of the C-4 H to the C-6 position during conversion of cholesterol into coprostanol indicated that Eubacterium HL followed the so-called indirect pathway with formation of 4-cholesten-3-one.

I observed that nearly 36% of ^{3}H remained in the C-4 position, whereas 64% of ^{3}H was transferred to the C-6 position of coprostanol. These two values are close to the deuterium distribution in [4-^{2}H]-cholesterol reported earlier (67% in an axial position, 33% in an equatorial position).

The indirect pathway hypothesis for formation of coprostanol implies that the microorganism could possess two enzyme systems: one that converts cholesterol into 4-cholesten-3-one, and the other that reduces 4-cholesten-3-one to coprostanol. It should be mentioned that production of coprostanol on a large scale did not yield small amounts of 4-

cholesten-3-one in this experiment.

Reduction of coprostanone by Eubacterium HL

According to Eyssen et al. (1973), Eubacterium ATCC 21,408 was capable of converting cholesterol into coprostanol; it also could convert 4-cholesten-3-one and coprostanone into coprostanol. I investigated, therefore, whether Eubacterium HL was able to reduce coprostanone to coprostanol in basic cholesterol growth medium described earlier.

Radioactive coprostanone was incubated with Eubacterium HL for 3 days in basic cholesterol growth medium at 37°C. The procedure used in this experiment were same as before. The data are presented in Table 11.

This experiment demonstrated that Eubacterium HL was able to reduce coprostanone into coprostanol. This experiment, together with the fact that more than 60% of 4-^{3}H of cholesterol was transferred by an intramolecular shift to the C-6 position of coprostanol during the process of biohydrogenation, tends to favor the theory of the indirect pathway for formation of coprostanol.

Table 11. Reduction of coprostanone by Eubacterium HL

Compound	^{3}H (dpm)[a]	^{14}C (dpm)[a]	$^{3}H/^{14}C$
Coprostanone blank[b]	1139	1102	1.05
Cholesterol	52	20	
Coprostanol	617	590	1.05
Coprostanone	330	320	1.03
Background[c]	46	18	

[a] Value is an average of 4 measurements.

[b] Coprostanone blank indicates coprostanone isolated from the uninoculated blank after 3 days of incubation.

[c] Background indicates the background of a Beckman L5-8000 liquid scintillation spectrophotometer. Background was not subtracted from other values in the table.

SUMMARY

The primary purpose of this research was to determine the pathway of cholesterol reduction to suggest whether one or more protein molecules are responsible for cholesterol reductase activity in Eubacterium sp. strain HL.

A significant conversion of [4-^{3}H, 4-^{14}C]-cholesterol to coprostanol was observed after incubation with Eubacterium HL isolated by Freier and Hartman (1991) in growth medium under anaerobic conditions. The coprostanol synthesized from [4-^{3}H, 4-^{14}C]-cholesterol by incubation of Eubacterium HL retained 97% of the tritium originally present in cholesterol. More than 60% of the tritium in cholesterol, however, had been transferred to the C-6 position in coprostanol. Furthermore, Eubacterium HL was capable of converting cholesterol to coprostanol, and of converting coprostanone to coprostanol. These observations support the hypothesis that t he major pathway for biohydrogenation of cholesterol by Eubacterium HL involves the intermediate formation of 4-cholesten-3-one followed by reduction of the latter to coprostanol. The conclusions from the present investigations are in agreement with a previous study that showed production of coprostanol by Eubacterium 21,408 involves the intermediate formation of 4-cholesten-3-one (Parmentier and Eyssen, 1973).

I too have demonstrated that the multistep mechanism is

followed by the cholesterol reductase of Eubacterium HL, and developed a method that may be used to screen other sourses of cholesterol reductase for their enzymes for the mechanisms. There is no evidence that a single step pathway is ever followed for this transformation. It should be mentioned that conversion of [4-^{3}H, 4-^{14}C]-cholesterol to coprostanol by incubation with Eubacterium HL in growth medium did not yield detectable amounts of 4-cholesten-3-one and coprostanone. This is an important result for future research. Use of cholesterol reductase to decrease the cholesterol content of foods will require transfer of a gene(s) coding for cholesterol reductase into safe, easy-to-grow organisms, such as E. coli, yeast, or lactobacillus, for the production of sufficiently large amounts of the reductase. Cloning of genes for commercial production of cholesterol reductase will be several-fold easier if only one gene rather than several genes are involved in the reduction of cholesterol to coprostanol.

REFERENCES

Arima, K., Nagasawa, M., Bea, M. and Tamura, G. 1969. Microbial transformation of sterols. Part 1. Decomposition by microorganisms. Ag. Biol. Chem. 33:1636-1643.

Bjorkhem, I. and Gustafsson, J. 1971. Mechanism of microbial transformation of cholesterol in coprostanol. Eur. J. Biochem. 21:428-432.

Breitamier, E. and Voelter, W. 1987. Pages 355-356 in ^{13}C NMR spectra of natural products in carbon-13 NMR spectroscopy. VCH Verlasgesellschaft mbH, D-6940 Weinheim.

Chevallier, F. 1967. Dynamics of cholesterol in rats studies by the isotopic equilibrium method. in Paoletti, R. and Kritchevsky, D. eds. Adv. lipid Res. Vol. 5. Academic Press, New York.

Eyssen, H. J. and Parmentier, G. G. 1974. Biohydrogenation of sterols and fatty acids by the intestinal microflora. Am. J. Clin. Nutr. 27:1329-1340.

Eyssen, H. J., Parmentier, G. G., Compernolle, F. C., Pauw, G. D. and Denef, M. 1973. Biohydrogenetion of sterol by Eubacterium ATCC 21,408-Novel species. Eur. J. Biochem. 36:411-421.

Freier, T. A. and Hartman, P. A. 1991. Isolation of unique cholesterol-reducing bacteria. Pages 262 in Abstracts 0-23 of the 91st Annual Meeting of the American Society for Microbiology. Dallas, Texas.

Groot, P. H. E. and Schlek, L. M. 1984. Effects of fat ingestion on high-density lipoprotein profiles in human sera. J. Lipid Res. 25:684-692.

Grundy, S. M., Ahrens, J. R. and Salen, G. 1968. Dietary β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. J. Lipid Res. 9:374-387.

Leyden, D. E. and Cox, R. H. 1977. Pages 207-208 in Analytical Applications of NMR. John Wiley and Sons, Inc., New York.

Lindsey, C. A. and Wilson J. D. 1965. Evidence for a contribution by the intestinal wall to the serum cholesterol of the rat. J. Lipid Res. 6:173-181.

Lockley, W. J. S., Rees H. H. and Goodwin, T. W. 1978. Synthesis of stereospecifically labelled [4-^{3}H]cholesterol. J. Lab. Comp. Radiopharmaceut. 15:413-423.

Mazschner, S. S. and Fine, J. B. 1989. Physical process for simultaneous deodorization and cholesterol reduction of fats and oils. U. S. patent 4804555.

Quintao, E., Grundy, S. M. and Ahrens, E. H. 1971. An evaluation of four methods for measuring cholesterol absorption by the intestine in man. J. Lipid Res. 12:221-232.

Parmentier, G. and Eyssen, H. 1973. Mechanism of biohydrogenation of cholesterol to coprostanol by bacterium ATCC 21,408. Biochim. Biophys. Acta 348:279-284.

Parish, E. J. and Chitrokorn, S. 1983. A simplified, one step synthesis of cholest-5-en-3-one. J. Am. Chem. 15:365-366

Rosenfeld, R. S. and Gallagher, T. F. 1964. Further studies of the biotransformation of cholesterol to coprostanol. Steroids 4:515-520.

Sadzikowski, M. R., Sperry, J. F. and Wilkins, T.D. 1977. Cholesterol reducing bacterium from human feces. Appl. Environ. Microbiol. 34:355-362.

Sawan, S. P., James, T. L., Gruenke, L. D. and Craig, J. C. 1979. Proton NMR assignments for cholesterol. Use of deuterium NMR as an assignment aid. J. Mag. Reson. 35:409-413.

Silverstein, R. M., Basser, G. C. and Morrill, T. C. 1981. Pages 257-274 in Spectrometric Identification of Organic Compounds. John Wiley and Sins, Inc., New York.

Still, W. C., Kahn, M. and Mitra, A. 1978. Rapid chromatographic technique. J. Org. Chem. 43:2923-2925.

GENERAL SUMMARY

The public has an abiding interest in the relation of diet and health. A recent publication from the National Research Council/National Academy of Sciences with the title "Diet and Health" (1989) points up this continuing public interest and concern about the relationships of these two subjects. The "cholesterol scare" has been a major factor causing public concern about consuming foods derived from animals. My research group expect to develop the technology to use a purified enzyme to safely produce milk, egg, and meat that has substantially lower concentrations of cholesterol. Knowledge of the characteristics of the cholesterol reductase will be highly useful for subsequent development of protocols for using the enzyme to decrease the cholesterol content of animal products used as food.

Researchers at Iowa State University (Freier and Hartman, 1991) have developed a strain of bacteria named Eubacterium sp. strain HL that someday may allow people to eat high-cholesterol foods without absorbing most of the cholesterol. Species of Eubacterium are normal inhabitants of the human colon, which should minimize concerns about adding a constituent enzyme of the bacteria to foods that will be consumed by people.

The conversion of cholesterol into coprostanol by intestinal microorganisms has been reported to occur by means of two different pathways. From studies on biohydrogenation of [4-^{3}H, 4-^{14}C]-cholesterol by Eubacterium HL, the mechanism of indirect pathway is of major importance for coprostanol formation. As is apparent, the present experiments do not provide direct evidence for the participation of mechanism of direct pathway in coprostanol formation. This could have implications in our future goals of isolating the cholesterol reductase and cloning the gene for commercial production of cholesterol reductase.

REFERENCES

American Heart Association Nutrition Committee. 1982. National of the diet-heart statement of the American Heart Association. Arteriosclerosis 4:177-191.

Barry, C. D., Dobson, C. M., Sweigart, D. A., Ford, L. S. and Williams, R. J. P. 1973. Nuclear magnetic resonance. Pages 173-191 In R. E. Sievers ed. Nuclear Magnetic Resonance Shift Reagents. Academic Press, New York.

Bennion, L. J. and Grundy, S. M. 1975. Effects of obesity and caloric intake on biliary lipid metabolism. J. Clin. Invest. 56:996-1011.

Bergstrom, S., Danielsson, H. and Samuelsson, B. 1960. Formation and metabolism of bile acids. in K. Bloch ed. Lipid Metabolism. John Wiley and Sons, New York.

Bhattacharyya, A. K. 1986. Differences in uptake and esterification of saturated analogues of cholesterol by rat small intestine. Am. J. Physiol. 251:G495-G500.

Bjorkhem, I. and Gustafsson, J. 1971. Mechanism of microbial transformation of cholesterol in coprostanol. Eur. J. Biochem. 21:428-432.

Borgström, B. 1969. Quantification of cholesterol absorption in man by fecal analysis after the feeding of a single isotope-labeled meal. J. Lipid Res. 10:331-337.

Brinkley, A. W., Gottesman, A. R. and Mott, G. E. 1982. Isolation and characterization of new strains of cholesterol-reducing bacteria from baboons. Appl. Environ. Microbiol. 43:86-89.

Connor, W. E. 1980. U. S. dietary goals, a pro view, with special emphasis upon the etioogical relationships of dietary factors to coronaty heart disease. Pages 44-81 In P. J. Garry, ed. Human Nutrition, Clinical and Biochemical Aspects. Am. Assoc. Clin. Chem. Washigton, D.C.

Dam, H. 1934. The formation of coprostanol in the intestine. 2. The action of intestinal bacteria on cholesterol. Biochem. J. 28:820-825.

Davignon, J. 1977. Pages 961-989 in Hypertension, Pysiopathology and Treatment. McGraw-Hill, New York.

Dehal, S. S., Freier, T. A., Young, J. W., Hartman, P. A. and Beitz, D. C. 1991. A novel method to decrease the cholesterol content of foods. Pages 203-220 in C. Haberstroh and C. E. Morris eds. Fat and Cholesterol Reduced Foods; Technologies and Strategies. Portfolio Publishing Co., Woodland, Texas.

Dietschy, J. M. and Milson, J. D. 1970. Regulation of cholesterol metabolism. 3. Excretion and degradation. New Eng. J. Med. 282:1241-1249.

Eyssen, H . J., Parmentier, G. G., Compernolle, F. C., Pauw, G. D. and Denef, M. 1973. Biohydrogenetion of sterol by Eubacterium ATCC 21,408-Novel species. Eur. J. Biochem. 36:411-421.

Farkas, J., Angel, A. and Avigan, M.I. 1973. Studies on the compartmentation of lipid in adipose cells. 2. Cholesterol accumulation and distribution in adipose tissue components. J. Lipid Res. 14:344-356.

Freier, T. A. and Hartman, P. A. 1991. Isolation of unique cholesterol-reducing bacteria. Pages 262 in Abstracts 0-23 of the 91st Annual Meeting of the American Society for Microbiology. Dallas, Texas.

Froning, G. W. 1991. Supercritical fluid extraction of cholesterol from dried egg. Pages 277-188 in C. Haberstroh and C. E. Morris eds. Fat and Cholesterol Reduced Foods; Technologies and Strategies. Portfolio Publishing Co., Woodland, Texas.

Gould, R. G. and Cook, R. P. 1958. The metabolism of cholesterol and other sterols in the animal organism. Pages 237-251 in R. P. Cook ed. Cholesterol, Chemistry Biochemistry and Pathology. Academic Press. New York.

Grundy, S. M. 1979. Dietary fats and sterols in nutrition, lipid and coronary heart disease: a global view. Pages 89-118 in R. I. Levy, B. M. Rifkind, B. H. Dennis, and H. Ernst, eds. Nutrition in Health and Disease. Vol 1. Raven Press, New York.

Grundy, S. M. 1983. Absorption and metabolism of dietary cholesterol. Annu. Rev. Nutr. 3:71-76.

Grundy, S. M. and Mok, H. Y. I. 1977. Determination of cholesterol absorption in man by intestinal perfusion. J. Lipid Res. 18:263-271.

Heltman, E. 1970. Pages 115-119 in Steroid Biochemistry, 1st edition. Academic Press. New York.

Hofmann, A. F. and Borgstrom, B. 1964. The intraluminal phase of fat digestion in man. J. Clin. Invest. 43:247-259.

Hulley, S. B. 1988. A national program for lowering high blood cholesterol. Am. J. Obstet. Gynecol. 158:1561-1566.

Johnson, T. L. and Somkuti, G. 1989. Properties of cholesterol dissimilation by Rhodococcus equi. J. Food Prot. 53:332-350.

Kannel, W. B., Lastelli, W. P. and Gordon, T. 1979. Cholesterol in the prediction of atherosclerotic disease. Ann. Internal Med. 90:85-91.

Linder, C. 1985. Nutritional biochemistry and metabolism. Pages 331-340 In Nutrition and Atherosclerosis. Elsevier Science, New York.

Macdonald, I. A., Bokkenhenser, V. D., Winter, J., McLernon, A. M. and Mosbach, E. H. 1983. Degradation of steroids in the human gut. J. Lipid Res. 24:675-700.

Marsheck, W. J. Kraychy, S. and Muir, R. D. 1972. Microbial degradation of acerola. Appl. Microbiol. 23:72-77.

McNamara, D. J., Proia, A. and Miettinen, T. A. 1981. Thin-layer and gas-liquid chromatographic identification of neutral steroids in human and rat feces. J. Lipid Res. 22:474-484.

Miettinen, T. A., Proia, A. and McNamara, D. J. 1981. Origins of fecal neutral steroids in rats. J. Lipid Res. 22:485-495.

National Research Council. 1989. Diet and Health, Implications for Reducing Chronic Disease Risk. National Academy Press, Washington, D.C.

Novak, R. A., Reightler, W. J., Pasin, G., King, A. J. and Zeidler, G. 1991. Supercritical fluid extraction of cholesterol from liquid egg. Pages 289-298 in C. Haberstroh and C. e. Morris eds. Fat and Cholesterol Reduced Foods; Technologies and Strategies. Portfolio Publishing Co. Woodland, Texas.

Page, I. H. 1954. Atherosclerosis. An Introduction. Circulation 10:1-27.

Quintao, E., Grundy, S. M. and Ahrens, E. H. 1971. Effects of dietary cholesterol on the regulation of total body cholesterol in man. J. Lipid Res. 12:233-247.

Small, D. M. 1977. Cellular mechanisms for lipid deposition in atherosclerosis. Part 1. New Eng. J. Med. 297:873-877.

Parmentier, G. and Eyssen, H. 1973 Mechanism of biohydrogenation of cholesterol to coprostanol by bacterium ATCC 21,408. Biochim. Biophys. Acta 348:279-284.

Rosenfeld, R. S. and Gallagher, T. F. 1964. Further studies of the biotransformation of cholesterol to coprostanol. Steroids 4:515-520.

Rosenfeld, R. S., Zumoff, B. and Hellman, L. 1967. Conversion of cholesterol into man to cholestanol via a 3-ketonic intermediate. J. Lipid Res. 8:16-23.

Sabine, J. R. 1977. Cholesterol. Marcel Dekrer Inc., New York.

Salen, G. and Shefer, S. 1983. Bile acid synthesis. Annu. Rev. Physiol. 45:679-685.

Snog-kjaer, A., Prange, J. and Dam, H. 1956. Conversion of cholesterol to coprostanol by bacteria. J. Gen. Microbiol. 14:256-260.

Strong, J. P. and Eggen, D. A. 1970. Risk factors and atherosclerotic lesions. Pages 355-364 in R. J. Jones ed. Atherosclerosis, Proceeding of the Second International Symposium. Springer-Verlog New York Inc., New York.

Turley, S. D. and Dietschy, J. M. 1982. Cholesterol metabolism and excretion. Pages 476-492 in I. Arias, H. Popper, D. Schachter and D. A. Shafritz, eds. The liver: Biology and Pathobiology. Raven Press, New york.

U. S. Department of Health and Human Services. 1988. The Surgeon General's report on nutrition and health. Washington, D.C. U.S. Department of Health and Human Services, Public Health Service, DHHS (PHS) Publication No. 88-50210.

Wilson, J. D., and Reinke, R. T. 1968. Transfer of locally synthesized cholesterol from intestinal wall to intestinal lymph. J. Lipid Res. 9:85-92.

Zubay, G. 1988. Biochemistry. Pages 168-170. MacMillan Publishing Co. New York.

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APPENDIX

Interpretation of One Dimensional NOE Experiment

Based on mass spectral data (p.31), total deuterium in cholesterol molecules can be calculated:

D1+2(D2)=56%+2(13%)=0.82.

Irradiation at C-4 of deuterated cholesterol (p.51) led to 0.94% enhancement at C-19 compared to 2.49% enhancement of unlabeled cholesterol. That means 62% decrease due to deuterium in 4β position. Therefore, 0.62/0.82=76% of the deuterium is in the 4β position, and 24% of deuterium in 4α position.

When C-19 was irradiated, it led to 76% as much NOE at C-4 in deuterated cholesterol was seen as compared to the unlabeled cholesterol. How to interpret this value? From mass spectral data and 1D NOE, we know deuterium has two different positions at C-4 in deuterated cholesterol. So irradiation at C-19 led to different NOE at C-4 of deuterated cholesterol.

A. ^{2}H α gives 200% NOE vs unlabeled cholesterol

B. ^{2}H β gives 0% NOE vs unlabeled cholesterol

C. unlabeled C-4 gives 100% vs unlabeled cholesterol

The solution is complete:

2A+C=0.76

A+B+C=1

A+B=0.82 (based on mass spectral data)

Now we get: A=0.29

B=0.53

C=0.18

Therefore, B/(A+B)=65% of the deuterium is in the 4β position, and A/(A+B)=35% of the deuterium is in the 4α position.

From these experiment, we think about 65-76% of the deuterium is in the 4β position, although these two values are not very accurate.

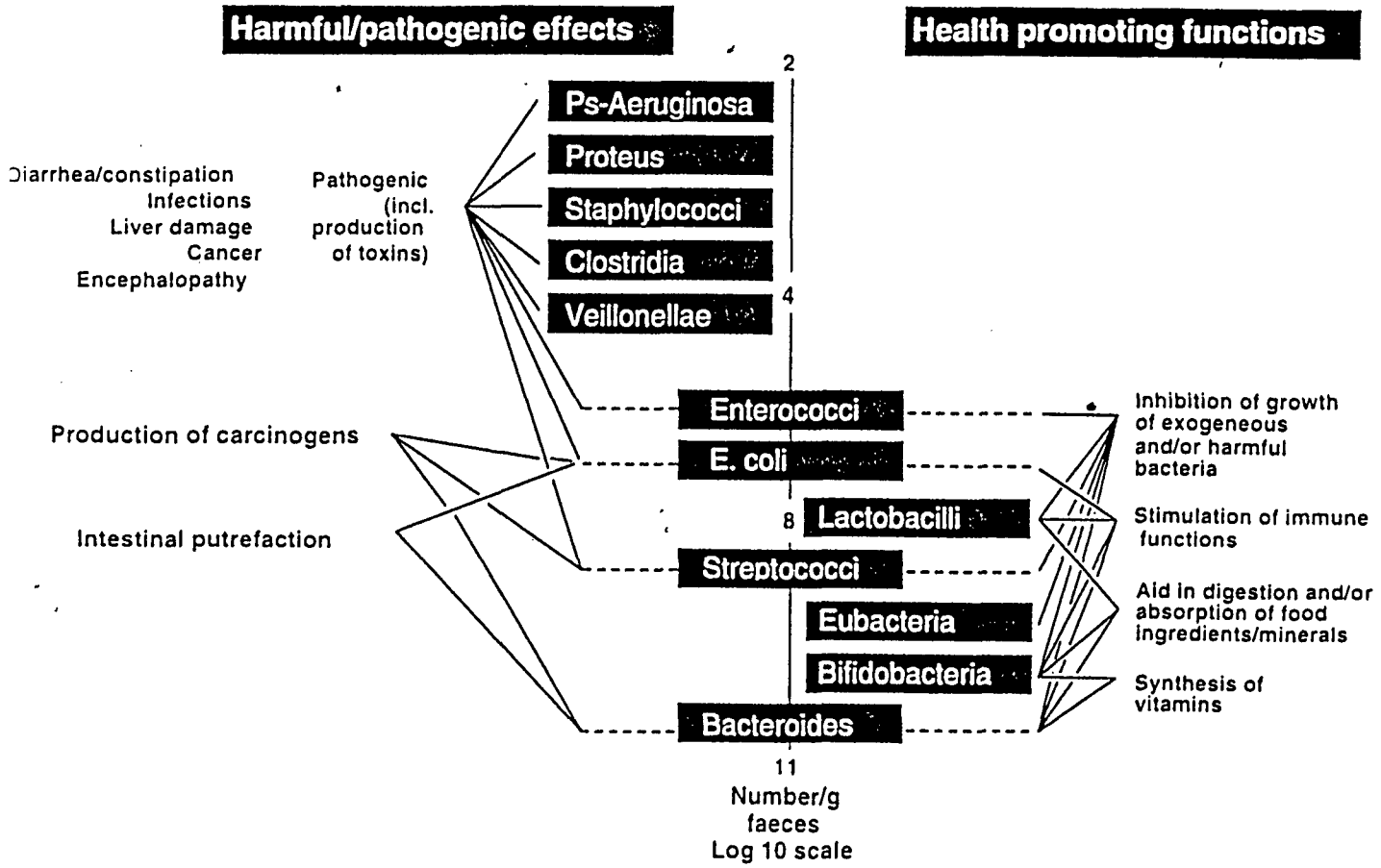

FIGURE 1 Generalized scheme of the composition and health effects of predominant human fecal bacteria. The figure shows approximate numbers of the different genera. The bacteria are generally split into those groups that have harmful or pathogenic influences on human health, those that have beneficial effects, and those that may have both. Potential reasons for the classification scheme are given.