Isolation and characterization of unique cholesterol-reducing anaerobes

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Iowa State University

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Isolation and characterization of unique cholesterol-reducing anaerobes

Freier, Timothy Alan, Ph.D.

Iowa State University, 1991
Isolation and characterization of unique cholesterol-reducing anaerobes

by

Timothy Alan Freier

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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1991
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEROL STRUCTURES</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>Health Benefits of Reduced Serum Cholesterol Levels</td>
<td>3</td>
</tr>
<tr>
<td>Cholesterol and Colon Cancer</td>
<td>4</td>
</tr>
<tr>
<td>Absorption of Sterols by the Human Digestive System</td>
<td>7</td>
</tr>
<tr>
<td>Cholesterol-reducing Bacteria</td>
<td>11</td>
</tr>
<tr>
<td>Mechanism of Cholesterol Reduction</td>
<td>22</td>
</tr>
<tr>
<td>Other Related Microbial Steroid Reactions</td>
<td>26</td>
</tr>
<tr>
<td>Eubacteria</td>
<td>38</td>
</tr>
<tr>
<td>Technologies for Reducing Cholesterol in Foods</td>
<td>39</td>
</tr>
<tr>
<td>PART I. ISOLATION OF CHOLESTEROL-REDUCING BACTERIA</td>
<td>46</td>
</tr>
<tr>
<td>Introduction</td>
<td>46</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>50</td>
</tr>
<tr>
<td>Bacteria</td>
<td>50</td>
</tr>
<tr>
<td>Growth media</td>
<td>50</td>
</tr>
<tr>
<td>Clear cholesterol-containing media</td>
<td>53</td>
</tr>
<tr>
<td>Isolation media</td>
<td>57</td>
</tr>
<tr>
<td>Growth of cholesterol-reducing bacteria in clear media</td>
<td>62</td>
</tr>
<tr>
<td>Isolation procedure</td>
<td>62</td>
</tr>
<tr>
<td>Sterol conversion assay</td>
<td>64</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>65</td>
</tr>
<tr>
<td>Media development</td>
<td>65</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Preliminary studies on the isolation of cholesterol-reducing bacteria</td>
<td>68</td>
</tr>
<tr>
<td>and on cholesterol reductase</td>
<td></td>
</tr>
<tr>
<td>Successful isolation of pure cultures of CRB</td>
<td>72</td>
</tr>
<tr>
<td><strong>PART II. CHARACTERIZATION OF A CHOLESTEROL-REDUCING BACTERIUM</strong></td>
<td>79</td>
</tr>
<tr>
<td>Introduction</td>
<td>79</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>81</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td>81</td>
</tr>
<tr>
<td><strong>Media</strong></td>
<td>82</td>
</tr>
<tr>
<td>Characterization of strain HL</td>
<td>83</td>
</tr>
<tr>
<td>Results</td>
<td>93</td>
</tr>
<tr>
<td>Discussion</td>
<td>114</td>
</tr>
<tr>
<td><strong>GENERAL SUMMARY</strong></td>
<td>122</td>
</tr>
<tr>
<td><strong>LITERATURE CITED</strong></td>
<td>124</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGMENTS</strong></td>
<td>134</td>
</tr>
</tbody>
</table>
STEROL STRUCTURES

5-ANDROSTEN-3β,17β-DIOL (Androstenediol)

5β-CHOLESTAN-3-ONE (Coprostan-3-one)

5-ANDROSTEN-3β-OL 17β-CARBOXYLIC ACID (Etienic acid)

5,7,22-CHOLESTATRIEN-24β-METHYL-3β-OL (Ergosterol)

5β-CHOLANIC ACID-3α,12α-DIOL (Deoxycholic acid)

5-CHOLESTEN-3β,7β-DIOL (7β-Hydroxycholesterol)

5,22-CHOLESTADIEN-24b-ETHYL-3β-OL (Stigmasterol)

5-CHOLESTEN-3α-OL (Epicholesterol)

5α-CHOLESTAN-3β-OL (Cholestanol)

5-CHOLESTEN-3β-OL (Cholesterolin)

5β-CHOLESTAN-3β-OL (Cholesterol)

4-CHOLESTEN-3-ONE (Cholestenone)

5α-CHOLESTAN-3β-OL (β-Cholestanol)

5-CHOLESTEN-3α-OL (β-Cholesterol)

5α-CHOLESTAN-3β-OL (Dihydrocholesterol)
GENERAL INTRODUCTION

Dietary cholesterol is of significant concern to a great number of people. Americans consuming a typical "Western" diet need to reduce their dietary intake of cholesterol to reduce their chances of suffering from coronary heart disease and stroke. Food industries are attempting to meet consumer demands for cholesterol-reduced products. One potential method to reduce cholesterol levels in foods is the use of enzymes to transform cholesterol into coprostanol, a sterol that is not absorbed by the human digestive system. It is possible that the use of cholesterol reductase might be relatively inexpensive and would be well-received by consumers.

It was established in the 1930s that intestinal bacteria convert cholesterol to the saturated product coprostanol. Other early studies showed that whereas cholesterol was absorbed readily by the human digestive system, coprostanol was not absorbed. However, because cholesterol is absorbed in a region of the intestine that is proximal to the area that cholesterol-reducing bacteria (CRB) typically colonize, intestinal bacteria probably have little or no effect on serum cholesterol levels.

Previous investigators were successful in isolating several strains of CRB. Most of these bacteria had characteristics that made common laboratory manipulations difficult. Requirements included the need for a lipid found
only in high concentrations in mammalian brain tissue and the need for complete anaerobiosis. These CRB also did not form colonies on solid plating media.

The present study was undertaken to develop new methods for the cultivation of previously isolated CRB, and to isolate new strains of CRB with characteristics that might facilitate their application to reducing the cholesterol content of foods.
LITERATURE REVIEW

Health Benefits of Reduced Serum Cholesterol Levels

Although there is great variation from individual to individual, it has been proven that the intake of dietary cholesterol directly affects serum cholesterol levels. A predictive equation developed by Hegsted (1986) indicated that for a person consuming a 2500 kcal diet, an increase of 100 mg per day of cholesterol would increase the serum cholesterol level by approximately 4 mg per dl. The most important health implication of elevated serum cholesterol is coronary heart disease (CHD). This disease is responsible for approximately one out of every three deaths in the United States (Levy, 1981). In 1958, the Western Electric Study was initiated. This study involved the detailed dietary and clinical histories of over 1900 middle-aged men (Shekelle et al., 1981). A 19-year follow-up study showed that a dietary reduction of 200 mg cholesterol per 1000 kcal resulted in a 37% reduction in total mortality. The large body of evidence linking elevated serum cholesterol with CHD has been recently reviewed (National Research Council, Committee on Diet and Health, 1989).

The American Heart Association and the National Heart, Lung, and Blood Institute have reviewed the epidemiologic, laboratory, and clinical studies linking cholesterol and CHD (Gotto, 1990). It was stated that there is a definite link...
between elevated serum cholesterol levels and CHD, that diet modification can lower the risk of CHD, and that lowering serum cholesterol levels would be beneficial to everyone.

Cholesterol and Colon Cancer

Several studies have indicated a possible link between serum cholesterol levels or dietary cholesterol intake and colon cancer. Colon cancer is a major cause of neoplastic disease in populations consuming a Western diet (high in saturated fat and cholesterol, low in complex carbohydrates and fiber). The incidence of colon cancer is much reduced in populations consuming a non-Western diet (Hill and Aries, 1971; Finegold et al., 1974).

It has been suggested that the intestinal microflora acts upon dietary factors to form cancer-promoting compounds. Clostridia that dehydrogenate the steroid nucleus were isolated with increased frequency from fecal samples taken from subjects living in areas with a high incidence of colon cancer (Goddard et al., 1975). These clostridia were also present more often in the feces of cancer patients than in the feces of control patients (Hill et al., 1975). Mastromarino et al. (1976) documented a significant increase in fecal microbial 7a-dehydroxylase and cholesterol dehydrogenase in the feces of colorectal cancer patients as compared to patients who did not have colorectal cancer. Studies of this type are complicated, however, by the
multiplicity of variables encountered when testing diseased subjects.

One possible explanation for the link between high-cholesterol diets and colon cancer is that the diet affects the type and numbers of intestinal bacteria that are capable of forming cancer-promoting compounds. Drasar and Jenkins (1976) determined that various dietary supplementations that alter serum cholesterol levels had no effect on the fecal flora. Finegold et al. (1977) compared a group of people at low risk for developing colon cancer with a group of people at high risk. The low-risk group had increased fecal counts of aerobic bacteria and lowered counts of certain anaerobic bacteria in comparison to the high-risk group. There also appears to be no difference between the numbers of various taxonomical groups of intestinal bacteria of patients with large bowel cancer and healthy controls (Mastromarino et al., 1978).

Bile acids are a group of metabolic end products that are affected by diet and the fecal microflora, and play a role in carcinogenesis. Cholesterol in the body is derived from two sources: exogenous cholesterol from the diet, and endogenous cholesterol that is synthesized in the liver and intestine. Cholesterol from both of these sources is metabolized in the liver, mainly to the primary bile acids, cholic acid and chenodeoxycholic acid. These bile acids are conjugated with taurine and glycine; the conjugates are secreted into the intestine. The microflora in the large
bowel is capable of deconjugating these bile acids. Approximately 80% of the free bile acids are then converted to deoxycholic acid and lithocholic acid by bacteria that express the enzyme 7α-dehydroxylase. These steroids are referred to as secondary bile acids.

Several lines of evidence have implicated secondary bile acids as causative agents in colon cancer. Bile acids have structural similarities with the polycyclic aromatic hydrocarbons, compounds that are known carcinogens. Epidemiological studies have linked the Western diet, increased concentrations of secondary bile acids, and colon cancer. Certain sterols and bile acids have shown tumor-promoting activity in experimental carcinogenesis studies. Secondary bile acids may inhibit the host’s ability to detoxify exogenous carcinogens. Finally, secondary bile acids can exhibit mutagenicity, transforming activity, and DNA-strand breakage (reviewed by Nair, 1988).

On the average, greater than 70% of fecal cholesterol is converted to coprostanol in the large intestine of healthy adult humans (Wilkins and Hackman, 1974). It has been suggested by several investigators that the microbial conversion of cholesterol to its reduced products may be associated with the development of colon cancer. These speculations have been based entirely upon epidemiological studies, and to date no direct evidence implicating the reduced products of cholesterol with colon cancer has been presented (Macdonald et al., 1983; Goldin, 1986).
Absorption of Sterols by the Human Digestive System

The transfer of sterols from the lumen of the gastrointestinal system to the circulatory system or lymph involves several steps. The process includes the transfer of the sterol into the mucosal cells, metabolic processing in the mucosa, and transfer into the lymph. Sterols that are absorbed can originate either from dietary sources or from endogenous sources that include bile, saliva, gastric secretions, and cells sloughed from the intestinal mucosa. Approximately 400 to 1000 mg of cholesterol, 100 to 500 mg of bile acids, and less than 2 mg of steroid hormones pass through the colon of the average human each day (Macdonald et al., 1983). The amount of cholesterol that is absorbed by the intestine varies greatly between individuals and is dependent upon other dietary components such as the amounts and degree of saturation of fats. In general, approximately 20 to 50% of total cholesterol is absorbed (Treadwell and Vahouny, 1977). Cholesterol and related sterols are absorbed primarily in the proximal region of the jejunum (Borgstrom, 1960).

When a sterol is absorbed, it is esterified in the intestinal mucosal cells. The ability to be esterified seems to be one of the major determining factors that distinguishes sterols that enter the lymph from those that do not. Sterols that are esterified must have a free hydroxyl group at C-3 of
ring A in the β position, and the A and B rings must be oriented trans:trans. A double bond at the C-5 position is not required, and a double bond or a hydroxyl group at C-7 of ring B or an alteration of the side chain inhibits esterification. Although most sterols are not absorbed if they are not efficiently esterified, epicholesterol is not esterified, but is absorbed, and dihydrocholesterol is esterified, but is absorbed only half as efficiently as cholesterol. Coprostanol is very poorly esterified (reviewed by Treadwell and Vahouny, 1977).

Some of the earliest studies on the absorption of sterols were carried out by Schoenheimer (1931). Large amounts of various sterols were fed to animals, and the thoracic duct lymph was analyzed. Sterols that were absorbed by the intestine would appear in the lymph. From these studies it was concluded that plant sterols and coprostanol were completely nonabsorbable.

Much of the information available on the absorbability of sterols resulted from balance studies. In this type of study, the amount of a sterol that is ingested by an animal is compared with the amount that is excreted in the feces, after correcting for sterol synthesis by the animal. When sitosterol, ergostanol, cholestanol, and coprostanol were fed to mice, there was no change in the sterol balance. Sterol synthesis in these animals remained the same as when they were fed a sterol-free diet. It was concluded that coprostanol was not absorbed by the intestinal system of the
The nonabsorbability of coprostanol by the intestinal systems of animals was questioned by Setty and Ivy (1960). When 28 mg per day of coprostanol was added to the diet of rats, an average of 48% of the sterol was absorbed. They also determined that 65% of cholesterol and 22% of cholestanol were absorbed. The methods used in these studies may account for the differences in coprostanol absorption reported by Setty and Ivy (1960) and those reported by other workers. Coprostanol was measured by the analysis of the difference between the total sterol, determined by digitonin precipitation of the mixture, and the amount of cholesterol, calculated from a color reaction with the Liebermann-Burchard reagent. It was subsequently found that this procedure was not accurate for coprostanol determination (Wells and Mores, 1961).

A more sensitive method for determining sterol absorption is through the administration of radiolabeled sterols. Rosenfeld and coworkers (1963) fed coprostanol-C\textsuperscript{14} and cholestanol-C\textsuperscript{14} to two human subjects. At the peak of plasma radioactivity, one subject had 3.6% of the administered coprostanol-C\textsuperscript{14} in the plasma, and the second subject had 3.5%. The amount of ingested cholestanol-C\textsuperscript{14} in the plasma of one subject was 4.7%. The second subject was not tested. The cholestanol that appeared in the plasma had been esterified, whereas none of the coprostanol was esterified.
Bhattacharyya (1986) fed fasted rats coprostanol and cholestanol. After 4 hours, the rats were sacrificed and the small intestine was dissected. The total intestinal tissue content of coprostanol was only 3.8% of the total dose fed, while the content of cholestanol was 14.6% of the dose fed. The amount of cholestanol that was esterified was 5 to 30 times higher than the amount of esterified coprostanol in all sections of the intestine tested.

In another experiment by the same investigator, the small intestine was removed from fasted rats, washed, everted, and formed into sacs. These sacs were then incubated in buffer with and without coprostanol or cholestanol. The percent uptake of cholestanol was significantly higher than that of coprostanol in all of the segments of the small intestine. The author stated that coprostanol was not absorbed because its uptake and esterification by the intestinal mucosal cells was extremely limited, while the uptake and esterification of cholestanol was not limited.

Several possible reasons for the difference in uptake were tested. It was thought that there may have been a difference in the solubility of the two sterols in mixed micelles, thereby limiting the availability of coprostanol to the mucosal cells. However, no difference was observed in the concentrations of the two sterols in the mixed micellar layer isolated from the intestinal contents of the sterol-fed animals. Another postulate was that the sterols formed
different structures in mixed micelles that altered their ability to diffuse out of the micelles. The rate of diffusion of coprostanol out of mixed micelles was observed to be significantly slower than the diffusion of cholestanol. This may be the reason for the lower uptake of coprostanol by intestinal mucosal cells compared to the uptake of cholestanol. Another reason may be the structural difference between the two sterols. The A/B ring structure of coprostanol is cis, which causes the C-3 hydroxy group to assume an equatorial position. Cholestanol and cholesterol each have trans A/B ring junctions, with the 3-hydroxy group in the axial position.

Cholesterol-reducing Bacteria

Schoenheimer stated in 1931, "Since koprosterol was not found in the tissues but always in larger amounts in the feces, we must still assume that it is a product of the bacterial action in the intestines even though we have not been able to reproduce the same in vitro. We must consider that up to the present it has not been possible to cultivate all of the intestinal bacteria and it is very probable that any one of the difficultly cultivatable anaerobes may be able to bring about this change. It is hoped that the near future may throw light on this question." It was not until 42 years later that a pure culture of a cholesterol-reducing bacterium was isolated (Eyssen et al., 1973).
In 1934, Dam observed that cholesterol was readily reduced to coprostanol (also called coprosterol and koprosterol) by mixed fecal cultures from humans or rats. If the feces were heated to 100°C for 20 min, no hydrogenation of cholesterol occurred. Rosenheim and Webster (1943) determined that the administration of the bacteriostatic agent succinylsulphathiazole to rats inhibited coprostanol formation in vivo.

Further attempts to isolate a cholesterol-reducing bacterium were reported by Snog-Kjaer et al. (1956). A medium was formulated that included 5% (w/v) desiccated hog or ox brain. Brain was used because it provided an easy way to produce a colloidal suspension of cholesterol. This medium was made partially anaerobic by the addition of the reducing agent cysteine hydrochloride and incubation in vacuo. Mixed cultures of fecal bacteria saturated 80 to 93% of the sterols when incubated in the brain medium for 8 d at 37°C. However, pure cultures of Clostridium welchii, Clostridium sporogenes, Bacterium bifidum, various streptococci and micrococci, Escherichia coli, and Aerobacter aerogenes failed to hydrogenate the cholesterol substrate. The investigators also noted that copper sulfate was not inhibitory to the anaerobic cholesterol-reducing bacteria, and that the reducing activity of mixed cultures of fecal anaerobes was maintained after storage at 15°C for 6 months.

Although the bacterium responsible for cholesterol conversion into coprostanol had not yet been isolated,
several studies were carried out to determine the effects on coprostanol formation of adding different compounds to the diet. Penicillin, sulfa suxidine, Tween 80, and sodium taurocholate depressed the excretion of coprostanol in rats. Aureomycin, streptomycin, and chloromycetin had no effect (Wells, 1957). When rats were fed a 40% lactose diet, total liver cholesterol was elevated compared to controls, and markedly less coprostanol was excreted in the feces (Wells and Cooper, 1958). This effect was thought to have been caused by a decrease in the intestinal pH from an average value of 7.65 in control rats to 5.80 in lactose-fed rats. Interestingly, when the lactose diet was supplemented with 2% calcium chloride, total liver cholesterol was decreased, and coprostanol was returned to normal levels in fecal samples. The authors postulated that calcium formed insoluble complexes with phospholipids or fatty acids, and that these complexes were involved in the cholesterol-absorption inhibition.

Crowther et al. (1973) investigated the effects upon the fecal flora of feeding a liquid diet to human volunteers. These researchers claimed that several pure cultures of bacteria converted cholesterol to coprostanol when the bacteria were grown in the brain medium of Snog-Kjaer et al. (1956). Of 20 strains of Clostridium spp. tested, 9 were positive, and converted 15 to 20% of the cholesterol to coprostanol. Eighteen strains of Bacteroides spp. and 12 strains of Bifidobacterium spp. were tested, with 12 and 9
strains positive, respectively. Some *Bacteroides* strains converted more than 50% of the cholesterol substrate after 7 d of incubation.

Sadzikowski and coworkers (1977) tried to repeat the experiments conducted by Crowther's group (1973) because the original strains of cholesterol-reducing bacteria had been lost. Using the same methods and media, Sadzikowski and coworkers (1977) were not able to isolate any cholesterol-reducing bacteria. The inability of subsequent investigators to demonstrate cholesterol reduction by pure cultures of similar strains of bacteria remains to be explained.

The first isolation of a pure culture of a cholesterol-reducing bacterium was most likely accomplished by Eyssen et al. (1973). A basal medium was formulated that contained freeze-dried beef brain powder as the cholesterol source. This medium was prereduced, and growth was carried out under a nitrogen atmosphere. Rat cecal contents were inoculated into this medium, and small amounts of coprostanol were observed after the medium was incubated. The sterols were extracted, and the samples were spotted on thin layer chromatography plates. Greater concentrations of coprostanol were observed after several successive transfers of the culture into fresh medium, and by the 15th transfer, 90% of the cholesterol was hydrogenated within 72 h. When those cultures were streaked on agar-containing plates of the same medium, none of the colony-forming bacteria could convert
cholesterol to coprostanol in liquid culture media. However, a mixed culture that contained only a *Clostridium perfringens* and a small Gram-positive bacterium did convert cholesterol to coprostanol. When this culture was passed through a 0.8 μm syringe filter, a pure culture of the small bacterium was obtained that reduced cholesterol in the brain-containing medium.

The cholesterol-reducing bacterium was small, anaerobic, and Gram positive in young cultures, but became Gram negative after 3 d of incubation. Many diploforms were observed in young cultures. The organism did not grow in many commonly used media, even after supplementation with serum or yeast extract. However, when 1 to 2 mg/ml cholesterol suspended in lecithin was added, these media supported growth for a limited number of transfers. After approximately 10 transfers, these media no longer supported growth of this bacterium. Long-term propagation could only be carried out on media containing brain powder. It was concluded that the organism had an obligate requirement for a delta-5 sterol.

The cholesterol-reducing bacterium grew optimally at 37° to 40°C, and did not produce spores. Tests for the production of indole, acetyl-methyl-carbinol, hydrogen sulfide or catalase were negative, and nitrate was not reduced to nitrite. The organism was not motile, and produced small amounts of acid and gas from glucose, lactose, galactose, and melibiose. No acid or gas was observed after growth in media containing maltose, fructose, raffinose,
mannose, trehalose, melezitose, xylose, or rhamnose. The bacterium was classified in the genus *Eubacterium* and was registered by the American Type Culture Collection as *Eubacterium* ATCC 21408.

In addition to cholesterol, *Eubacterium* 21408 also reduced 4-cholesten-3-one to coprostanol. Sitosterol, campesterol, stigmasterol, 5-androsten-3β-ol-17-one, and 5-pregnen-3,20β-diol were converted to their corresponding 5-hydrogenated derivatives. There was no conversion of cholesteryl acetate or cholesteryl chloride.

Several studies were initiated to determine the role of cholesterol-reducing bacteria in the intestine. When the feces of conventional rats or of gnotobiotic rats inoculated with a cholesterol-reducing bacterium were tested, cholesterol was extensively reduced to coprostanol. However, in cecectomized rats, this activity was lost within 2 d of the operation (Eyssen et al., 1972). *Eubacterium* 21408 was eliminated from the intestine of cecectomized rats. The change in pH or Eh of the intestinal contents was found not to be the reason for the loss of the bacterium. It was postulated that the cecum was necessary to slow the intestinal transit time enough to allow the growth of the slowly-developing eubacterium.

Crowther et al. (1973) determined that in humans the intestinal transit time was not crucial in maintaining cholesterol-reducing bacteria. When healthy volunteers were fed a fat-free liquid diet, the percentage of neutral
steroids excreted in the feces as coprostanol decreased. This decrease in the excretion of coprostanol may have been caused by an alteration of the bacterial species present in the gut, or because the enzyme responsible for cholesterol reduction was synthesized at a lower level due to a lack of substrate. Fecal transit time in these volunteers was actually longer than in controls (Crowther et al., 1973).

The percentage of cholesterol converted to coprostanol in healthy volunteers on a North American diet was categorized into two distinct patterns: low conversion and high conversion (Wilkins and Hackman, 1974). In a group of 31 volunteers, fecal samples from 8 people contained less than 30% of coprostanol as total animal sterols, while the fecal samples from the other 23 volunteers contained greater than 60% coprostanol. Because all the volunteers were consuming similar diets and the patterns were stable with time, it was thought that the differences must be at the level of the microbial ecology of the colon. Factors affecting cholesterol conversion may have been the composition of the intestinal microflora, the physical state of the cholesterol molecule in the intestine, or the intestinal transit time (Wilkins and Hackman, 1974).

The effect of various sugars on the conversion of radiolabeled cholesterol to coprostanol in human fecal homogenates was tested (Subbiah et al., 1974). When lactose, galactose, fructose, sucrose, or glucose were added to the homogenates, the percent of cholesterol converted to
coprostanol decreased significantly. It was noted that this decreased conversion may have been caused by a shift in pH or by a catabolite repression effect. The exact mechanism was not elucidated.

Working with pure cultures of *Eubacterium* 21408, Eyssen et al. (1974) observed that lactose did not inhibit growth of the bacterium. However, when the bacterium was cocultured with a *Clostridium* sp., an *Escherichia coli*, or a *Streptococcus faecalis* in the presence of lactose, it was inhibited in its growth. The decrease in pH caused by the fermentation of lactose by the other bacteria was thought to have been responsible for the inhibition of *Eubacterium* 21408.

Sadzikowski and coworkers (1977) were the first researchers to isolate a cholesterol-reducing bacterium from human feces. A human fecal sample was serially diluted and inoculated into a pork brain-containing prereduced medium. The highest dilution that formed coprostanol upon incubation was again serially diluted and inoculated into the medium. After several consecutive serial dilutions, the mixed culture was treated with clindamycin, erythromycin, penicillin G, and phenethyl alcohol. The only remaining bacterium visible upon microscopic examination of the resulting coprostanol-producing culture was a Gram-positive diplobacillus.

The cholesterol-reducing bacterium isolated from human feces by Sadzikowski et al. (1977) was very similar to the
bacterium isolated by Eyssen's group (1973). The isolate did not form colonies on a variety of aerobic or anaerobic agar plating media, and did not grow in any of the conventional broths that were tested. The human isolate also had an obligate requirement for cholesterol.

In an attempt to explain the differences in coprostanol excretion between high and low converters observed by Wilkins and Hackman (1974), the cholesterol-reducing bacterium of human origin and Eubacterium 21408 were incubated with several strains of bacteria to check for inhibition. An organism tentatively identified as Fusobacterium russii caused a 40% inhibition of coprostanol formation by Eubacterium 21408. However, Sadzikowski's isolate was only slightly inhibited (Sadzikowski et al., 1977). It was concluded that one reason that some people convert a greater percent of cholesterol to coprostanol is that their cholesterol-converting flora respond differently to antagonistic bacteria that are present in the intestine.

Using techniques similar to those used by previous investigators (Eyssen et al., 1973; Sadzikowski et al., 1977), a cholesterol-reducing bacterium was isolated from baboon feces (Mott and Brinkley, 1979). This isolate was designated Eubacterium 403, and was nearly identical to the previous isolates. All of the cholesterol-reducing bacteria would grow for only a limited number of transfers in brain-free media supplemented with cholesterol in lecithin. For long-term maintenance, the addition of homogenized brain
or lipid extracts of brain was required. By separating an organic solvent extract of brain into its components, it was determined that the bacteria required a plasmalogen, plasmenylethanolamine (PLE) (Mott and Brinkley, 1979). PLE is similar to phosphatidylethanolamine, except that it contains an alkenyl ether linkage at C-1. When pure PLE was added to a medium that contained cholesterol dispersed with lecithin, the eubacteria grew, formed fibers, and solidified the medium. The cultures also catabolized PLE as it was observed that the PLE disappeared with cell growth. It was also determined that coprostanol formation was directly related to the PLE concentration.

Using a simplified medium containing PLE, it was possible to study the metabolic characteristics of cholesterol-reducing bacteria. Mott et al. (1980) studied in detail the biochemical characteristics of *Eubacterium* 21408 and 403. These strains were negative for nitrate reduction, indole production, and gelatin and starch hydrolyses. There was no fermentation of the 22 carbohydrates tested. Both strains were positive for esculin hydrolysis and did not possess an arginine dihydrolase pathway. In addition to PLE, 5 other alkenyl ether lipids supported the growth of the eubacteria in a cholesterol-lecithin medium. Allocholesterol and 4-cholesten-3-one were reduced to coprostanol. When the medium contained 2 mg per ml of 4-cholesten-3-one, coprostanone was the major product after incubation. But if the level of this sterol was reduced to 0.2 mg per ml, the
major product was coprostanol. The authors concluded that the cholesterol-reducing bacteria resembled *Eubacterium lentum*, but because they lacked the arginine dihydrolase pathway and had a unique requirement for certain sterols and alkenyl ether lipids, they could not be classified in any existing species within the genus *Eubacterium*.

A major problem in performing various laboratory procedures with the cholesterol-reducing bacteria was the inability to grow the bacteria on a solid medium. Brinkley et al. (1980) developed a cholesterol-brain agar medium (CBA) that sustained the growth and colony formation of these bacteria when used in an anaerobic chamber. The medium contained 5% cholesterol dispersed with 0.1% lecithin and 3% freeze-dried brain. This very high concentration of cholesterol was thought to be necessary to overcome the limited diffusion of water-insoluble sterols through the agar matrix. Casamino Acids and yeast extract were also necessary for growth. Trypan blue dye was added to aid in the visualization of the colonies. The colonies were very small, had a fibrous appearance, and did not decolorize the trypan blue as many other anaerobes did.

The cholesterol-brain agar medium was used to isolate several new strains of cholesterol-reducing bacteria (Brinkley et al., 1982). Feces or intestinal contents were obtained from baboons, diluted, and plated on CBA. After 3 to 5 d of incubation, colonies that had the characteristic appearance of previously isolated cholesterol-reducing
bacteria were picked and inoculated into brain-containing media. Using this method, 9 new strains of cholesterol-reducing bacteria were isolated. All of these new strains formed tiny colonies on brain heart infusion agar supplemented with sodium pyruvate and yeast extract. This finding was significant because it indicated that not all cholesterol-reducing bacteria have an obligate growth requirement for cholesterol and PLE. The strains reduced cholesterol to coprostanol when grown in cholesterol-lecithin medium containing PLE. Two of the strains could reduce cholesterol in the absence of PLE, while the other 7 strains required PLE to reduce cholesterol. These two strains also produced succinate. All of the strains produced small amounts of acetate. Unlike previously isolated strains of cholesterol-reducing bacteria, several of the new isolates produced acid from various carbohydrates. Every stain of cholesterol-reducing bacterium tested metabolized pyruvate, and produced acetoin. In the presence of pyruvate, the strains produced CO$_2$ concentrations of from 9 to 20% of the headspace gas. The isolates were negative for starch and gelatin hydrolysis, did not reduce nitrate, and did not produce indole, urease, or hydrogen.

Mechanism of Cholesterol Reduction

Early studies of the conversion of cholesterol to coprostanol were conducted by using fecal homogenates that
contained a mixture of bacterial cultures. In an attempt to elucidate the mechanism of this reaction, various radiolabeled cholesterol substrates were incubated with feces, and the coprostanol product was then analyzed. A dual-labeled cholesterol compound (cholesterol-3\textsuperscript{d},4-\textsuperscript{14}C) was fed to human volunteers. When coprostanol excreted in the feces was assayed, the ratio of deuterium to \textsuperscript{14}C remained the same. This indicated that, during the conversion of cholesterol to coprostanol, the hydrogen group at C-3 remained intact. Incubation of the labeled cholesterol with fecal material confirmed the results. However, when the group at C-3 was oxidized to the corresponding ketone, the coprostanone still contained an appreciable level of deuterium. It was concluded that the conversion of cholesterol to coprostanol must be a more complicated reaction than the simple reduction of the 5-6 double bond (Rosenfeld et al., 1954). Further experimentation established that the deuterium that had been removed from the C-3 position was moved to C-5 and C-6, and possibly exclusively to C-6 (Rosenfeld et al., 1956).

Rosenfeld and Gallagher (1964) determined that when cholesterol-3-\textsuperscript{3}H was transformed to coprostanol-\textsuperscript{3}H by human fecal suspensions, most of the tritium was retained at C-3, suggesting that the hydroxyl group at C-3 was not oxidized to a ketone during the formation of coprostanol. However, in some experiments, a significant amount of the tritium was lost or transferred to new positions of the coprostanol
molecule. The authors concluded that the reduction of cholesterol to coprostanol could be achieved by at least two different microorganisms by at least two chemical pathways. One pathway involved the intermediate formation of coprostanone, while another did not.

Björkhem and Gustafsson (1971) anaerobically incubated [4β-3H,4-14C]- and [3α-3H,4-14C]cholesterol with cecal contents from rats. When the 3α-3H compound was converted into coprostanol, 50% of the tritium was lost. When [4-14C]cholesterol with no tritium label was incubated in the presence of [3α-3H]β-sitosterol, the 14C-labeled coprostanol contained significant amounts of tritium in the C-3α position. This indicated that some of the tritium that remained in the C-3 position may have been introduced in a reductive step following an initial oxidation step. These experiments provided evidence that an intermediate ketone was involved in cholesterol reduction. When the 4β-3H compound was incubated with cecal contents, 40% of the tritium was lost. Of the tritium that remained, most had been transferred to the C-6 position. This indicated that the double bond at the C-5 position of cholesterol was isomerized to the 4 position during coprostanol formation.

The only attempt to purify the enzyme(s) responsible for the biohydrogenation of cholesterol to form coprostanol was carried out by Björkhem and coworkers (1973). They obtained a crude enzyme preparation by freeze-pressing the cecal contents of rats. After centrifugation at 100000 X g,
3-oxo-delta-4-steroid 5β-reductase activity was found mainly in the supernatant. About half this activity could be precipitated between 25 and 50% saturation with ammonium sulfate. Approximately 70% of the activity was lost when the extract was stored at 4°C for 48 h. Attempts to further purify the enzyme by Sephadex chromatography, DEAE-cellulose chromatography, and hydroxyapatite chromatography were not successful because of the rapid loss in enzyme activity. Freeze-pressing was the only method of cell breakage that did not destroy enzyme activity. The other methods that were tried included sonification, treatments with detergents, treatment with lysozyme-EDTA, and sand-milling.

Although the conversion of cholesterol to coprostanol was studied, the only activity found in cell-free extracts of rat cecal contents was the reduction of 4-cholesten-3-one, progesterone, testosterone, and 7a-hydroxy-4-cholesten-3-one to the corresponding 3-keto-5β-steroids (Björkhem et al., 1973). There was no activity with cholesterol, pregnenolone, or dehydroepiandrosterone as substrates. The delta-4-reductase used NADH as a cofactor, but not NADPH. The mechanism of reduction involved the transfer of hydrogen from the 4β-position of NADH to the 5β-position of the steroid. There was a sharp pH optimum at 6.5.

Experiments similar to those carried out by Björkhem and Gustafsson (1971) on rat cecal contents were carried out by Parmentier and Eyssen (1974), except *Eubacterium* ATCC 21408 was used instead of a mixed culture. They observed a 65%
loss of tritium when $[3a-3^H,4-^{14}C]$cholesterol was converted into coprostanol. However, when preformed $[3a-3^H,4-^{14}C]$coprostanol was incubated with the bacterium, 40% of the tritium was lost. The authors concluded that reversible oxidation-reduction reactions were being carried out at the 3-hydroxyl group, and that this information could not be used to determine the mechanism of cholesterol reduction. When $[4B-3^H,4-^{14}C]$cholesterol was incubated with *Eubacterium* 21408, 81% of the tritium was retained, but greater than 90% of the retained label was located at the C-6 position of coprostanol. This indicated that the reduction of cholesterol by this bacterium involved the intramolecular shift of tritium from C-4 to C-6, suggesting that the intermediate formation of 4-cholesten-3-one was the major pathway for the conversion of cholesterol to coprostanol by *Eubacterium* ATCC 21408.

**Other Related Microbial Steroid Reactions**

The study of related microbial steroid reactions is of great value when investigating cholesterol reduction to coprostanol, because there may be many similarities. Anaerobic bacteria in the intestine act on cholesterol, bile acids, and steroid hormones. Some reactions that may have similar characteristics to the conversion of cholesterol to coprostanol will be considered.
It was demonstrated that *Clostridium paraputrificum* could transform 4-androsten-3,17-dione to 3a-hydroxy-5β-androstan-17-one with 5β-androstan-3,17-dione as an intermediate (Glass et al., 1979). The initial reaction was the complete reduction of the double bond at C-4, which was carried out by early log phase cells. The next reaction was the reduction of the keto group at C-3, and occurred most rapidly as the culture entered stationary phase. The product of this second reaction did not appear until 2 h after the formation of the intermediate. When crude cell extracts were assayed, the reaction proceeded as it did with growing whole cells, and no exogenous cofactors were required. No special precautions were taken to maintain anaerobic conditions during the harvesting and breakage of the cells.

To test for cofactor requirements, crude cell extracts of *C. paraputrificum* were treated with a Sephadex G-25 column to remove low molecular weight compounds (Glass et al., 1979). It was observed that the addition of NADH with FAD or FMN or the addition of NADPH with FAD greatly increased the activity of the enzyme system. These data suggested the presence of an electron transport system.

Quantitatively, the most important steroid reaction carried out by intestinal anaerobic bacteria is the 7a-dehydroxylation of cholic acid and chenodeoxycholic acid to form the secondary bile acids deoxycholic and lithocholic acids. This reaction has a significant impact on the
physiology of the host, and has been studied in great depth. *Clostridium leptum* was assayed in an anaerobic washed whole cell system radiochromatographically by Stellwag and Hylemon (1979). A pH optimum of 6.5 to 7.0 was determined for 7a-dehydroxylase. There was a 10-fold increase in the 7a-dehydroxylation of cholic acid during the transition from logarithmic to stationary growth phase, and the increase occurred both with and without cholic acid in the growth medium. The reaction showed a high degree of substrate specificity and was very sensitive to inhibition by molecular oxygen. The authors noted that the reaction seemed to occur in or on the cytoplasmic membrane and speculated that 7a-dehydroxylation was coupled to an energy generating system in *Clostridium leptum*.

*Eubacterium* sp. strain VPI 12708 was determined to have inducible 7a-hydroxylase activity (White et al., 1980). The activity was demonstrated both in washed whole cells and in cell-free extracts. The induction of the enzyme was highly specific and required a free carboxy group and an unhindered 7a-hydroxy group on the B ring of the bile acid. It was also determined that cholic acid induced NADH:flavin oxidoreductase activity. It appeared that the NADH:flavin oxidoreductase may have been supplying reduced flavin that was required by the 7a-dehydroxylase. The cholic acid-inducible NADH:flavin oxidoreductase was purified and further characterized (Lipsky and Hylemon, 1980). The enzyme had an absolute specificity for NADH as the electron donor
but could use a variety of electron acceptors, including 2,6-dichlorophenolindophenol, $K_3Fe(CN)_6$, methylene blue, menadione, riboflavin, FMN, and molecular oxygen.

As is the case with most cholesterol reductases studied, 7α-dehydroxylase is believed to act by a multiple-step pathway. The initial step of 7α-dehydroxylation of bile acids was thought to be the diaxial transelination of water resulting in the elimination of the 7α-hydroxy group and the 6β-hydrogen. The double bond at C-6 was then believed to be reduced at the 6α and 7β position (Samuelsson, 1960). Surprisingly, White and coworkers (1981) determined that NAD$^+$ was required for enzyme activity in anaerobic dialyzed cell-free extracts, rather than a reduced cofactor. They also found that the delta-6 intermediate did not accumulate and that the 7α-dehydroxylase activity and the delta-6 reduction activity eluted from gel filtration columns as a single peak. It was also determined that *Eubacterium* 12708 could 7β-dehydroxylate ursodeoxycholic acid to lithocholic acid (White et al., 1982). The investigators suggested that a single enzyme or enzyme complex may have catalyzed the multiple-step reaction. Further study by the same group (White et al., 1983) suggested that 7-dehydroxylase was regulated by the NAD$^+$-NADH ratio in the bacterial cell.

7α-Dehydroxylation of bile acids was also demonstrated in resting cells of an unidentified Gram-positive, nonsporing, anaerobic bacterium by Masuda and Oda (1983). With this bacterium, when the glucose concentration in the
growth medium was increased from 0.3 to 1.0%, enzyme activity was completely lost, even though there was an enhancement of cell growth. It was suggested that a decrease in pH may have been responsible, or that glucose may have repressed 7a-dehydroxylase production.

Masuda et al. (1984) studied 7a-dehydroxylation by resting cells of a Eubacterium lentum-like bacterium (strain c-25). The organism could grow only under strict anaerobic conditions. However, when a cell suspension was exposed to atmospheric oxygen for 1 hour, less than 10% of the 7a-dehydroxylase activity was lost. When the cells were ruptured by sonic oscillation, enzyme activity was completely lost. The pH optimum for activity against cholic acid was 7.3, while the optimum against chenodeoxycholic acid was 7.7, suggesting the presence of 2 distinct enzymes.

Cell extracts of Eubacterium 12708 produced a novel highly polar bile acid metabolite when grown in the presence of primary bile acids (Coleman et al., 1987a). It was determined that this compound contained a nucleotide with a pyrophosphate group covalently linked to the bile acid at the C-24 position. It was thought that this metabolite was an intermediate in the 7-dehydroxylase reaction. This new evidence led to a modification in the proposed pathway of 7-dehydroxylation. It was proposed that the mechanism involved an initial two-step oxidation of a primary bile acid nucleotide to produce a 3-oxo-delta-4,6-steroid nucleotide. There is then a three-step reduction of the intermediate, and
the bound nucleotide is relinked to 7-dehydroxylase, releasing the secondary bile acid.

Because the 7-dehydroxylase of *Eubacterium* 12408 was inducible, it was possible to determine that four new polypeptides were synthesized after exposure to cholic acid (White et al., 1981; Paone and Hylemon, 1984). The greatest activity corresponded with a polypeptide that had a molecular weight of 27000 (PP-27). PP-27 was purified, the amino-terminal end of the molecule was sequenced, and the corresponding mixed oligonucleotide probe was synthesized. This probe hybridized with a 2.2-kilobase EcoRI fragment. Size fractionation of a total digest of genomic DNA enriched for the 2.2-kilobase fragment. This enriched fragment was ligated into the bacterial plasmid pUC8 and used to transform *Escherichia coli* HB101. The use of the probe in a colony hybridization procedure allowed the detection of the 7-dehydroxylase gene in the transformants. The entire gene was cloned on a 1150-base-pair TaqI fragment. Western blot analysis indicated that PP-27 was expressed in *E. coli*. This was the first reported cloning of a bile acid metabolism gene and the first gene cloned from a *Eubacterium* sp. (Coleman et al., 1987b). PP-27 was shown to be associated with 7-dehydroxylation, although purified PP-27 did not exhibit enzymatic activity. It was thought that PP-27 was involved in at least one of the oxidation-reduction reactions of 7-dehydroxylation (Coleman et al., 1988).
To further elucidate the mechanism of 7-dehydroxylation, (3β-³H)- and (5-³H)-labeled cholic acid were used as substrates for whole cells and extracts of *Eubacterium* 12708 (Björkhem et al., 1989). The majority of the label was lost during 7-dehydroxylation, suggesting the formation of a 3-oxo-delta-4 intermediate. It was thought that some ³H was retained because it was transferred from the labeled bile acid to a cofactor in an oxidative step, and then the same cofactor was used in a reductive step. This mechanism appeared to be very similar to the mechanism for the conversion of cholesterol to coprostanol (Björkhem and Gustafsson, 1971).

An enzyme system that is closely related to 7-dehydroxylase is 21-dehydroxylase. A strain of *Eubacterium lentum* was isolated from human feces that catalyzed the 21-dehydroxylation of 11-deoxycorticosterone to progesterone (Feighner et al., 1979). The enzyme had a broad pH range with an optimum of 6.4 to 6.8. In crude cell extracts, activity was rapidly and irreversibly inhibited by atmospheric oxygen (Feighner and Hylemon, 1980). Extracts that were stored anaerobically under argon gas at 4°C lost over 40% of their activity within 48 hours. When glycerol or reduced glutathione was added to the crude extracts, the activity was partially stabilized. This stabilization was not observed when 2-mercaptoethanol or L-cysteine was added. Regardless of the addition of glycerol or reducing agents, all activity was lost after 5 days. Anaerobically dialyzed
cell extracts required both a reduced pyridine nucleotide (NADH or NADPH) and an oxidized flavin (FAD or FMN). It was observed that an NAD(P)H:FMN oxidoreductase provided reduced flavin for the 21-dehydroxylase. These two enzymes were not physically associated and were regulated by independent control systems. Sparging washed whole cell suspensions with hydrogen gas increased 21-dehydroxylase activity 35-fold. Preliminary evidence indicated that an oxygen-labile hydrogenase was present in whole cells and cell extracts. It was thought that the 21-dehydroxylase was located on the membrane or that there was a neutral steroid transport system in the E. lentum strain studied.

Another enzyme system that has been studied and may have similarities to the cholesterol reductase system is 7a-hydroxysteroid dehydrogenase. This enzyme is known to occur in Escherichia coli, Bacteroides fragilis, Clostridium absonum, Clostridium limosum, and Brevibacterium fuscum (Prabha et al., 1989). 7a-Hydroxysteroid dehydrogenase was purified from E. coli and characterized (Prabha et al., 1989). This enzyme had a broad specificity but required bile acids that had a hydroxyl group at the C-7 position. NAD$^+$ was used as a cofactor. Although the enzyme did not require metal ions, there was increased activity in the presence of Na$^+$, K$^+$, Ca$^{2+}$, and Mn$^{2+}$. Detergents and oxidizing groups were inhibitory to the enzyme, and reducing agents were stimulatory.
A lecithinase-lipase-negative *Clostridium* sp. was shown to have NADP-dependent 3β-, 7α- and 7β-hydroxysteroid dehydrogenase activities (Edenharder et al., 1989). All three dehydrogenases could be detected in the supernatant and membrane fractions after solubilization with Triton X-100, suggesting that the enzymes were membrane bound. The 3β-hydroxysteroid dehydrogenase had a pH optimum of 7.5 and was synthesized constitutively. A molecular weight of 104000 was estimated by molecular sieve chromatography. In cell-free extracts, activity could easily be demonstrated in the reductive direction by using a 3-keto substrate. However, it was not possible to demonstrate the oxidation of 3β-hydroxy bile acids. No special precautions to exclude oxygen were necessary during the preparation of the crude cell extract or the enzyme assay. The activity of the extract was unchanged when stored at 4°C for up to 3 h. Activity was still detectable after 48 h of storage at 4°C. Reducing agents stabilized the enzyme, while EDTA had no effect. Activities of 7α- and 7β-hydroxysteroid dehydrogenases required induction. The *Clostridium* sp. studied showed oxidative activity of 7α-hydroxysteroid dehydrogenase, but extracts could only be demonstrated to work in the reductive direction. The 7β-hydroxysteroid dehydrogenase also would only reduce a 7-keto group when in a cell-free extract. The authors could not explain why enzymes that exhibited reversible oxidation-reduction activities in vivo were shown to be irreversible in vitro.
A 7α-hydroxysteroid dehydrogenase was purified from *Eubacterium* 12708. The enzyme was a tetramer of identical subunits, with a molecular weight of approximately 124000. The enzyme was NADP-dependent, was inhibited by sulfhydryl reactive compounds, and was not affected by metal ion chelators (Franklund et al., 1990).

7β-Hydroxysteroid dehydrogenase activity was demonstrated in a strain of *Eubacterium aerofaciens* (Hirano and Masuda, 1982). A crude cell extract was prepared by sonicating the bacteria, and the enzyme was partially purified by column chromatography. All the steps subsequent to growing the bacteria were done aerobically. The enzyme was dependent on NADP⁺ as a cofactor and was expressed constitutively. An optimum pH of 10.5 and a molecular weight of 45000 were determined. Enzyme activity was stabilized when dithiothreitol or thioglycolate was added to the reaction mixture, and the addition of sulfhydryl inhibitors such as *p*-chloromercuribenzoate or iodoacetate abolished enzyme activity. Divalent cations or EDTA had no effect on enzyme activity.

Two strains of *Eubacterium* sp. were isolated that catalyzed the 16α-dehydroxylation-reduction of 16α-hydroxysteroids to form 17-iso-steroids (Bokkenheuser et al., 1980). This system appeared to be comprised of two enzymes, a 16α-hydroxyprogesterone dehydroxylase and a 16-dehydroprogesterone reductase (16-DHPR) (Winter et al., 1982). Using resting cells, it was determined that 16-DHPR
activity was stimulated by hemin, hydrogen, or pyruvate (Glass and Burley, 1985). When hydrogen or pyruvate were present, the *Eubacterium* sp. also reduced the delta-4 double bond of progesterone and dehydroxylated the 3α-hydroxy group. It was thought that, in this bacterium, hemin was used in an electron transport system that supplied electrons to 16-DHPR that was coupled to the oxidation of pyruvate and hydrogen.

A cell-free extract with 16-DHPR activity was obtained from a *Eubacterium* sp. (Watkins and Glass, 1991). Anaerobic precautions were taken during the growth, harvesting, and breakage of the bacterium. The activity was inducible and required a steroid with a delta-16 double bond and a C-20 ketone. When pyruvate was used as an electron donor, coenzyme A and methyl viologen were required for 16-DHPR activity. It was determined that a pyruvate:methyl viologen oxidoreductase oxidized pyruvate to generate reduced methyl viologen that supplied electrons to 16-DHPR. Flavins or NAD(P)H could not replace pyruvate as electron donors.

16-DHPR activity was found in both the cytoplasmic and membrane fractions of crude cell extracts; however, the specific activity was approximately twice as high in the membrane fraction as in the cell extracts. The 16-DHPR activity was stable for at least one week when stored anaerobically at 0°C. When attempts were made to solubilize 16-DHPR from the membrane with Triton X-100, all activity was lost. The pyruvate:methyl viologen oxidoreductase activity was detected in the cytoplasm but not in the membrane.
fraction. The 16-DHPR had a very broad pH optimum that extended from 5.0 to 8.0. The highest enzyme activity was obtained when the reaction mixture contained 10% methanol. The activity was inhibited by dithiothreitol.

Desmolase is another steroid-altering enzyme that has been demonstrated in a Eubacterium sp. A bacterium that was classified as Eubacterium desmolans was isolated from cat feces (Morris et al., 1986). This bacterium synthesized a desmolase that cleaved the side chain of 17α-hydroxysteroids between C-17 and C-20. It also produced a 20β-hydroxysteroid dehydrogenase that reduced the keto group at C-20.

In summary, in addition to cholesterol reductase activity, eubacteria or eubacteria-like organisms have been shown to have the following activities against steroids: 7α-dehydroxylase (White et al., 1980), delta-6 reductase (White et al., 1982), 21-dehydroxylase (Feighener et al., 1979), 7α-hydroxysteroid dehydrogenase (Franklund et al., 1990), 7β-hydroxysteroid dehydrogenase (Hirano and Masuda, 1982), 16α-dehydroxylase, 16-dehydroprogesterone reductase, progesterone delta-4 reductase, 3α-dehydroxylase (Bokkenheuser et al., 1980), and desmolase and 20β-hydroxysteroid dehydrogenase (Morris et al., 1986). Most of the strains that acted on a particular substrate did not need the substrate for growth, and growth was often not enhanced by the addition of the substrate to the growth medium. The reason that these organisms produce such an array of specialized enzymes, many of which are constitutive,
is not known (Glass et al., 1979; Morris et al., 1986).

Eubacteria

The word "eubacterium" is derived from the Greek words for beneficial bacterium. In the context of this review, it is used to designate a bacterial genus. The genus name should not be confused with the other common usage of the word, which denotes the eubacterial kingdom. In that context, it stands for the kingdom of "true" bacteria in contrast to another kingdom of bacteria, called archaeabacteria.

Members of the genus *Eubacterium* are nonsporing, Gram-positive rods that are obligately anaerobic and chemoorganotrophic. Eubacteria produce mixtures of organic acids from carbohydrates or peptones. The most common acidic end products are butyric, acetic, and/or formic acids. Eubacteria do not produce propionic or lactic acid as the major end product, or succinic acid with lactic acid and small amounts of acetic or formic acids in the presence of CO₂. They also do not produce acetic and lactic acids as the major end products. The production of acidic end products is the major differentiating factor that distinguishes the genus *Eubacterium* from the closely related genera *Propionibacterium, Lactobacillus, Actinomyces*, and *Bifidobacterium* (Moore and Moore, 1984).
Eubacteria have been isolated from cavities of man and other animals, plant and animal products, soft tissue infections, and soil. Their sensitivity to oxygen varies, and many strains need to be cultivated on prereduced media. The optimal growth conditions include an incubation temperature of 37°C and a pH near neutrality (Moore and Moore, 1984). In the human intestine, eubacteria are usually found in concentrations of $10^9$ to $10^{12}$ per ml of fecal material (Goldin, 1986). They are rarely found in the stomach, jejunum, or ileum and exist primarily in the colon. In a study of the anaerobic fecal flora of the baboon, Brinkley and Mott (1978) enumerated $3.3 \times 10^{10}$ eubacteria per gram of dry fecal matter. These bacteria comprised 16.7% of the total number of viable organisms recovered.

Technologies for Reducing Cholesterol in Foods

As the consumer demand for reduced-cholesterol foods increases, industries are investigating several methods for supplying this demand. One of the new methods that is just beginning to be used is steam-stripping. This process uses steam distillation to remove greater than 90% of cholesterol from butter oil and lard (Sperber, 1989). This process will have few regulatory problems and should be relatively inexpensive, making it one of the best candidates for widespread use in the near future.
Supercritical CO\textsubscript{2} extraction is a process that has been used for many years for various purposes, such as the extraction of caffeine from coffee. In this type of extraction, a very high pressure at relatively low temperatures is used to bring a material into a supercritical state. When CO\textsubscript{2} is in this state, it behaves both as a liquid and as a gas, and it dissolves cholesterol in foods. This process has been used to reduce cholesterol levels in butterfat by up to 98\% (Sperber, 1989). The greatest challenge in applying this technology is in developing cost-efficient methods to treat foods on an industrial scale.

Enzymes have been used to treat foods since ancient times. The uses included the production of fermented liquors, leavened bread, the conversion of milk to curds and whey, and meat tenderization with papain from papaya fruit and tree leaves (reviewed by Neidleman, 1991). Some of the more important enzymes used in the food industry today include amylases, proteases, glucose oxidase, pectinase, hemicellulase, invertase, rennin, lipase, catalase, and lactase. When compared to chemical processes, enzymatic methods are less harsh, form fewer by-products, are easier to control, and are considered "natural" by the consumer.

To date, very little research has been done on enzymatically reducing the cholesterol content of foods. Besides our current work on cholesterol reductase (Dehal et al., 1991; Freier and Hartman, 1991), the only publications known to this author on the subject of enzymatic reduction of
food cholesterol involved cholesterol oxidase.

The isolation of bacteria from soil with the ability to oxidize cholesterol was first accomplished by Turfitt (1944). He demonstrated that nocardiae were the predominant cholesterol oxidizers in soil. A very broad range of aerobic bacteria have been shown to have cholesterol oxidizing abilities. A *Streptomyces* sp. was isolated on a medium that contained cholesterol as the sole carbon source (Brown and Peterson, 1966). It was able to utilize 80 to 100% of the cholesterol in a 0.1% cholesterol medium within 6 d. Most of the carbon in cholesterol was converted to CO₂, but some appeared in the cellular material. More recently, 726 cholesterol-degrading bacteria were isolated and compared taxonomically (Ferreira and Tracey, 1984). The majority of the cholesterol oxidizers were classified as *Rhodococcus* spp., and a few as *Mycobacterium* spp. and *Nocardia* spp.

The first attempt to use cholesterol-oxidizing bacteria to reduce the cholesterol content of a food was made by Aihara et al. (1988). A strain of *Rhodococcus equi* that had been isolated from butter was grown in a minimal medium. Sterile egg yolk was added to this medium in various concentrations, ranging from 0.3 to 1.7 mg per ml. Cholesterol degradation was then monitored by thin-layer chromatography. After 3 d of incubation, the cholesterol content of the medium had decreased, but, at concentrations above 0.9 mg of egg yolk per ml, the degradation was incomplete. The only detectable intermediate was
4-cholesten-3-one, and the authors suggested that the cholesterol was rapidly converted to nonsteroid compounds with almost no accumulation of steroid intermediates.

Several strains of cholesterol-oxidizing bacteria were compared for their ability to rapidly degrade cholesterol with no accumulation of intermediates (Johnson and Somkuti, 1990). It was observed that, when cholesterol was introduced to an actively growing strain of _R. equi_, it was rapidly degraded with no accumulation of intermediates. If resting cells were used, 4-cholesten-3-one and 1,4-cholestadiene-3-one and other unidentified intermediates were detected. The oxidation enzyme system was stimulated by the presence of cholesterol in the growth medium but was constitutively expressed. Cell-free extracts were prepared by sonication from the most efficient cholesterol-degrading strains. The most efficient extract could degrade up to 9.4 μg of free cholesterol per minute per mg protein. However, when this extract was incubated with egg yolk cholesterol, only 1.9 μg of cholesterol was degraded per minute per mg protein. The investigators believed that the cholesterol-degrading enzymes may have had a limited accessibility to the egg yolk cholesterol. Up to 40% of the egg yolk cholesterol was degraded by an extract after 1 h of incubation. Using cholesterol in cream as the substrate, only 2.4% of the cholesterol was degraded in 1 h; 7.0% was degraded after 15 h. It was concluded that more data are needed on the toxicity of the products generated by
cholesterol-oxidizing systems. In addition, the system studied could not realistically be used to reduce the cholesterol content of foods unless more efficient enzymes or better ways of making the cholesterol accessible to the enzymes are developed.

There are several ways that enzymatic reactions involving water-insoluble substrates can be made more efficient. When a cell paste of a cholesterol-oxidizing Nocardia sp. was suspended in carbon tetrachloride, cholesterol was converted to 4-cholesten-3-one at a more rapid rate than when the reaction was carried out in an aqueous system (Buckland et al., 1975). The cells could be easily separated from the reaction mixture and reused several times.

Kazandjian and coworkers (1986) developed a system for the determination of cholesterol concentrations in an organic solvent system. They coupled peroxidase with cholesterol oxidase and tested for activity in a toluene solvent system. They determined that activity was maintained by both enzymes. Yoshimoto et al. (1987) modified cholesterol oxidase with a synthetic copolymer of polyoxyethylene allylmethyldiether and maleic acid anhydride. This modified enzyme was soluble in organic solvents. In benzene, the modified cholesterol oxidase formed 4-cholesten-3-one with an activity of 0.6 µmol per min per mg protein.

Many enzymes have increased activities in water-organic solvent two-phase systems. Several hydroxysteroid
dehydrogenases had greatly increased activities in two-phase systems consisting of ethyl acetate or butyl acetate in an aqueous buffer (Carrea et al., 1988). A very efficient system was devised for hydroxysteroid dehydrogenases that consisted of immobilized enzyme, a two-phase buffer, and an NAD(P)(H)-regenerating system.

Another type of system that has been recently developed for the enzymatic conversion of water-insoluble substrates is the detergentless microemulsion. This system consists of a hydrocarbon, isopropanol, and water (Khmelnitsky et al., 1988). When cholesterol oxidase was tested in a detergentless microemulsion, large quantities of cholesterol were converted to 4-cholesten-3-one with 100% yield. The separation of reaction product and the regeneration of enzyme were easily accomplished.

The activities of steroid-reactive enzymes can be greatly increased in organic solvent-containing systems. Unfortunately, most of these systems use chemicals that are toxic or carcinogenic and would not be ideal for the treatment of foods. Supercritical CO₂ is a much more viable option. CO₂ has a critical temperature of 31.1°C, which is a suitable temperature for treating most biochemicals. Proteins are insoluble in supercritical CO₂, so enzymes are easily removed from the reaction. CO₂ is inert, inexpensive, and nontoxic, making it an excellent candidate for food treatment. Randolph et al. (1988) showed that cholesterol oxidase was active in supercritical CO₂ and that nearly 100%
of the cholesterol in their preparations was converted to 4-cholesten-3-one within 1 h.

There are also several other ways that an enzymatic conversion of cholesterol could be made more efficient. When resting cells of a cholesterol-oxidizing strain of *Rhodococcus erythropolis* were irradiated with a low level of ultrasonic energy, the kinetic rates of biotransformation were significantly enhanced (Bar, 1988). Another way cholesterol conversion could be improved is by using immobilized cells. Sonomoto et al. (1982) achieved increased activity and stability of 11α-hydroxylation of progesterone when *Rhizopus stolonifer* mycelia were entrapped in a photo-crosslinked resin. Tiny magnetic particles were used to immobilize a cholesterol side-chain degrading *Mycobacterium* sp. (Flygare and Larsson, 1987). This allowed the repeated use of the bacterial cells, because cells could be retrieved from the reaction mixture with a magnet.

An immobilized cell method was developed for a continuous steroid reduction by a strict anaerobe (Abramov et al., 1990). Cells of *Clostridium paraputrificum* were immobilized in polyacrylamide-hydrazide beads. The gel-entrapped cells could tolerate the presence of up to 30% ethylene glycol that was added as a cosolvent. A system was optimized that could continuously reduce 1,4-androstadiene-3,17-dione to its 3-keto-5β and 3α-hydroxy-5β products with a yield of 98% for at least 120 h. It is possible that a similar technique could be used to reduce cholesterol to coprostanol in foods.
PART I. ISOLATION OF CHOLESTEROL-REDUCING BACTERIA

Introduction

To date, a total of 13 cholesterol-reducing bacteria (CRB) have been isolated and characterized (Eyssen et al., 1973; Sadzikowski et al., 1977; Mott and Brinkley, 1979; Brinkley et al., 1982). All of these bacteria have been classified as strains of Eubacteria sp. All of the strains were isolated from the fecal contents of rats, humans, or baboons.

Two methods have been used to isolate CRB. The first method employed enrichment in cholesterol-containing media, repeated dilution to extinction of coprostanol-producing cultures, filtration, and use of selective agents (Eyssen et al., 1973; Sadzikowski et al., 1977; Mott and Brinkley, 1979). These methods were used because the CRB did not form colonies on any type of plating medium that was tried. The second method involved using an anaerobic chamber to plate dilutions of samples on a solid medium that contained a high level of cholesterol homogenized with brain tissue (Brinkley et al., 1982).

Because of the methods used and the sources that were sampled, most of the characterized strains of CRB had similar properties. The bacteria required strict anaerobic conditions for growth, and all but two strains required a plasmalogen (plasmenylethanolamine) to reduce cholesterol to
coprostanol (Brinkley et al., 1982). Many of the strains also had an obligate requirement for cholesterol or a related sterol for growth (Eyssen et al., 1973; Sadzikowski et al., 1977; Mott and Brinkley, 1979). The CRB grew slowly, and they formed colonies on solid media poorly or not at all.

Because previously isolated strains of CRB were very fastidious, common laboratory procedures could not be used to study them. The growth media that were used contained brain or insoluble lipids that interfered with obtaining a clean bacterial pellet after centrifugation. The cultures did not form colonies on agar plates, making reisolation and enumeration difficult. For these reasons, various methods for producing clear media that may sustain CRB were formulated, and new enrichment and isolation techniques were combined with more diverse sample types to isolate bacteria with better characteristics.

Several methods to solubilize cholesterol were tested. Cholesterol has been esterified with adipic acid and reacted with polyethylene glycol to yield a water-soluble derivative (Proksch and Bonderman, 1978). Many investigators have used cholesterol-lecithin vesicles or liposomes as the source of cholesterol for cholesterol-requiring bacteria (Kahane and Razin, 1977; Stanton, 1987). Cyclodextrins are doughnut-shaped molecules composed of 6 to 8 glucose units linked by α-1,4-glycosidic bonds. Cyclodextrins have been used to solubilize sterols (Hesselink et al., 1989; Bar, 1990); when hydrophobic molecules such as cholesterol enter a hydrophobic
cavity within the cyclodextrin, they become much more soluble in water.

Several different plating methods were used to obtain isolated colonies. Typically, plating media contain agar as the solidifying agent. However, some bacteria are inhibited by agar. Gelrite (Schweizerhall, Inc., South Plainfield, NJ) is an agar substitute, composed of gellan gum from Pseudomonas clodea, that has been used for the cultivation of bacteria that are inhibited by agar (Harris, 1985). Solid plating media for anaerobes are usually either poured into petri plates that are incubated in anaerobic chambers or jars or made into roll tubes (Holdeman et al., 1977). Another technique is to contain a solid medium in specially constructed bottles under an oxygen-free atmosphere (Hermann et al., 1986). This latter method is called the Wolfe bottle technique.

Certain compounds have been shown to be stimulatory to the growth of various stains of Eubacterium spp. or to promote coprostanol production. L-Arginine and L-lysine stimulated the growth of Eubacterium nodatum, Eubacterium timidum, and Eubacterium brachy (Hill et al., 1987). Riboflavin enhanced the 7a-dehydroxylase activity of a Gram-positive intestinal anaerobe (Masuda et al., 1983). Glycyrrhizin and p-nitrophenyl-mono-β-D-glucuronide (pNPG) stimulated the growth of Eubacterium sp. strain GLH when grown in a carbohydrate-free medium (Akao et al., 1988). When CaCl₂ was added to the diet of rats, the percentage of
coprostanol in the feces increased (Wells and Cooper, 1958). Brinkley et al. (1982) observed that pyruvate stimulated CRB.

Selective agents and various media formulations were tested for their ability to inhibit the background flora. Watanabe et al. (1986) used a medium that contained cholesterol as the sole carbon source to enrich for and isolate cholesterol-oxidizing bacteria. Bile salts have been used to selectively inhibit non-enteric bacteria. Deoxycholic acid inhibits flagellation and swarming in Proteus spp. (D'Mello and Yotis, 1987). Bile salts also increased the cholesterol uptake of a strain of Lactobacillus acidophilus under anaerobic conditions (Gilliland et al., 1985). Snog-Kjaer and coworkers (1956) observed that coprostanol is formed by mixed cultures of bacteria in the presence of 0.15 mg per ml CuSO₄, a compound that is inhibitory to many microbes. Sadzikowski et al. (1977) used 0.5% phenethyl alcohol to eliminate Gram-negative rods that were present in a mixed cholesterol-reducing culture. This compound has also been used to inhibit the swarming of Proteus spp. (Kopp et al., 1966). A cholesterol-reducing Eubacterium sp. was capable of growing in media containing clindamycin, 1.6 µg per ml; erythromycin, 3 µg per ml; and penicillin, 2 U per ml (Sadzikowski et al., 1977).

New methods and sample sources were used to isolate novel cholesterol-reducing bacteria with characteristics that may make industrial-scale food treatment possible.
Materials and Methods

Bacteria

Eubacterium ATCC 21408 (Eyssen et al., 1973) and Eubacterium sp. from rats and Eubacterium sp. from a human (Sadzikowski et al., 1977) were obtained from the culture collection of the Virginia Polytechnic Institute and State University. The bacteria were transferred every two weeks into an anaerobic brain-containing medium. Mixed cultures and pure cultures of new isolates of cholesterol-reducing bacteria were maintained by weekly transfers in base cholesterol plus CaCl₂ (BC) medium.

Growth media

Standard brain medium (SBM) was prepared as described by Brinkley et al. (1982), except it was boiled under N₂ gas that was purified by passage through a column of heated copper (Hungate technique) as described by Holdeman et al. (1977). The medium was cooled, dispensed to 16 X 100 mm culture tubes (9 ml per tube), sealed with butyl rubber stoppers, placed in a press, and sterilized in an autoclave at 121°C for 15 min. Standard pork brain (SPB) medium was prepared as described by Sadzikowski (1977) except that it was boiled, dispensed, and sterilized in the same manner as SBM. Thioglycolate medium with brain (TMB) contained per liter: thioglycolate medium without dextrose or indicator (Difco, Detroit, MI), 24 g; lyophilized pork brain, 30 g;
yeast extract (Difco), 10 g; sodium thioglycolate, 1.3 g; and resazurin, 1 mg. TMB was homogenized in a Waring blender (Waring Products Div., New Hartford, CT) for 5 min under a stream of N₂ gas and then prepared anaerobically in the same manner as SBM. Several compounds were added to TMB medium to test for increased coprostanol production by CRB. These included the following (per liter): 0.5 g of cysteine•HCl•H₂O substituted for sodium thioglycolate, 93 mg of riboflavin, 5 g of L-lysine, 5 g of L-arginine, and 50 mM potassium phosphate buffer, pH 7.0.

A base cholesterol medium (BM) was prepared as described by Brinkley and Mott (1982) except that cholesterol and lecithin were homogenized in distilled, purified water (dH₂O) in a Waring blender under a stream of N₂ gas, the other components were added, and then the medium was prepared anaerobically in a manner similar to SBM. Plasmenylethanolamine was not added. BM contained the following components per liter: lecithin (type IV-S, Sigma Chemical Co., St. Louis, MO), 1 g; cholesterol, 2 g; casitone, 10 g; yeast extract, 10 g; sodium thioglycolate, 0.5 g; K₂HPO₄, 40 mg; KH₂PO₄, 40 mg; and resazurin, 1 mg. The pH was adjusted to 7.2 with 3 N KOH before boiling.

The efficiency of coprostanol production was tested with mixed cultures grown in several modifications of BM. BM was prepared with and without each of the following: potassium phosphate buffer, sodium pyruvate, sodium formate (0.4 g per liter), sodium selenite (0.17 mg per liter), and calcium
chloride (0.1 g per liter). BM was also inoculated and added to tubes containing Tween 80 (Fisher Scientific Co., Fair Lawn, NJ) as described by Holdeman et al. (1977). In addition, various concentrations of sodium formate (0.04 g to 8.4 g per liter) and of CaCl₂·2H₂O (0.01 g to 100 g per liter) were tested. BM that contained 1 g per liter CaCl₂·2H₂O, and that did not contain potassium phosphate buffer, was called BC medium. BM was prepared with each of the following: 1.2 mM glycyrrhizin (Sigma), 2.4 mM p-nitrophenol mono-β-D-glucuronide (pNPG; Sigma), or 10 ml per liter VPI hemin solution with 200 µl per liter VPI vitamin K₁ solution (Holdeman et al., 1977).

To test for the conversion of sterols other than cholesterol by mixed cultures of CRB, the following sterols (Sigma) were substituted for cholesterol in BM: stigmosterol, ergosterol, androstenedione, and 4-cholesten-3-one.

Thioglycolate medium with cholesterol (TC) contained the same concentration of homogenized lecithin and cholesterol as BM, but contained (per liter): Thioglycolate Medium without dextrose or indicator (Difco), 24 g; sodium pyruvate, 0.5 g; and resazurin, 1 mg. After the pH was adjusted to 7.2, the medium was heated to boiling in a microwave oven, dispensed to screw-capped tubes, and sterilized. Just before inoculation, TC tubes were steamed 5 min in an autoclave or were placed in boiling water for 5 min to partially drive off oxygen and rapidly cooled to 37°C.
Supplemented brain-heart-infusion broth (BHI-S) contained the following components (per liter): BHI broth (Difco), 37 g; yeast extract, 5 g; resazurin, 1 mg; cysteine·HCl·H₂O, 0.5 g; VPI hemin (Holdeman et al., 1977), 1 ml; and VPI vitamin K₁ (Holdeman et al., 1977), 0.2 ml. BHI-S broth was prepared anaerobically by using the same method as was used for SBM. The cysteine, hemin solution, and vitamin K₁ solution were added after boiling. Medium M10 was prepared as modified by Brinkley and Mott (1978), except it was used as a broth that was made by using Hungate techniques (Holdeman et al., 1977). M10 medium was also prepared with homogenized cholesterol (0.2%) and lecithin (0.1%). Peptone yeast extract (PY) and PY plus glucose (PYG) media were prepared as described in the Anaerobe Laboratory Manual (Holdeman et al., 1977).

Clear cholesterol-containing media

Various methods of producing clear cholesterol-containing media that would support the growth of CRB were formulated. A roll-tube medium that contained polyoxyethyl-cholesteryl sebacate (water-soluble cholesterol, Sigma) was prepared (WSRT). This medium contained the following components per liter: water-soluble cholesterol, 4 g; Casamino Acids (Difco), 8 g; Folch Brain Extract (Sigma), 0.8 g; yeast extract, 8 g; K₂HPO₄, 4 g; agar, 16 g; and resazurin, 1 mg. The medium was boiled 5 min under O₂-free N₂ gas and tempered to 46°C. Cysteine·HCl·H₂O
was added (400 mg). The medium was dispensed to roll tubes that were sealed with butyl rubber stoppers, placed in a press, and sterilized in an autoclave for 15 min at 121°C. Roll tubes were then made as described by Holdeman et al. (1977).

A liposome-containing medium (LIP) was prepared by dissolving lecithin (97% phosphatidyl choline, Behring Diagnostics, La Jolla, CA), 916 mg; cholesterol (Sigma), 200 mg; sodium cholate (Sigma), 760 mg; and Folch Brain Extract (Sigma), 18 mg, in 30 ml of 95% ethanol. The ethanol was evaporated under a stream of N₂ gas. Distilled water (dH₂O), 220 ml, was added, and the mixture was sonicated for 5 min at 100 watts (Braun-sonic 1510, B. Braun Co., Melsungen). The following components (base medium) were then added: Casamino Acids, 2 g; yeast extract, 2 g; K₂HPO₄, 1 g; sodium thioglycolate, 0.1 g; and resazurin, 0.2 mg. The pH was adjusted to 7.2, and the medium was boiled, dispensed, and sterilized in the same manner as for SBM.

Supplemented proteose peptone broth (SPP) was prepared in the manner described by Hill et al. (1987) for the growth of Eubacterium spp., except a sterile liposome preparation was substituted for horse serum. The liposomes were prepared by evaporating 3 ml of 50% plasmalogen phosphatidyl ethanolamine (Sigma) under N₂ gas in a flask. Three grams of water-soluble cholesterol (Sigma) dissolved in 60 ml dH₂O was added, and the flask was agitated until the lipids were suspended. The liposomes were filtered through a 0.22 μm
pore size syringe filter (Costar Corp., Cambridge, MA),
bubbled aseptically with N₂ gas for 30 min, and added to
sterile, reduced SPP (940 ml) to produce SPPL broth.

A thioglycolate medium with liposomes (T-LIP) was
formulated. Cholesterol, 200 mg, and phosphatidylcholine
(97% pure, Behring Diagnostics), 440 mg, were dissolved in 5
ml of chloroform and placed in a screw-capped vial. The
solvent was evaporated under a stream of N₂ gas, 10 ml dH₂O
were added, and the vial was sonicated in a water bath
sonicator for 15 min. This liposome preparation was added to
the following components dissolved in 100 ml of dH₂O:
Thioglycolate Medium without dextrose or indicator (Difco),
4.8 g; yeast extract, 2 g; sodium thioglycolate, 0.2 g; and
resazurin, 0.1 mg. The medium was reduced and sterilized as
described for SBM.

A medium containing an albumin emulsion of cholesterol
and oleic acid (ACQ; Treadwell and Vahouny, 1977) was
prepared. Phosphatidyl ethanolamine containing 50%
plasmalogen (Sigma), 5 ml, was evaporated in a test tube
under a stream of N₂ gas. Oleic acid, 5 ml, was added and
the tube was mixed on a vortex mixer until the lipid was
dissolved. Cholesterol, 0.4 g, was added, and the mixture
was bubbled with N₂ gas for 20 min. Albumin (Bovine,
Fraction V, Sigma), 0.4 g, was added and the emulsion was
mixed on a vortex mixer. The other components of the base
medium (as described above for LIP medium) were dissolved in
220 ml dH₂O, and slowly added to the cholesterol emulsion
with stirring. The medium was boiled, dispensed, and sterilized by using the same method as for SBM.

A medium was prepared that contained β-cyclodextrin: cholesterol (2:1 molar ratio) in base medium (CDBM), prepared anaerobically. The β-cyclodextrin (Sigma), 1.2 g, was dissolved with the other components in 100 ml of dH₂O. Cholesterol, 0.2 g, dissolved in 3 ml of 95% ethanol, was added in a dropwise manner to the stirred medium. The complete medium was then boiled under N₂ gas, dispensed to tubes, and sterilized.

Serum contains a large amount of cholesterol that is soluble in water. When serum was boiled to drive off oxygen, a precipitate formed. To avoid precipitation, an anaerobic serum-containing medium was formulated by using Oxyrase (Oxyrase, Inc., Ashland, OH) to eliminate the need to boil the medium to attain a low oxidation-reduction potential. The medium (SOT) contained the following components: Thioglycolate Medium without dextrose or indicator (Difco), 4.8 g; yeast extract, 2 g; sodium thioglycolate, 0.25 g; resazurin, 0.2 mg; and dH₂O, 100 ml. After the medium was sterilized in an autoclave and cooled to room temperature, 100 ml of sterile, inactivated fetal bovine serum (Hazelton Research Products, Denver, PA), and 0.33 ml of Oxyrase were added, and the flask was sealed with a rubber stopper. The medium was incubated overnight at 37°C and was then dispensed to sterile tubes under N₂ gas.
Ex-Cyte I (Miles, Inc., Kankakee, IL) is a sterile bovine lipoprotein medium supplement that contains 10 mg per ml cholesterol in aqueous solution. Ex-Cyte I Lactose Pyruvate (XLP) medium contained the following components per liter: Casitone, 10 g, yeast extract, 5 g, sodium thioglycolate, 0.5 g, CaCl₂·2H₂O, 0.1 g, and resazurin, 1 mg. This medium was boiled under N₂ gas, cooled, sealed in a flask, sterilized, and cooled to room temperature. A solution containing 5 g of sodium pyruvate and 5 g of lactose dissolved in 35 ml dH₂O was filter-sterilized by using a 0.22 μm syringe filter and was added to 20 ml of Ex-Cyte I in a sterile flask. This mixture was bubbled with sterile N₂ gas for 30 min and then aseptically added to the other sterile, reduced medium components.

Isolation media

An anaerobic medium (1C) was formulated that contained cholesterol as the sole carbon source. This medium was similar to medium I of Watanabe et al. (1986) that was used aerobically to enrich for cholesterol-oxidizing bacteria. One liter contained: NH₄Cl, 1 g; K₂HPO₄, 0.25 g; MgSO₄·7H₂O, 0.25 g; NaCl, 5 mg; FeSO₄, 0.5 mg; and cholesterol, 1 g. The pH was adjusted to 7.0; the medium was homogenized with a Waring blender and boiled under N₂ gas. After cooling, 2.5 ml of a 10% solution (w/v) of Na₂S·9H₂O was added; the medium was dispensed to tubes and sterilized in the same manner as was used for SBM.
BHI-S was modified by the addition of Oxgall (Difco) at a concentration of 3 g per liter and cholesterol at 2 g per liter (BHI-SOC). Oxgall and cholesterol were homogenized in dH$_2$O in a Waring blender. This homogenate was then added to the remaining components, and the medium was prepared as described above for BHI-S. Oxgall and cholesterol were added in a similar manner to AC broth (Difco) that was then prepared (aerobically) according to the manufacturer’s instructions (AC-OC). Oxgall was also added (0.5%, w/v) to SPB broth (SPBO).

Batches of TMB medium were made that contained one of the following: CuSO$_4$, 0.15 mg per ml; phenethyl alcohol, 0.5%; clindamycin, 1.6 µg per ml; erythromycin, 3 µg per ml; and penicillin, 2 U per ml. Phenethyl alcohol (0.5%) was also added to BM broth plus 0.5% sodium pyruvate.

To screen for possible cholesterol-reducing lactobacilli, MRS broth (Difco) was prepared according to the manufacturer, except that it was made anaerobically and with the addition of Oxgall and cholesterol (MRS-OC) by using the same method as for the preparation of BHI-SOC. Cysteine·HCl·H$_2$O was added (0.5 g per liter) after boiling. LBS broth (Difco) was also prepared with the addition of Oxgall, cholesterol, and cysteine (LBS-OC).

Several media and methods were adopted for the isolation of CRB by using solid anaerobic media. Cholesterol brain agar (CBA) was prepared according to Brinkley and coworkers (1980). This medium was made aerobically, sterilized, and
poured into sterile petri dishes. The dishes were then placed in an anaerobic vacuum jar that was flushed with N₂ gas and then evacuated under vacuum several times. After drying overnight, the plates were inoculated, placed back into the jar that was made anaerobic as was described for drying the plates, and incubated at 37°C. This medium was also prepared anaerobically as described for SBM; after it was boiled and cooled, it was dispensed (8 ml per tube) to 18 x 142 mm roll tubes that contained 0.12 g agar (Difco) as described by Holdeman et al. (1977). After sterilization, roll tubes were fashioned.

CBA supplemented with 0.5% sodium pyruvate was also used in a method that included an Oxyrase overlay technique. Sodium lactate (60% syrup), 4.7 ml, and agar, 20 g, were added to 1 liter of 20 mM sodium phosphate buffer, pH 7.5. The mixture was heated in a microwave oven and dispensed 15 ml per tube to screw-capped tubes. The tubes were sterilized in an autoclave (121°C for 15 min), and cooled to 45°C. To each tube, 0.15 ml of Oxyrase was aseptically added, and the molten Oxyrase solution was poured over an inoculated CBA plate. The plates were then incubated (in air) at 37°C. The Oxyrase overlay was also prepared with the addition of 1 mg per ml cycloheximide, 1.6 μg per ml clindamycin, and 3 μg per ml erythromycin. The cycloheximide was added after boiling but before sterilization. The tubes were sterilized in an autoclave for 12 min at 121°C. Clindamycin and erythromycin were added as filter-sterilized (0.22 μm pore size) solutions
after the overlay was sterilized.

Modified lecithin/cholesterol Tween 80 roll tubes (MLT) contained per liter: lecithin (Sigma, type II-S), 0.3 g; cholesterol, 0.2 g; casitone, 16 g; yeast extract, 5 g; NaCl, 2.5 g; L-cystine, 0.25 g; sodium thioglycolate, 1 g; KH$_2$PO$_4$, 1.2 g; K$_2$HPO$_4$, 3.8 g; resazurin, 1 mg; sodium pyruvate, 0.5 g; and Tween 80, 0.2 ml. This medium was prepared anaerobically and dispensed, 8 ml per tube, to roll tubes (25 x 140 mm) containing 0.13 g of agar. Tubes were sterilized in an autoclave (121°C for 15 min). MLT was also made with 0.5% phenethyl alcohol and with twice the normal concentration of agar (MLTP) to inhibit swarming bacteria. This medium was also prepared with the omission of Tween 80, and the addition of 0.1% (w/v) of deoxycholic acid (Sigma).

BM was prepared as previously described and dispensed (10 ml per tube) to 25 x 140 mm roll tubes that contained 0.15 g agar, sterilized, and cooled at an angle to form agar slants. BC agar (BCA) contained the following components per liter: lecithin (type IV-S, Sigma), 10 g; cholesterol, 20 g; casitone, 10 g; yeast extract, 10 g; trypan blue (Allied Chemical Co., NY), 100 mg; BiTek agar (Difco), 17.5 g; and CaCl$_2$·2H$_2$O, 1 g. The lecithin and cholesterol were homogenized with dH$_2$O in a Waring blender for 10 min under a stream of N$_2$ gas, the other components were added, the pH was adjusted to 7.2, and the medium was sterilized in an autoclave. Flasks containing 500 ml of medium were sterilized for 45 min at 121°C. After sterilization, the
medium was stirred as it cooled to 45°C; then, it was poured into petri dishes and dried overnight in a vacuum desiccator. The plates were then inoculated and incubated in a GasPak jar containing a GasPak Plus anaerobic system envelope with palladium catalyst (Becton Dickinson Microbiology Systems, Cockeysville, MD) with a layer of silica gel desiccant in the bottom of the jar and incubated at 37°C. This medium was also prepared with the substitution of 0.7% Gelrite (Schweizerhall, South Plainfield, NJ) for agar (BCG). In addition, BCA and BCG media were prepared as prereduced media and used in a Wolfe bottle method (Hermann et al., 1986).

A modification of the lecithin agar (MLA) used by Chrisope et al. (1976) was used for the isolation of CRB. MLA medium was prepared as described for lecithin agar, except fraction A contained lecithin, 3 g; cholesterol, 6 g; and dH2O, 135 ml, and was homogenized in a Waring blender for 10 min. Fraction B contained casitone, 3 g; yeast extract, 3 g; BiTec agar (Difco), 5.4 g; trypan blue, 30 mg; and dH2O, 150 ml. The pH of fraction B was adjusted to 7.2. Fraction C contained CaCl2·2H2O, 0.3 g, dissolved in 15 ml dH2O. These 3 fractions were sterilized in an autoclave (121°C for 20 min) in separate containers and then tempered to 45°C in a water bath. The fractions were then carefully combined with gentle swirling in the order A + (B + C), then poured into sterile petri plates. The agar plates were dried overnight at room temperature under vacuum in a desiccator that contained CaCl2 pellets. After inoculation, the plates were
placed in a GasPak jar containing a GasPak Plus anaerobic system envelope with palladium catalyst and incubated at 37°C.

**Growth of cholesterol-reducing bacteria in clear media**

Pure cultures of CRB were grown in cholesterol- or brain-containing media. They were then transferred to one of the clear cholesterol-containing media and incubated at 37°C for 1 week. The cultures were transferred weekly in the clear media for 5 weeks. After the fifth transfer, the cultures were inoculated into a brain-containing medium, incubated for 1 wk, and extracted. The extracts were assayed for coprostanol.

**Isolation procedure**

Samples were collected in 25 x 140 mm tubes, sealed with butyl rubber stoppers, and immediately transported to the laboratory where they were diluted and inoculated into various media. Cultures were incubated at 37°C for 7 to 21 d, then extracted and assayed for coprostanol. The highest dilution that tested positive for coprostanol was again serially diluted, incubated, and assayed. This was repeated several times to reduce background bacteria.

Anaerobic digester and raw sewage samples were collected from a municipal waste treatment plant. Samples were also collected from freshly voided feces of a baby goat and from an adult goat. The adult goat had been fed a high-
cholesterol (2% w/w) diet. The samples were serially diluted in VPI anaerobic diluent (Holdeman et al., 1977) to $10^{-10}$, then inoculated into medium 1C, BHI-SOC broth, BHI-S broth, SPBO broth, MRS-OC broth, and LBS-OC roll tubes. The samples were also serially diluted in sterile 0.1% peptone blanks and inoculated into AC-OC broth. In addition, dilutions of adult goat feces were inoculated into TMB broth, BHI-SOC broth, and modified M10 broth. Rumen fluid from the adult goat was serially diluted in VPI diluent and inoculated into SPB broth, modified M10 broth, and BHI-SOC broth.

Freshly voided horse feces were collected, diluted, and inoculated into LIP medium, BHI-SOC broth, TMB broth, and SPPL broth. Samples from human and cat feces were serially diluted in TMB broth containing sodium pyruvate and CuSO$_4$.

The following samples were diluted and incubated in BM broth: sponge from a salt water aquarium, sediment samples from rivers, streams, and ponds in the Ames, IA area, and samples of commercially packaged, frozen pork brains.

Samples from soil, cattle and sheep confinement-area runoff, hog sewage lagoon, rotting hay, wet tree leaves, sediment from ponds, streams, and drainage ditches, standing water, rotting wood, and wet grass were collected. These samples were diluted in TC medium and incubated at 37°C for 1 week.

The highest coprostanol-producing dilutions were again serially diluted in the same medium in which they were originally inoculated or in BC broth. After this was
repeated several times, the cultures were transferred weekly.

Samples that coagulated the cholesterol-containing media and that tested positive for coprostanol were inoculated onto various plating media for isolation of cholesterol-reducing bacteria. Isolated colonies of every morphological type evident were picked with a sterile platinum needle and inoculated into SBM, SPB, TMB, BM, or BC broths. These cultures were incubated for 7 to 21 days (37°C) and then tested for coprostanol production. Coprostanol-positive cultures were Gram-stained (Holdeman et al., 1977) and restreaked to a solid plating medium.

**Sterol conversion assay**

Samples that originated from sources expected to contain cholesterol esters were first saponified. Samples (2 ml) were placed in 18 X 150 mm screw-capped tubes. Ethanol (95%), 6 ml, and KOH (33%), 6 ml, were added, and the tubes mixed on a vortex mixer. They were then placed in a water bath at 55°C for 30 min. After cooling, 10 ml of hexane was added to each tube, and the tubes were vigorously mixed. Distilled water (4 ml) was added, the tubes were mixed again, and then the tubes were subjected to centrifugation for 10 min at 1000 X G to separate the phases. The top (hexane) layer was removed to a vial and evaporated under air at room temperature. The lipids were resuspended in 2 ml of chloroform. Samples that were not expected to contain cholesterol esters were simply extracted with hexane as
Samples were spotted on silica gel thin-layer chromatography (TLC) plates (Si250-PA, J. T. Baker, Inc., Phillipsburg, NJ). The plates were developed in hexane-ethyl acetate (80:20). Sterol spots were visualized by overnight exposure to iodine vapors in a sealed container. Permanent records of TLC plates were made by placing the developed plates on a clear glass plate that was positioned on a photoduplication machine.

Results and Discussion

Media development

Brain-containing media (SBM and TMB) were difficult to prepare using Hungate techniques (Holdeman et al., 1977) because the homogenized media foamed excessively during boiling. However, if caution was exercised and a large headspace was allowed in the boiling flask, the medium could be prepared. The three strains of cholesterol-reducing eubacteria all grew in the brain-containing media. The media coagulated after 3 to 5 days of incubation. When samples were saponified, extracted, and assayed by TLC, approximately 50 to 90% of the cholesterol had been converted to coprostanol.

To facilitate the isolation of cholesterol reductase, attempts were made to grow the eubacteria in a clear medium. No colonies were evident when the eubacteria were streaked to
solidified roll tubes of WSRT agar. Eubacteria grown in SBM or TMB media were inoculated (10% inoculum) into the following media: LIP, SPPL, T-LIP, ACO, CDBM, SOT, and XLP. The cultures were incubated for 1 week and then transferred (1% inoculum) into fresh media. This was repeated 5 times; then, the cultures in each type of medium were assayed for coprostanol production and inoculated into a brain-containing medium. After 5 transfers, there was no obvious turbidity in any of the clear media, the cultures were negative for coprostanol production, and no subsequent growth or coprostanol production was observed after inoculation into SBM or TMB media. No growth was evident when CRB were inoculated and transferred in BHI-S, modified M10, PY, or PYG media.

The water-soluble cholesterol preparation (Sigma) may have been toxic to the eubacteria, or may be in a form that is not utilizable by the bacteria. This may be why no growth was observed on WSRT or SPPL media. According to Eyssen et al. (1973), their cholesterol-reducing eubacterium required at least 1 to 2 mg of cholesterol per ml of growth medium. LIP medium contained a high concentration of cholesterol (7.6 mg per ml) that remained dissolved in the medium. It also contained Folch Brain Extract that would be expected to contain plasmeneylethanolamine. The lack of growth in this medium may be explained by the presence of cholic acid in toxic amounts, or the cholesterol may not have been in a form that was accessible to the bacteria. T-LIP, ACO, and CDBM
media each contained 2 mg per ml of cholesterol and no cholic acid, but did not contain plasmenylethanolamine. Mott and Brinkley (1979) determined that the CRB that they studied required plasmalogen to grow and reduce cholesterol. It was hoped that a different form of cholesterol would negate the requirement for plasmalogen, or that the eubacteria could be gradually "weaned" from this requirement. Unfortunately, this was not successful. Media that contained serum or Ex-Cyte I also did not support the growth of the three strains of CRB tested. The reason may have been the lack of plasmalogen, or the Eh of the medium may not have been sufficiently low. XLP medium did support the growth of a new cholesterol-reducing isolate, *Eubacterium* sp. strain HL. The isolation and characterization of two isolates, one of which was studied in detail, will be discussed further in Part II of this dissertation.

When pure cultures of CRB were grown in TMB medium supplemented with riboflavin, L-lysine, or L-arginine, coprostanol production was not appreciably increased. Coprostanol production was greater in TMB that contained sodium thioglycolate as the reducing agent than in TMB containing cysteine. Coprostanol was also produced more efficiently in TMB medium when potassium phosphate was deleted from the formulation, than when the medium contained potassium phosphate.

Cholesterol conversion by stable mixed cultures of CRB in BM was not stimulated by sodium pyruvate, sodium formate,
sodium selenite, Tween 80, glycyrrhizin, pNPG, hemin, or vitamin K. The intensity of the TLC spot corresponding to coprostanol increased with increasing concentrations of CaCl₂ up to 1 mg per ml. At concentrations of CaCl₂ greater than 1 mg per ml, coprostanol production was inhibited. Mixed cultures of CRB were capable of converting stigmasterol, ergosterol, and 4-cholesten-3-one to their corresponding 3-β-hydroxyl, 5-β derivatives. Androstenedione was not reduced.

**Preliminary studies on the isolation of cholesterol-reducing bacteria and on cholesterol reductase**

In an attempt to isolate cholesterol-reducing bacteria with unique properties, several sources were sampled that had not previously been investigated. An anaerobic sewage digestor was sampled. The environment within the digestor was very anaerobic, and cholesterol and related plant sterols were present. Samples from the digestor did contain coprostanol, but no stable mixed cultures or pure cultures of CRB were obtained from this source. Raw sewage was sampled because CRB from a diversity of sources may have been present, and bacteria capable of tolerating limited amounts of oxygen would have survived in this type of environment. Aerotolerance would be a desirable characteristic of a CRB to be used either experimentally or on an industrial scale. Unfortunately, no aerobic, aerotolerant, or anaerobic CRB were isolated from this source.
It was proven that coprostanol that is produced in the rumens of sheep was of bacterial origin (Ashes et al., 1978). However, no attempt was made by those workers to isolate the CRB. For this reason, the ruminal contents of goats were sampled and inoculated into several types of media. Coprostanol was evident after 2 transfers but was not produced in subsequent transfers.

An adult goat was fed a high-cholesterol diet in the hopes of enriching the relative numbers of cholesterol-reducing bacteria in vivo. It was observed that the amount of coprostanol excreted in the feces did greatly increase, although the ratio of coprostanol to cholesterol probably remained the same. A mixed culture was obtained from the feces of this animal. When the culture was repeatedly transferred in cholesterol-containing media, it continued to produce coprostanol.

Horse feces were sampled because horses have an extensive large intestine that would be expected to be a rich environment for CRB. Several stable mixed cultures were obtained that contained CRB from horse feces. Samples that were inoculated into BHI-SOC and TMB media were positive for coprostanol by TLC assay after 10 to 15 transfers. The CRB were apparently resistant to the concentration of Oxgall used in BHI-SOC medium. However, after numerous transfers and serial dilutions, several distinct morphologies were present in Gram stains, and a pure culture could not be obtained.
Stable mixed cultures were also obtained from samples of human and cattle feces, a marine sponge, sediments from rivers, streams, and ponds, standing water, a hog sewage lagoon, and commercial frozen pork brains. Many stable, mixed cultures were obtained after dilution in TC medium, which was not a prereduced medium. This method was used in attempts to isolate bacteria that were not strict anaerobes. The mixed culture from the marine sponge produced nearly 100% coprostanone from cholesterol, and only very faint coprostanol bands were evident on TLC plates. Nearly all of the stable mixed cultures of CRB produced 4-cholesten-3-one and coprostanone in addition to coprostanol.

Samples from several sources were inoculated into an anaerobic medium (IC) that contained cholesterol as a sole carbon source. After several transfers in this medium, growth was not evident, and samples that were assayed by TLC were negative for coprostanol. Unlike bacteria that oxidize cholesterol, CRB probably do not break the ring structures of cholesterol and cannot assimilate the carbon that exists in the cholesterol molecule.

Stable mixed cultures of CRB were inoculated onto various types of solidified media to attempt to isolate pure cultures of CRB. Hundreds of trials were conducted. When roll-tube techniques were used, isolated colonies could not be obtained because the entire surface of the medium became covered with bacterial growth, probably from swarming bacteria. The addition of phenethyl alcohol and doubling the
agar concentration did not rectify the problem. Cholesterol-containing roll tubes were also not clear, so visualizing colonies was difficult. CRB on BM agar slants and on agar- or Gelrite-solidified media in Wolfe bottles also were rapidly overgrown by background bacteria. Because the CRB that had been isolated previously were relatively slow-growing bacteria, the solidified media were incubated for at least one week. When Oxyrase overlays were made on CBA plates, many plates became contaminated. When various antibiotics were added to the overlay, the contamination problem was solved, but none of the isolated colonies that were picked and inoculated to cholesterol-containing media produced coprostanol. Although approximately 1000 colonies were picked, grown in cholesterol-containing media, extracted and assayed by TLC, no CRB were isolated using any roll tube, agar slant, Wolfe bottle, or Oxyrase overlay technique.

In an attempt to selectively enrich for CRB, several selective agents were incorporated into various media. Stable mixed cultures of CRB continued to grow and reduce cholesterol in the following media: BHI-SOC, SPBO, TMB with phenethyl alcohol, and TMB with clindamycin and erythromycin. Cultures also grew in BM medium plus phenethyl alcohol and in media containing deoxycholic acid, and CuSO₄. Samples that were inoculated into media selective for lactobacilli tested negative for coprostanol production after incubation.

Because of the difficulties encountered in trying to obtain pure cultures of CRB, attempts were made to purify
cholesterol reductase from mixed cultures. Various mixed cultures were grown in large volumes in several different types of growth media. The cells were harvested by centrifugation, and washed in buffer. Buffers were used that varied in pH, reducing agents, molarity, and degree of anaerobiosis. Cells were broken by several techniques, including sonication, French press, enzymatic lysis, agitation with glass beads, and treatment with detergents and organic solvents. Broken cells were treated with and without the addition of various protease inhibitors, including Pepstatin A, Aprotinin, Leupeptin, and phenylmethylsulfonyl fluoride (all from Sigma). Cell-free extracts were assayed using cholesterol solubilized by several methods as the substrate. Reactions were carried out under $H_2$, $N_2$, $CO_2$, $Ar$, or air. Cofactors, including FAD, NAD, NADH, NADP, and NADPH, were added alone and in combinations. No cholesterol reductase activity was observed in a cell-free fraction using any of the techniques listed above.

Successful isolation of pure cultures of CRB

A stable mixed culture containing CRB that originated from a hog sewage lagoon was diluted in BC medium and spread to BCA plates that were incubated at 37°C in a GasPak jar. After 5 d of incubation, colonies were picked and inoculated into BC broth. The use of pre-dried plates and a layer of silica gel dessicant in the GasPak jar eliminated overgrowth of the plate by swarming bacteria. After 1 wk of incubation,
several of the BC broth tubes had coagulated (Figure 1). When these cultures were assayed by TLC, approximately 80% of the cholesterol had been converted to coprostanol. A Gram stain of the cultures revealed the presence of a single morphological type of cells that appeared as small, Gram-positive rods. When these cultures were restreaked to BCA or MLA plates, a single morphological type of colony grew on the plates (Figure 2). The colonies were small (approximately 1 mm), and appeared bright white and fibrous against the dark blue background of the trypan blue-containing agar. Growth was similar on BCA and MLA plates, but MLA plates had a smoother, more homogeneous surface, making it easier to differentiate the small colonies from particles of lipid. Using the same methods, a pure culture of a CRB originating from standing water was isolated. Both CRB strains had similar cellular and colonial morphologies, and efficiently converted cholesterol to coprostanol in BC medium.

The same method that was used to isolate the two new strains of CRB described in the previous paragraph was repeatedly tried using the other stable mixed cultures. However, no colonies resembling the colonies formed by the CRB were observed when the other stable mixed cultures were plated. It is likely that the CRB in these cultures had a growth requirement for plasmalogen, as was observed for other CRB (Mott and Brinkley, 1979). The requirement for plasmalogen may have been furnished by other anaerobic
Figure 1. Photograph of BC medium. Left: tube inoculated with strain HL and incubated for 5 d at 37°C. Right: uninoculated tube.
Figure 2. Colonies of strain HL on an MLA agar plate after incubation for 3 d at 37°C in a GasPak jar.
bacteria in the mixed culture. It has been reported that *Bacteroides succinogenes* uses isobutyric and valeric acids as precursors for the synthesis of long-chain fatty acids and fatty aldehydes. These compounds are then used to synthesize ethanolamine plasmalogen (Wegner and Foster, 1963). The pathway for the formation of plasmenylethanolamine in *Clostridium butyricum* has been elucidated (MacDonald and Goldfine, 1990). Cells of CRB that were physically disassociated from plasmalogen-producing bacteria on the agar plates may not have grown because of the lack of available plasmalogen.
PART II. CHARACTERIZATION OF A CHOLESTEROL-REDUCING BACTERIUM

Introduction

Previously isolated strains of cholesterol-reducing bacteria (CRB) have been characterized as strains of *Eubacterium* sp. (Eyssen et al., 1973; Sadzikowski et al., 1977; Mott and Brinkley, 1979; Brinkley et al., 1982). All of these strains had been isolated from the fecal contents of rats, humans, or baboons, and most had very similar characteristics. Because these bacteria had characteristics that made them difficult to work with in the laboratory, new methods and sample sources were explored for the isolation of novel CRB. The isolation of these new strains was described in Part I of this dissertation.

The ability to tolerate limited amounts of oxygen would be a valuable attribute of a CRB, because many laboratory procedures would be facilitated. Anaerobic bacteria are probably sensitive to oxygen because they lack protective enzymes and are susceptible to attack by superoxide radicals, $\text{H}_2\text{O}_2$, oxidized thiols, and peroxided lipids. These cause irreversible damage to the DNA and other cellular components, or excessive NADH oxidation that limits the bacterium's reducing power (Brusa et al., 1989). When 30 strains of freshly isolated intestinal anaerobic bacteria belonging to the genera *Eubacterium*, *Peptostreptococcus*, and *Coprococcus* were tested, they were all killed within 100 to 120 min
(Brusa et al., 1989). Three species of anaerobic intestinal bacteria were compared; a great variation existed in oxygen sensitivity. After 2 h of aeration, no viable cells of *Peptococcus magnus* were detected. *Bacteroides fragilis* survived aeration up to 5 h, and *Clostridium perfringens* survived up to 10 h (Walden and Hentges, 1975). Although no data have been published as to the aerotolerance of previously isolated CRB, all the manipulations used to isolate and grow the bacteria were done by using techniques designed for cultivating strict anaerobes.

Another property of previously isolated CRB that made them recalcitrant to common laboratory procedures was their requirement for a plasmalogen for growth and cholesterol reduction (Mott and Brinkley, 1979). Plasmenylethanolamine is expensive to synthesize and is not commercially available. It is found in high concentrations in mammalian brain tissue, so most CRB were grown in brain-containing media. These media were very turbid, were not homogeneous, and were difficult to make anaerobic. Besides containing cholesterol and plasmalogen, brain tissue also contains many other components, making biochemical characterization of bacteria difficult when using these media. It is impossible to harvest cells of CRB from brain-containing media for enzymatic assays because a thick, viscous pellet is formed, and the brain tissue cannot be separated from the cells.

The isolation and characterization of unique CRB is also important because many questions remain to be answered about
their ecological roles in the rumen and intestines of animals. Many questions also remain about the physiology of these bacteria. Isolation of strains of CRB with unique properties may help answer some of these questions.

Materials and Methods

Bacteria

A new strain of CRB, designated Eubacterium sp. strain HL, was isolated, as described in Part I. The bacterium was maintained by weekly transfer in BC medium (see Materials and Methods, Part I), and incubating at 37°C. Strain HL, and another unidentified pure culture of a CRB, were frozen for long-term preservation. A modification of the procedure developed by Teather (1982) was used. B medium was prepared as described in Part I, except that the concentration of sodium thioglycolate was doubled and a volume of glycerol equal to the volume of dH2O was added before boiling. The B plus glycerol medium was dispensed, 1.5 ml per tube, to 13-mm diameter test tubes, and the tubes were sealed with butyl rubber stoppers. An overnight culture of the bacterium to be preserved, grown in BC medium, was aseptically and anaerobically dispensed (2 ml per tube) to the sterile, anaerobic B plus glycerol tubes. The cultures were briefly mixed by vortexing, and the junction of the test tube and the stopper was wrapped with parafilm. The tubes were frozen at -20°C. According to Teather (1982),
bacteria stored in this manner can be repeatedly thawed, sampled, and refrozen without significant loss of viability and can be stored for at least 2 years.

Media

The basal medium used in the biochemical characterization was similar to the medium used by Brinkley et al. (1982) and contained the following components per liter: Casitone (Difco), 10 g; yeast extract, 10 g; lecithin (type IV-S; Sigma), 1 g; sodium thioglycolate, 0.5 g; cholesterol, 2 g; K$_2$HPO$_4$, 40 mg; KH$_2$PO$_4$, 40 mg; CaCl$_2$·2H$_2$O, 1 g; and resazurin, 1 mg. The cholesterol and lecithin were homogenized with 300 ml of distilled water (dH$_2$O) in a Waring blender for 5 min at high speed under a stream of O$_2$-free Ar. The homogenized lipids were added to the remaining components dissolved in 700 ml of dH$_2$O. The pH was adjusted to 7.2. The medium was reduced by using the Hungate technique (Holdeman et al., 1977). The medium was boiled for 5 min under a stream of Ar that had been purified of contaminating O$_2$ by passage over hot, reduced copper. After cooling to below room temperature (with swirling to keep the lipids dispersed), the medium was dispensed (4 ml per tube) to 13-mm diameter tubes. The tubes were sealed with butyl rubber stoppers, placed in an anaerobic press, and sterilized in an autoclave for 15 min at 121°C. The tubes were inverted several times while cooling to keep the lipids dispersed. Brain heart infusion agar (Difco) was prepared with the
addition of 0.5% sodium pyruvate, 1% lecithin, and resazurin (1 mg per liter). Rogosa SL broth (Difco) was prepared according to the manufacturer's instructions, except 0.2% cholesterol and 0.1% lecithin were homogenized and added in the same manner as used for the preparation of BC medium.

For the determination of nitrate reduction, a medium was formulated that contained the following components per liter: Beef extract (Difco), 3 g; peptone (Difco), 5 g; KNO₃, 1 g; lecithin (type IV-S; Sigma), 1 g; and cholesterol, 2 g. The lecithin and cholesterol were homogenized, and the medium was prepared in the same manner as the basal medium described above, except it was dispensed to 16-mm diameter tubes (9 ml per tube). To test for growth, samples of nitrate reduction medium that had been inoculated with strain HL and incubated for 3 d at 37°C were extracted, and the sterols were separated by thin-layer chromatography (TLC) as described in Part I.

Characterization of strain HL

To determine the optimal medium for the production of cholesterol reductase, several formulations were compared. BC medium was prepared as described in Part I. BC medium was also prepared but with one-tenth the normal concentrations of lecithin, cholesterol, and CaCl₂ (BC.1 medium). BC.1 medium was also prepared with 0 mM, 10 mM, and 25 mM PIPES buffer (Sigma), pH 7.0. After the media were sterilized and cooled, filter-sterilized solutions of sodium pyruvate and lactose
that had been bubbled with Ar for 30 min were added, alone and in combination, to BC.1 media containing the three concentrations of PIPES. The final concentrations of sodium pyruvate were 0, 0.02, and 0.5%. The final concentrations of lactose were 0, 0.5, and 1.0%. BC.1 medium with no buffer, 0.5% sodium pyruvate, and 0.5% lactose was designated SGM medium.

The optimal medium for the production of cholesterol reductase was determined by incubating resting cells of strain HL grown in various media, with a reaction mixture that contained radiolabeled cholesterol. Strain HL was grown in BC medium and BC.1 medium containing various combinations and concentrations of PIPES, lactose, and sodium pyruvate for 2 days. The bacterium was harvested by centrifugation at 10000 × g at 4°C for 30 min. The pellet was resuspended in a volume of buffer (50mM PIPES, 0.5% sodium thioglycolate, pH 7.0) corresponding to 10% of the original growth medium. Samples (1 ml) of the cell suspension were incubated for 1 h at 37°C with 160 μl of radiolabeled cholesterol substrate. The substrate consisted of a sonicated suspension of phosphatidylcholine, 10 mM; cholesterol, 7.5 mM; [1a,2α(n)-3H]cholesterol (specific activity 44.3 Curies per mmole; Amersham Corp., Arlington Heights, IL), 20 μl; and buffer, 20 ml. After incubation, the reaction mixture was extracted with chloroform-methanol (2:1; v:v). Sterols were separated by TLC, using silica gel-H plates (0.75 mm thickness). The plates were developed in hexane-ethyl
acetate (75:25). Sterol spots were visualized by using a 0.2% ethanolic solution of 2,7-dichlorofluorescein. Spots corresponding to authentic cholesterol and coprostanol standards (Steraloids Inc., Wilton, NH) were scraped from the plates, and radioactivity was determined by using a Beckman LS-8000 liquid scintillation spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). Net counts per minute (CPM) are reported as the average total CPM of duplicate or triplicate samples minus the average total CPM of the corresponding reaction mixture that contained bacteria that had been boiled for 10 min.

Several parameters of the resting-cell assay were optimized. Strain HL was transferred to fresh BC medium (10% inoculum) and incubated overnight. This culture was used to inoculate SGM medium (1% inoculum). To ascertain the best time to harvest strain HL for enzymatic assays, the SGM culture was incubated, and samples were removed at various time intervals for use in the resting-cell assay. In another experiment, a 48-h culture of HL in SGM medium was used in the resting-cell assay. Cells were incubated with the reaction mixture for periods of 1, 2, and 3 h. The effects of pyruvate in the growth medium were determined by inoculating strain HL into SGM medium containing various concentrations of pyruvate (0 to 2%) and assaying the resting cells. Cells were also grown in SGM medium without pyruvate, and then incubated in resting-cell reaction mixtures that contained from 0 to 1% sodium pyruvate.
Strain HL was inoculated into SGM medium containing from 0 to 1% yeast extract. After 3 transfers (1% inoculum), the cells in overnight cultures were enumerated by a direct count method (Packard and Ginn, 1985). Because of problems in counting caused by clumping in SGM medium, 1-ml samples of culture were added to 1 ml of a 10% solution of Tween 80 in dH₂O. Samples (10 μl) of each culture were then placed on a microscope slide, dried, heat fixed, stained with crystal violet solution for 2 min, rinsed with dH₂O, dried, and counted. Cultures of strain HL grown in regular SGM and in SGM without yeast extract were diluted and spread to plates of MLA (described in Part I) to determine colony forming units (CFU). The CFU of strain HL after 4 transfers in Rogosa SL broth, BC medium, SGM, and BC plus 10 mM MES, pH 5.5, were also determined by using MLA plates.

To assay for growth and coprostanol production at various pH values, BC was prepared without buffer, and the pH was adjusted with HCl or KOH to values ranging from 5.5 to 8.0. An overnight culture of strain HL in BC medium was inoculated (1% inoculum) into the media. After incubation for 18 h at 37°C, the media were extracted, and the extracts were assayed by TLC. BC was also prepared in each of the following buffers (50 mM): acetate, pH 5.0; citrate-phosphate, pH 5.6; phosphate, pH 6.4 and pH 7.2; Tris-HCl, pH 7.6; and Tris-HCl, pH 8.0. In addition, BC was formulated with the following buffers (10 mM; Sigma): MES, pH 5.5; PIPES, pH 7.0; and EPPS, pH 8.0.
To test for the ability of strain HL to grow in the absence of added cholesterol, BC medium was prepared without cholesterol (BLEC medium). Strain HL grown in BC medium was inoculated into BLEC medium and serially diluted. After 1 wk of incubation at 37°C, the highest dilution that had a grainy appearance was transferred to fresh BLEC medium. Strain HL was transferred 10 times in BLEC medium to confirm growth without cholesterol. This culture was then inoculated into BC medium, incubated 3 d, and assayed for coprostanol production by TLC.

For the determination of phospholipase activity, samples of strain HL grown in BLEC medium (2 ml) were extracted with 3 ml of chloroform-methanol (1:1). For phosphatidyl choline determination, TLC plates (Si250-PA; J. T. Baker Inc., Phillipsburg, NJ) were spotted with 10 or 20 μl samples, and developed with chloroform-methanol-glacial acetic acid-water (100:56:20:10). For free fatty acid determination, TLC plates were developed with hexane-ethanol-acetic acid (80:20:1). Lipid spots were visualized with iodine vapors after overnight exposure in a sealed container.

The ability of strain HL to grow in a cholesterol- and lecithin-free medium was determined by inoculating the bacterium into a lipid-free medium and observing turbidity after several transfers. Cultures were transferred every 3 d for a total of 5 times; then, these cultures (1% inoculum) were inoculated into BC broth. After 1 wk of incubation, the BC broth cultures were observed for coagulation and assayed
by TLC. The media tested included BC broth without cholesterol or lecithin (B medium), and B medium plus one of the following: 0.8% glycerol, 1.0% lactose, 0.02 or 0.5% sodium pyruvate, 0.05% sodium oleate and 0.02% sodium pyruvate, or VPI hemin and vitamin K_1 solutions (Holdeman et al., 1977). In a similar manner, the growth of strain HL in a clear, cholesterol-containing medium (XLP medium, prepared as described in Part I) and in B medium plus 0.2 mg per ml of 5-androstene-3β-ol-17β-carboxylic acid (Sigma) was tested.

The efficiency of coprostanol production by strain HL was determined by comparing the intensity of the coprostanol spots on TLC plates after growing the bacterium in various media. BC medium was prepared with the addition of one of the following: formate/fumarate solution (Holdeman et al., 1977), 0.5% amygdalin, 1 mM dithiothreitol, 1.0% glucose, 0.75% lactic acid, 1.0% lactose, 0.5% sodium acetate, or 0.5% sodium pyruvate. BC medium was also prepared with the substitution of an equimolar concentration of MgCl_2·6H_2O for CaCl_2·2H_2O. To determine growth and cholesterol reduction at various temperatures, tubes of BC medium were inoculated (1%) with strain HL, incubated at room temperature (about 25°C), 30, 37, 45, and 55°C, and then assayed by TLC.

To investigate the growth and cholesterol-reducing capabilities of strain HL in media containing various phospholipids, BC medium with 0.02% sodium pyruvate was prepared as described above, except without cholesterol, lecithin, or CaCl_2 (BP medium), and dispensed to 13-mm
diameter tubes (4.5 ml per tube). The following phospholipids were dissolved separately (45 mg each) in 5 ml of 95% ethanol at 60°C that contained 90 mg of cholesterol: phosphatidyl inositol (Sigma; approximately 50% pure), phosphatidyl choline (Sigma; approximately 40% pure), phosphatidyl choline (Calbiochem, Behring Diagnostics, La Jolla, CA; 97% pure), and phosphatidyl glycerol (Sigma; 98% pure). Cholesterol (90 mg) was also dissolved alone in 5 ml of ethanol. The ethanolic solutions were sterilized by passage through 0.22-μm pore size syringe filters. A 2-d culture of strain HL grown in BLEC medium was serially diluted to 10⁻⁵ in replicate tubes of BP medium. Each phospholipid/cholesterol solution and the cholesterol solution were added (50 μl per tube) to duplicate tubes of the 10⁻⁵ dilutions of strain HL. A sterile, anaerobic solution of CaCl₂·2H₂O in dH₂O (9%, w/v) was also added (50 μl per tube). The cultures were incubated at 37°C for 2 wk, then extracted, and assayed by TLC.

Similar experiments were performed to determine if putative intermediates were reduced to coprostanol. 4-Cholesten-3-one (Sigma, 90 mg) or cholesterol (90 mg) were each dissolved with 45 mg of 40% phosphatidyl choline in 5 ml of 95% ethanol at 60°C, and added (50 μl) to tubes containing 4.5 ml of BP medium prepared without pyruvate (B medium). Strain HL that had been transferred (1% inoculum) 4 consecutive times in BLEC medium was inoculated (1%) to tubes of B medium containing 4-cholesten-3-one with phosphatidyl
choline, or cholesterol with phosphatidyl choline. The tubes were incubated 1 wk, extracted, and assayed by TLC. The biohydrogenation of coprostanone to coprostanol by strain HL was also determined. The compound [4-\(^3\)H, 4-\(^13\)C]-coprostanone was prepared as previously described (Ren, D., 1991. M.S. thesis, Iowa State University, Ames, IA). This substrate was added to BC medium that was inoculated with strain HL and incubated at 37°C for 3 d. The sterols were extracted twice with 2 ml of a chloroform- methanol (2:1) solution. The organic phase was concentrated to approximately 100 µl. TLC and liquid scintillation counting were performed as described for the resting cell assay, except that sterol spots corresponding to authentic coprostanone and coprostanol standards were scraped into scintillation vials for isotopic measurements.

A similar technique was used to determine the amount of cholesterol converted to coprostanol with time. [4-\(^3\)H]-cholesterol (3 µl, in 95% ethanol; 2.6 µCi) and [4-\(^14\)C]-cholesterol (3 µl, in 95% ethanol; 2.5 µCi) were added to 9 ml of BC medium that was inoculated with 1 ml of an overnight culture of strain HL. Samples were removed each day for 7 days, and the amount of radioactivity in coprostanol was assayed as described above.

Strain HL was tested for reduction of nitrate, production of indole, hydrolysis of starch, gelatin, and esculin, and the fermentation of amygdalin, arabinose, cellobiose, erythritol, fructose, glucose, glycogen,
inositol, lactate, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, ribose, salicin, soluble starch, sucrose, and xylose. Filter-sterilized solutions of substrates were added to sterile basal medium to yield the final concentrations recommended by Holdeman et al. (1977). The test media were inoculated with 0.1 ml of strain HL grown in BC medium. The cultures were incubated at 37°C for 7 d, at which time the pH of the inoculated media were compared to uninoculated controls. The reduction of nitrate, production of indole, and the hydrolysis of starch, gelatin, and esculin were tested as described in the Anaerobic Laboratory Manual (Holdeman et al., 1977).

For the determination of H₂ and CO₂ production, media were dispensed (20 ml per tube) to 16 x 150 mm tubes that were sealed with rubber septum serum caps. BC, BC plus 0.5% lactose, BC plus 0.5% sodium pyruvate, BC plus 0.5% lactose and 0.5% sodium pyruvate, and SGM media were inoculated (1% inoculum) with strain HL grown in BC medium. Lactose and pyruvate were added as filter-sterilized solutions that were made partially anaerobic by bubbling with Ar for 10 min. The cultures were incubated at 37°C for 7 d. Headspace gas was analyzed on a Gow Mac series 580 gas chromatograph (Bridgewater, NJ) equipped with a thermal conductivity detector and a Hewlett-Packard 3393A integrator (Avondale, PA). Samples were removed from the culture tubes with a gas-tight syringe and injected into the sample loop (0.5 ml). N₂ was the carrier gas for H₂ determination, and He was the
carrier gas for CO₂ determination. The H₂ and CO₂ concentrations in the headspace of the cultures were compared with the concentration in the headspace over uninoculated media.

To determine fermentation products, strain HL was grown in BC medium. The BC culture was used to inoculate BC medium plus 0.5% sodium pyruvate, BC medium plus 0.5% sodium pyruvate and 0.5% lactose, BC medium plus 1.0% glucose, and SGM medium. After 4 d of incubation at 37°C, the presence of butanol, ethanol, isopropanol, methanol, and propanol were determined by injection of culture supernatants into a Hewlett-Packard gas chromatograph model 5890 equipped with a capillary column (5% phenyl-methyl silicone, 10 m x 0.53 mm x 2.65 μm film thickness), a 7673A automatic sampler, an HP3396 Series II integrator, and a hydrogen flame ionization detector. The carrier gas was N₂, and the column temperature was 40°C. The formation of acetic, butyric, caproic, formic, fumaric, isobutyric, isovaleric, lactic, oxalic, phenylacetic, propionic, and succinic acids by strain HL were determined by using the method for preparing butyl esters described by Salanitro and Muirhead (1975). Butylated samples were analyzed on the same gas chromatograph system described for determining alcohols, except the column temperature was increased 8°C per min between 55 and 190°C.

In addition to standard biochemical tests, strain HL was evaluated by using the An-IDENT system (Analytab Products, Plainview, NY). The An-IDENT system contains tests for the
following activities: indole production, N-acetyl-glucosaminidase, α-glucosidase, α-arabinosidase, β-glucosidase, α-fucosidase, phosphatase, α-galactosidase, β-galactosidase, hydrolysis of indoxyl-acetate, arginine utilization, leucine, proline, tyrosine, arginine, alanine, histidine, phenylalanine, and glycine aminopeptidases, pyroglutamic acid arylamidase, and catalase. A bacterial suspension was obtained from 3-d-old cultures grown on MLA plates that were incubated in a GasPak jar (described in Part I). The procedures outlined by the manufacturer were followed.

Aerotolerance was determined by incubating inoculated MLA plates in a GasPak jar for 5 d and then exposing the plates to air at room temperature. Isolated colonies were picked at various times after exposure to air and were inoculated into BC broth. After 1 wk of incubation, the cultures were extracted and analyzed by TLC for coprostanol production. Strain HL was also inoculated to MLA plates that were placed in a candle jar, an evacuated vacuum jar, and in air, and incubated at 37°C for 2 wk. The plates were then examined for growth.

Results

Strain HL grew and converted approximately 90% of the radiolabeled cholesterol in BC medium to coprostanol in 5 days (Figure 1). When strain HL was grown in BC medium for 2
Figure 1. Percentage changes in cholesterol and coprostanol concentrations as incubation proceeded. Strain HL was incubated in BC medium containing radiolabeled cholesterol.
d and the sterol spots visualized on TLC plates, a significant amount of the cholesterol had been converted (Figure 2, lane 3).

The results of preliminary studies, using a resting-cell assay with radiolabeled cholesterol, indicated that medium BC.1 yielded higher cholesterol reductase activity than medium BC. BC.1 medium was further optimized for enzyme yield by testing various combinations and concentrations of PIPES, lactose, and pyruvate. The highest cholesterol reductase activity was obtained in BC.1 medium with 0% PIPES, 0.5% lactose, and 0.5% sodium pyruvate (SGM medium; data not shown).

The results of resting-cell sterol conversion assays are shown in Figures 3-6. The highest cholesterol-reducing activity by strain HL was evident between 24 and 48 h of growth (Figure 3). After up to 3 h of incubation of the reaction mixture, cholesterol reductase activity continued to increase in a nearly linear manner (Figure 4). The addition of pyruvate to the growth medium greatly stimulated cholesterol-reductase activity (Figures 2 and 5). Pyruvate also stimulated activity when added to the enzyme reaction mixture (Figure 6).

Difficulties were encountered in making direct counts of cells of strain HL grown in SGM medium with various concentrations of yeast extract because of the small size of the bacterium and the presence of particulate constituents of the medium. Counts of approximately $3.4 \times 10^7$ cells per
Figure 2. TLC of strain HL incubated 2 d in BC medium containing various carbohydrates, lactate, or pyruvate. Lane 1: amygdalin; Lane 2: glucose; Lane 3: normal BC medium; Lane 4: pyruvate; Lane 5: lactate; Lane 6: standards (10 μg per spot)
Figure 3. Net radioactivity of coprostanol as a function of the incubation time of Eubacterium HL in SGM medium.
Figure 4. Net radioactivity of coprostanol as a function of the time of incubation of the assay reaction mixture.
Figure 5. Net radioactivity of coprostanol as a function of the pyruvate concentration of the growth medium.
Figure 6. Net radioactivity of coprostanol as a function of the pyruvate concentration of the assay reaction mixture.
ml were obtained for SGM medium without yeast extract, and approximately 4.8 X 10^7 cells per ml for SGM medium containing 1% yeast extract. Colony counts on MLA plates were 2 X 10^5 CFU per ml for cultures grown in SGM without yeast extract, and 1.8 X 10^7 CFU per ml when 1% yeast extract was added to the growth medium. After 4 consecutive transfers, then 24 h of incubation, CFU per ml on various media were: Rogosa SL broth plus cholesterol and lecithin, 0; BC medium, 3.5 X 10^6; SGM, 2.6 X 10^7; and BC medium plus 10 mM MES, pH 5.5, 5.5 X 10^6.

The effects of pH on coprostanol production were tested with strain HL. When the bacterium was grown in BC medium containing organic or inorganic buffers, the best coprostanol production as visualized by TLC assay occurred at about pH 7.0 (lanes 5, 7, 9, and 11, Figure 7). The amount of coprostanol produced at this pH was approximately the same as the amount of coprostanol produced at pH 7.2 in BC medium without a buffering system (lane 5, Figure 7). After 48 h, there was no coprostanol produced in BC medium buffered to pH 5.5 with MES (lanes 1 and 2, Figure 7) or to pH 8.0 with EPPS (lanes 3 and 4, Figure 7). The results of coprostanol production in unbuffered BC medium in which the pH was adjusted to various levels are shown in Figure 8. The greatest amounts of coprostanol were formed at pH values of 7.5, 7.2, and 7.0 (lanes 3-5, Figure 8). After incubation, the pH values of these media were 6.4, 6.3, and 6.2, respectively.
Figure 7. TLC of cultures of strain HL incubated in BC medium with various additions, sampled at 24 and 48 h. Lanes 1 and 2: MES, pH 5.5, 24 and 48 h; Lanes 3 and 4: EPFS, pH 8, 24 and 48 h; Lane 5: no additions, 48 h; Lanes 6 and 7: PIPES, pH 7.0, 24 and 48 h; Lanes 8 and 9: 0.5% sodium acetate, pH 7.0, 24 and 48 h; Lanes 10 and 11: PIPES, pH 7.0, 1 mM DTT, 24 and 48 h; Lane 12: standards, a is cholesterol, b is coprostanol, c is 4-cholesten-3-one, and d is an unknown contaminant.
Figure 8. TLC of cultures of strain HL incubated 18 h in BC medium at various initial pH values. Lane 1: coprostanol standard; Lane 2: pH 8.0; Lane 3: pH 7.5; Lane 4: pH 7.2; Lane 5: pH 7.0; Lane 6: pH 6.8; Lane 7: pH 6.5; Lane 8: pH 6.0; Lane 9: pH 5.5. Spot a is unknown, b is cholesterol, and c is coprostanol.
When strain HL was grown in BLEC medium (no cholesterol), growth was apparent because the medium assumed a grainy appearance. After transferring the bacterium up to 10 times in BLEC medium, inoculation into BC medium resulted in rapid and efficient cholesterol reduction, indicating that strain HL was capable of growing in a cholesterol-free medium and that the cholesterol-reducing ability was maintained in the absence of substrate. When strain HL was inoculated into B medium (no cholesterol or lecithin), no growth (turbidity) was evident, and after several transfers, then inoculation into BC medium, no growth or cholesterol reduction resulted. The results of TLC assays for phospholipase activity are illustrated in Figure 9. When strain HL was grown in BLEC medium, lecithin was metabolized, as evidenced by the disappearance of lecithin and the appearance of free fatty acids.

When a mixture of formate/fumarate was added to BC medium, there was no obvious stimulation of coprostanol production (data not shown). Other substrates were also examined (Figure 2). After 2 d of incubation, the amount of coprostanol produced by strain HL was approximately equal in BC medium with and without a variety of fermentable substrates. However, coprostanol production was slightly stimulated by the addition of sodium pyruvate to BC medium (lane 4, Figure 2) and, as shown in Figure 7 (lane 8), after 24 h coprostanol production was stimulated by the addition of sodium acetate to the growth medium. DTT was inhibitory
Figure 9. TLC for phospholipids and free fatty acids (FFA) of strain HL incubated 1 d in BLEC medium. Phospholipids: Lane 1, culture; Lane 2, uninoculated broth; Lane 3, 40% pure phosphatidyl choline; Lane 4, 99% pure phosphatidyl choline from soybean; Lane 5, 99% pure phosphatidyl choline from egg yolk. FFA: Lane 1, culture; Lane 2, uninoculated broth; Lane 3, oleic acid
(compare lanes 6 and 10, Figure 7), although some coprostanol was produced after 48 h. As observed with mixed cultures of CRB (Part I), \( \text{CaCl}_2 \) greatly stimulated coprostanol formation (data not shown). When \( \text{MgCl}_2 \) was substituted, coprostanol production was not stimulated (data not shown). No cholesterol was reduced when cultures were incubated at room temperature or at 55°C (Figure 10). The most efficient conversion of cholesterol to coprostanol by strain HL in BC medium occurred at 37°C (Figure 10).

When cholesterol was added as an ethanolic solution to BP medium in the absence of phospholipid, no cholesterol reduction occurred (Figure 11). The greatest amount of coprostanol was produced when cholesterol was added to BP medium in combination with a 40% pure preparation of phosphatidyl choline (Sigma type IV-S soybean lecithin). Cholesterol was also converted to coprostanol when a preparation of nearly pure phosphatidyl choline (97% phosphatidyl choline) was added with cholesterol. No coprostanol was produced in BP medium by strain HL when cholesterol was added with phosphatidyl inositol, or phosphatidyl glycerol. When 4-cholesten-3-one or cholesterol were added with a 40% pure preparation of phosphatidyl choline to BP medium, 100% of the sterols (as detected visually) were converted to coprostanol (Figure 12). When radiolabeled coprostanone was added to BC broth inoculated with strain HL, approximately 50% of the coprostanone was converted to coprostanol in 3 d (Ren, D. 1991. M.S. thesis,
Figure 10. TLC of culture extracts after incubation of strain HL for 4 d in BC medium at various temperatures. Lane 1: room temperature; Lane 2: 30°C; Lane 3: 37°C; Lane 4: 45°C; Lane 5: 55°C; Lane 6: standards
Figure 11. TLC of strain HL incubated for 14 d in cholesterol-containing BP medium with and without the addition of various phospholipids. Lane 1: no phospholipid; Lane 2: 97% pure phosphatidyl choline; Lane 3: 40% pure phosphatidyl choline; Lane 4: phosphatidyl inositol; Lane 5: phosphatidyl glycerol; Lane 6: standards.
Figure 12. TLC of cultures of strain HL after 1 week incubation in B medium plus 2 mg per ml sterol. Lane 1: 4-cholesten-3-one. Lane 2: cholesterol. Lane 3: cholesterol, uninoculated. Lane 4: 4-cholesten-3-one, uninoculated. Lane 5: standards
Iowa State University, Ames, IA)

Strain HL did not grow in media without cholesterol and lecithin, even when supplemented with glycerol, lactose, pyruvate, oleate, hemin, or vitamin K₃ (data not shown). Growth was also not observed when strain HL was inoculated into a basal medium containing the water-soluble sterol 5-androstene-3β-ol-17β-carboxylic acid (data not shown). However, strain HL did grow in XLP medium. This medium contained a bovine lipoprotein fraction as a soluble source of cholesterol, and was supplemented with lactose and pyruvate. After 5 successive transfers in XLP medium, growth and coprostanol production occurred when strain HL was transferred into BC medium. Growth in XLP broth was evidenced as a uniform turbidity, with no grainy appearance as was noted for growth of strain HL in BLEC medium. After 3 d of incubation in XLP or BC media, dilutions of strain HL were plated to MLA plates and incubated in a GasPak jar. Plate counts indicated that a cell density of about $3 \times 10^5$ was achieved in XLP medium compared to about $3 \times 10^8$ in BC broth.

Strain HL grew and reduced cholesterol in nitrate medium, but was negative for nitrate reduction. Indole production was negative in BC medium, and starch and gelatin were not hydrolyzed. Esculin was hydrolyzed. Much acid was produced by the fermentation of amygdalin, lactose, and salicin. Strain HL also weakly fermented arabinose, cellobiose, fructose, glucose, mannose, and melibiose. The
pH of the basal medium with no added carbohydrates also decreased from an average value of 6.95 to an average value of 6.18. The production of H₂ and CO₂ by strain HL in various media is listed in Table 1. The percentages of headspace gas volumes occupied by H₂ and CO₂ ranged from 4.5-7.2 and 0.9-1.8, respectively. The amounts of short-chain fatty acids produced by strain HL when grown in various media are listed in Table 2. Amounts of acetic acid produced ranged from 0.105 to 1.079 meq/100 ml. Between 0 and 0.196 meq/100 ml of formic acid was produced, and amounts of succinic acid ranged from 0.027 to 0.392 meq/100 ml. No alcohols were produced.

When strain HL was tested using the An-IDENT system, a positive reaction was evident for β-glucosidase, and for the hydrolysis of indoxyl-acetate. All of the other tests were negative.

Strain HL survived exposure to air for up to 48 h. No growth was evident on inoculated MLA plates incubated in a candle jar, in an evacuated vacuum jar, or in air.
Table 1. Average percentage of the headspace gas volume occupied by $H_2$ or $CO_2$ of various media after inoculation with strain HL and incubation for 4 d at 37°C

<table>
<thead>
<tr>
<th>Media</th>
<th>Percentage of headspace gas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$H_2$</td>
</tr>
<tr>
<td>BC</td>
<td>4.5 ± 0.1$^a$</td>
</tr>
<tr>
<td>BCG$^b$</td>
<td>7.1 ± 1.0</td>
</tr>
<tr>
<td>BCP$^c$</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>BCLP$^d$</td>
<td>7.2 ± 1.0</td>
</tr>
</tbody>
</table>

$^a$ Mean of 2 observations ± standard deviation.
$^b$ BC medium plus 1.0% glucose.
$^c$ BC medium plus 0.5% pyruvate.
$^d$ BC medium plus 0.5% lactose and 0.5% sodium pyruvate.
Table 2. Average amounts of metabolic end products produced by strain HL in various media after incubation for 4 d at 37°C

<table>
<thead>
<tr>
<th>Media</th>
<th>Acetic acid</th>
<th>Formic acid</th>
<th>Succinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>0.105 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0.027 ± 0.002</td>
</tr>
<tr>
<td>BCG</td>
<td>0.196 ± 0.005</td>
<td>0.196 ± 0.030</td>
<td>0.106 ± 0.004</td>
</tr>
<tr>
<td>BCP</td>
<td>0.859 ± 0.001</td>
<td>0.135 ± 0.014</td>
<td>0.118 ± 0.003</td>
</tr>
<tr>
<td>BCLP</td>
<td>1.079 ± 0.011</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.392 ± 0.007</td>
</tr>
<tr>
<td>SGM</td>
<td>0.395 ± 0.005</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.070 ± 0.008</td>
</tr>
</tbody>
</table>

<sup>a</sup> Media abbreviations are defined in Table 1, and in Materials and Methods (Part II).

<sup>b</sup> Mean of 2 observations ± standard deviation. Units are meq/100 ml.

<sup>c</sup> The areas of these peaks were not calculated by the gas chromatograph integrator; however, a small formate peak could be observed visually on inoculated media samples. No formate peak was observed on uninoculated media samples.
Discussion

The values for the amount of cholesterol reduced to coprostanol after 2 d of incubation were different when values calculated from the radiolabeled assay were compared to values estimated from the visual examination of TLC plates. These discrepancies resulted for several reasons. First, quantitative values could not be determined from the visualization technique, although rough estimates could be made. Also, the growth of strain HL may have been slower in the medium containing the radiolabeled substrate than in the normal growth medium, because of the unavoidable introduction of some oxygen when the radiolabeled substrate was added to the medium, and the presence of a small amount of ethanol. Finally, both assays are subject to substantial variation, although replicates were run to reduce these errors.

The observation that resting cells of strain HL grown in BC.1 medium or SGM had higher cholesterol reductase activity than cells grown in BC medium may be explained by the competition between the nonradiolabeled and radiolabeled cholesterol.

When strain HL was grown in media that contained cholesterol and lecithin, or lecithin in the absence of cholesterol, a very obvious coagulation of the medium occurred (see Figure 1, Part I). The mechanism that results in coagulation is not known. It may have been caused by the production of free fatty acids that resulted from the
hydrolysis of phosphatidyl choline. Whatever the reason for the coagulation, it was a quick and reliable indicator of coprostanol production because cholesterol reductase activities were always high in media that had coagulated and low in media that had not coagulated. There also appeared to be a relationship between coagulation and cell numbers.

Mott and Brinkley (1979) observed that most of the previously isolated strains of CRB required plasmenylethanolamine to reduce cholesterol to coprostanol. They determined that the plasmalogen was metabolized, as evidenced by its disappearance from the growth medium. The end products of the metabolism of plasmalogen were not reported. It was also observed that Eubacterium ATCC 21408 had sphingomyelinase activity. However, none of the metabolites of sphingomyelin supported growth of the bacterium (Mott and Brinkley, 1979). The ways by which phospholipase or sphingomyelinase activity are related to cholesterol-reducing activity are not known. One possibility is that these compounds or their metabolites alter the bacterial membrane in some way to make cholesterol more accessible to cholesterol reductase. They also may affect the type of micelle structure that the cholesterol substrate is imbedded in to increase the availability of cholesterol.

When glycerol or free fatty acids were added with cholesterol to a basal medium, strain HL did not grow or reduce cholesterol. This suggests that the actual enzymatic hydrolysis of phosphatidyl choline was necessary for strain
HL to reduce cholesterol. The addition of CaCl$_2$ had a stimulatory effect on coprostanol formation by strain HL. It has been reported that the phospholipid substrate must have a net positive charge for enzymatic action by phospholipase C (Ottolenghi, 1967). The positive charge is supplied by Ca$^{2+}$, an activator of phospholipase C. Perhaps the hydrolysis of phosphatidyl choline provides a cofactor, or acts directly in coprostanol formation. These questions can more easily be answered when a cell-free cholesterol reductase preparation is available.

Brinkley and coworkers (1982) reported that several strains of CRB could grow on supplemented brain heart infusion agar plates incubated in an anaerobic chamber. When the strains were repeatedly subcultured, they maintained their ability to reduce cholesterol. However, when I plated strain HL on the same medium used by Brinkley et al. (1982) and incubated the plates in a GasPak jar, no colonies were observed. However, MLA medium prepared without cholesterol (but with lecithin) did support the growth of strain HL, and the colonies were similar in appearance to colonies on MLA medium with cholesterol (described in Part I, illustrated in Figure 2, Part I). It is possible that the requirement for lecithin for colony formation is unique to strain HL.

Strain HL grew and retained its cholesterol-reducing ability after ten transfers in BLEC (cholesterol-free) medium. It is possible that a very minute amount of a phytosterol with a structure similar to cholesterol was
present in the crude soybean lecithin that was used in BLEC medium. However, no sterols with the retention value of cholesterol were detected by TLC assay of BLEC medium. The TLC assay used was sensitive enough to detect approximately 20 μg sterol per ml of culture medium. The amount of cholesterol or phytosterol that may have been present in the crude lecithin was well below the 1 mg per ml that was required for the maintenance of *Eubacterium* ATCC 21408 (Eyssen et al., 1973).

Some question remains as to the exact mechanism of biohydrogenation of cholesterol to coprostanol by strain HL (Figure 13). If coprostanol is produced via a multiple-step pathway, two of the intermediates are probably 4-cholesten-3-one and coprostanone. Strain HL converted both of these intermediates into coprostanol. However, no evidence of these intermediates was observed after incubation of strain HL in cholesterol-containing media, either by the standard TLC assay or by the more sensitive radiolabeled cholesterol TLC assay. Information obtained by using a dual-labeled cholesterol substrate suggested that coprostanol formation by strain HL involved the transfer of a hydrogen at C-4 to C-6. The most likely explanation for these observations was that there was an intramolecular hydrogen transfer with the transient formation of a double bond at C-4 (Ren, D. 1991, M.S. thesis, Iowa State University, Ames, IA). Apparently, coprostanol is formed by strain HL with the intermediate formation of 4-cholesten-3-one and possibly
Figure 13. Possible reaction sequences for cholesterol reduction to coprostanol
coprostanone, but no intermediates accumulate. It is possible that cholesterol reductase from strain HL exists as a complex of physically linked enzymes, or that cholesterol reductase is a single enzyme capable of carrying out several different reactions.

Stain HL closely resembles other members of the genus *Eubacterium*. Strain HL appears to be quite similar to strains 103 and 104 that were isolated by Brinkley et al. (1982) and were classified as *Eubacterium* sp. These strains had carbohydrate fermentation patterns similar to strain HL, produced β-glucosidase, hydrolyzed esculin, and metabolized pyruvate. Strains 103, 104, and HL all produced acetic acid and succinic acid.

Strains 103 and 104 grew on supplemented BHI agar plates with no cholesterol or lecithin, while strain HL did not grow in the absence of lecithin. Strains 103 and 104 reduced cholesterol to coprostanol in the absence of plasmalogen but were only tested in media that contained lecithin and cholesterol. The requirement of lecithin for cholesterol reduction and the presence of phospholipase activity were not tested in strains 103 and 104. Strains 103 and 104 produced from 1 to 11% CO₂ in the headspace of brain medium and 9 to 20% CO₂ in brain medium supplemented with pyruvate; no H₂ was produced (Brinkley et al., 1982). This was in contrast to strain HL, which produced approximately 1% CO₂ (a value that did not increase with pyruvate addition) and up to approximately 7% H₂. Strain HL also varied from strains 103
and 104 in its production of formate, although the methods used by Brinkley and coworkers may not have detected formate production (Brinkley and Mott, 1978).

Another possible difference between strains 103 and 104 and strain HL is the aerotolerance of the strains. Strain HL required anaerobic conditions to grow, but survived long exposures to atmospheric oxygen. Unfortunately, data on the aerotolerance of strains 103 and 104 were not published, and the strains were not available for comparison. Strain HL was negative for catalase activity, and the way that this bacterium is protected from oxygen merits further study.

When strain HL was grown in a medium containing 2 mg per ml cholesterol (BCLP), or 0.2 mg per ml cholesterol (SGM), decreases in the amounts of acetic and succinic acids were observed in the medium with less cholesterol. This difference was probably caused by a stimulation of the metabolism of strain HL by cholesterol, or by an increase in cell numbers in the presence of increased cholesterol concentrations.

When sodium pyruvate was present in the growth medium, strain HL produced significantly more acetic acid than in media without pyruvate, while the amounts of formic acid and succinic acid remained approximately the same. Pyruvate was metabolized by strain HL. This was evidenced by its disappearance from the growth medium as determined by GC analysis (data not shown). When pyruvate was added to the growth medium of strain HL that was harvested and used as the
enzyme source for radiolabeled cholesterol reduction, activity was stimulated. Pyruvate also stimulated cholesterol reductase activity when it was added as a component of the reaction mixture. It is possible that pyruvate acts directly as a cofactor in cholesterol reduction. Other workers reported a similar observation; pyruvate acted as an electron donor for 16-dehydroprogesterone reductase in cell extracts of Eubacterium sp. strain 144 (Watkins and Glass, 1991). More definitive data will become available on the interrelationships of pyruvate with cholesterol reductase when activity can be assayed in cell-free extracts of strain HL.

The significance of cholesterol reduction by strain HL or other CRB to the physiology of the bacteria remains to be elucidated. Eyssen et al. (1973) speculated that cholesterol acted as a terminal electron acceptor in CRB, thereby supplying energy by means of electron transport. However, several strains do not require cholesterol for growth, suggesting that some CRB can use alternate electron acceptors, or generate energy by another mechanism (Brinkley et al., 1982). The spirochete Treponema hydysenteriae required cholesterol for growth and reduced cholesterol to cholestanol (Stanton, 1987). Cholestanol was incorporated into the cellular lipids. The ability of several CRB to grow in the absence of cholesterol precludes its necessity as a cellular membrane component, at least in certain strains.
GENERAL SUMMARY

In Part I, several different media and methods were used to attempt to grow previously isolated strains of cholesterol-reducing bacteria (CRB) in clear media to facilitate the preliminary steps of cholesterol reductase isolation. It was concluded that the existing strains of CRB could not be used as sources of cholesterol reductase. The existing strains were too fastidious, and cholesterol reductase activities were low. Therefore, new media and methods were used to isolate bacteria with more desirable characteristics.

Stable mixed cultures were obtained from a variety of samples. Most of these sample types had never before been studied as possible sources for obtaining CRB. Two strains of CRB were isolated, one from a hog sewage lagoon and another from standing water.

In Part II, the hog sewage lagoon isolate, Eubacterium sp. strain HL, was characterized. This isolate has several characteristics that make it a better candidate for future uses in reducing the cholesterol content of foods when compared to previous isolates of CRB. These characteristics include the ability to grow without cholesterol or plasmalogen, aerotolerance, and the ability to form colonies on a solid medium incubated in GasPak jars. The metabolism of strain HL appears to be different from other Eubacterium spp. Perhaps the greater diversity of CRB now available will...
eventually lead to a better understanding of the role that cholesterol reduction plays in the physiology of CRB and to industrial applications of these unique bacteria.


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