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Mechanism of cholesterol reduction to coprostanol by *Eubacterium coprostanoligenes* ATCC 51222

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The mechanism of reduction of cholesterol to coprostanol by Eubacterium coprostanoligenes *ATCC 51222 was studied by incubating the bacterium with a mixture of* α- *and* β-*isomers of [4-^{3}H,4-^{14}C]cholesterol. Coprostanol, isolated after incubation of [4-^{3}H,4-^{14}C]cholesterol in a growth medium under anaerobic conditions, retained 97% of the tritium originally present in cholesterol. The majority of this tritium (64%), however, was in the C-6 position in coprostanol, which showed that the conversion of cholesterol into coprostanol by* E. coprostanoligenes *involved the intermediate formation of 4-cholesten-3-one followed by the reduction of the latter to coprostanol. In resting cell assays in which washed bacterial cells were incubated with micellar cholesterol in phosphate buffer at 37°C, both 4-cholesten-3-one and coprostanone were produced in addition to coprostanol. Furthermore, 5-cholesten-3-one, 4-cholesten-3-one, and coprostanone were converted efficiently to coprostanol by* E. coprostanoligenes. *These results support the hypothesis that the major pathway for reduction of cholesterol by* E. coprostanoligenes *involves the intermediate formation of 4-cholesten-3-one followed by reduction of the latter to coprostanol through coprostanone as an intermediate. (Steroids* **61**:33–40, 1996)

Keywords: dual-labeled cholesterol; coprostanol; cholesterol reduction; *Eubacterium;* NMR

Introduction

The conversion of cholesterol to coprostanol by intestinal microorganisms has been reported to occur by two pathways (Figure 1). One pathway involves the intermediary formation of 4-cholesten-3-one and coprostanone.[1–3] In the other pathway, cholesterol is transformed into coprostanol by the direct reduction of the 5–6 double bond.[4]

In previous work with cecal contents from rats, coprostanol, isolated after incubation with [4β-^{3}H,4-^{14}C]cholesterol, retained 60% of the tritium.[1] Most of this tritium had been transferred to the C-6 position, showing that the conversion of cholesterol into coprostanol involved isomerization of a 5–6 double bond to a 4–5 double bond. Rosenfeld and Gallagher incubated [3α-^{3}H]cholesterol with human feces and observed that the resulting coprostanol retained most of the label of C-3, indicating a direct reduction of the double bond.[4] Björkhem and Gustafsson demonstrated, however, that during the microbial conversion of [3α-^{3}H]cholesterol to coprostanol the 3α-tritium could be removed and then reinserted with high efficiency into the same position.[1] Thus, it was not possible to distinguish between the two reduction pathways when using [3α-^{3}H]cholesterol as a substrate. The study of Björkhem and Gustafsson[1] invalidated the conclusion of Rosenfeld and Gallagher.[4] They concluded, however, that both pathways were of equal importance,[1] whereas Parmentier and Eyssen determined that the indirect pathway predominated.[3]

Recently, Freier et al. isolated a pure culture of a cholesterol-reducing bacterium, *Eubacterium coprostanoligenes* ATCC 51222.[5] This isolate is a small, anaerobic, nonsporing, Gram-positive rod that reduces about 90% of the cholesterol in the growth medium to coprostanol.

In the present investigation, the objective was to elucidate the pathway(s) of cholesterol reduction by *E. coprostanoligenes.* The reactions were carried out with [4-^{3}H,4-^{14}C]cholesterol as the substrate in a growth medium incubated under anaerobic conditions. Resting cell assays also were conducted as an alternative method to study the reduction pathway.

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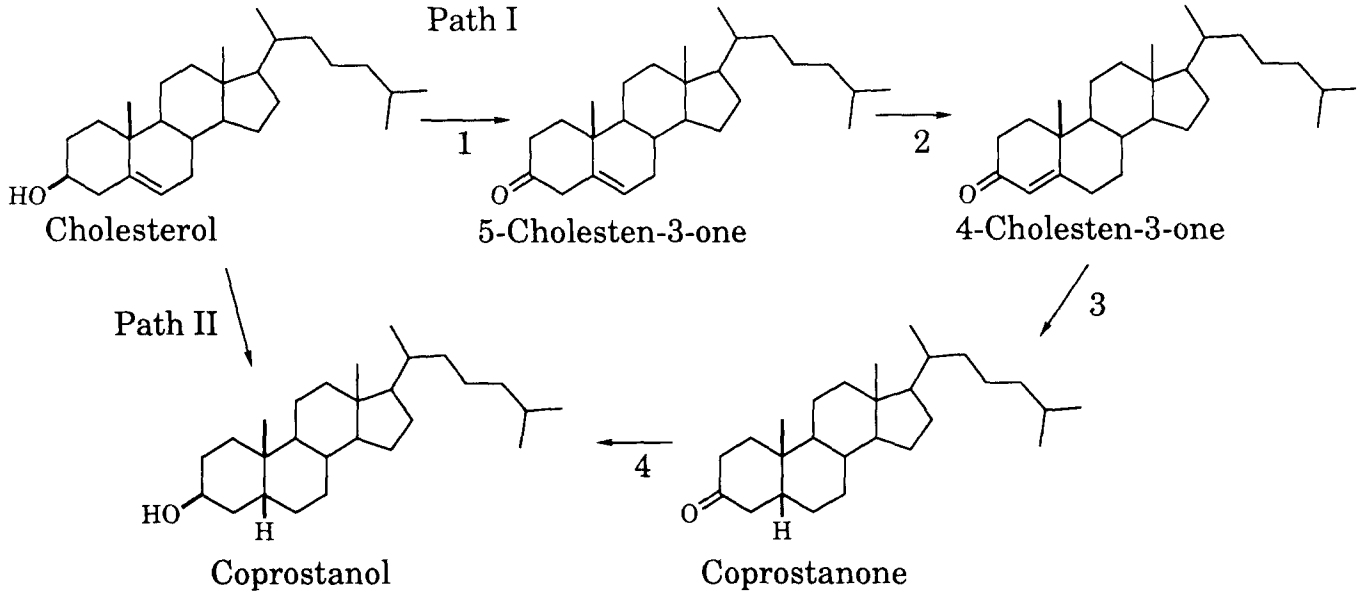

Figure 1 Proposed reaction pathways for enzymatic conversion of cholesterol to coprostanol (adapted from Björkhem and Gustafsson, 1971).[1] Path I. Indirect pathway: 1, oxidation; 2, isomerization; 3, reduction; 4, reduction.

Experimental

General overview of methods

[4-^{14}C]Cholesterol was purchased from Amersham Inc. (Arlington Heights, IL, USA), and [4-^{3}H]cholesterol was prepared by using modifications of published procedures for preparation of unlabeled cholesterol.[6] Cholesterol derivatives were prepared initially with deuterium label and then spectroscopically characterized. Tritiated derivatives were prepared by using optimized procedures. Stereochemistry of introduction of label was determined by NMR.

[4-^{3}H,4-^{14}C]Cholesterol was incorporated into the basal cholesterol (BC) medium[5] and incubated for five days with *E. coprostanoligenes.* The coprostanol was isolated by thin-layer chromatography (TLC). The amount of ^{3}H and ^{14}C in the cholesterol substrate and coprostanol product was determined by dual-label counting techniques with a liquid scintillation counter. The results were interpreted to determine the specific metabolic pathway for coprostanol synthesis in this bacterium. Possible intermediates also were incubated with the bacteria. Resting cell assays of cholesterol reduction were conducted by the procedures of Li et al.[7]

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

The scheme for synthesis of [4-^{2}H]cholesterol is shown in Figure 2. This sequence incorporates ^{2}H in the 4-position as a mixture of α- and β-isomers, but involves much more readily available reagents, and convenient procedures than those of Lockley et al.[6] To initiate the preparation of cholesta-3,5-dienyl-3-acetate, 4-cholesten-3-one (0.5 g, 1.30 mmol, Kodak Inc., Rochester, NY, USA) was dissolved in isopropanol acetate (2.5 mL, 22.7 mmol) with H_2SO_4 (0.002 mL, 0.03 mmol). After 1 h of reflux under N_2, the acetone that had formed was removed from the vapor phase via a needle through the septum with an aspirator vacuum protected by a $CaSO_4$ drying tube. At the end of the second hour, anhydrous sodium acetate (0.02 g, 0.24 mmol) was added to quench the reaction. The mixture was concentrated at decreased pressure. The residual blue-green fluorescent oil was diluted with a few microliters of chloroform and then decanted from the sodium acetate into about 6 mL of methanol. Additional chloroform was added to bring the oil into solution in the refluxing methanol. Seeding and slow cooling of this solution yielded 0.32 g of cholesta-3,5-dienyl-3-acetate (58%) with a melting point of 75.5–77°C. Synthesis of [4-^{2}H]cholesterol was initiated by adding $NaBH_4$ (30 mg, 0.79 mmol) and NaOH (8.5 mg, 0.21 mmol) to 1 mL of 1,2-dimethoxyethane. Then D_2O (0.03 mL, 1.66 mmol) was added to the mixture, followed by cholesta-3,5-dienyl-acetate (45 mg, 0.11 mmol). The mixture was stirred for 96 h at room temperature, extracted with hexane, and washed with water four times. After drying with $MgSO_4$, hexane was evaporated under decreased pressure. The residue was separated by flash chromatography with CH_3CN/CH_2Cl_2 (1:13, v/v).[8] The yield was 22 mg (59%).

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

[4-^{2}H]Cholesterol (0.0064 g, 0.017 mmol) was dissolved in 0.56 mL CH_2Cl_2, and anhydrous $CaCO_3$ powder (0.007 g, 0.070 mmol) was added to the solution. Pyridinium chlorochromate (0.012 g, 0.571 mmol) was added, and the mixture was stirred for 30 min under N_2 at 25°C. A saturated NaCl solution (5 mL) was then added. The suspension was filtered and then thoroughly extracted with diethyl ether (10 mL) three times. The ether layer was filtered through anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure to yield [4-^{2}H]-5-cholesten-3-one (0.006 g; 93% yield). Analysis by TLC showed a single spot at R_f 0.55 in ethyl acetate/hexane (4:6, v/v).[9]

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

[4-^{3}H]Cholesterol was prepared in the same manner as [4-^{2}H]cholesterol, with the substitution of 0.04 mL of 50 mCi/mmol tritiated water (Amersham Inc., Arlington Heights, IL, USA) for D_2O. The product obtained after purification by column chromatography on silica gel (0.040–0.063 mm) had a specific radioactivity of 8.7×10^{-4} mCi/μL. [4-^{14}C]Cholesterol was purchased from Amersham Inc. (Arlington Heights, IL, USA) and had a specific radioactivity of 70 mCi/mmol. The mixture of [4-^{3}H]cholesterol with [4-^{14}C] cholesterol is referred to as [4-^{3}H,4-^{14}C]cholesterol.

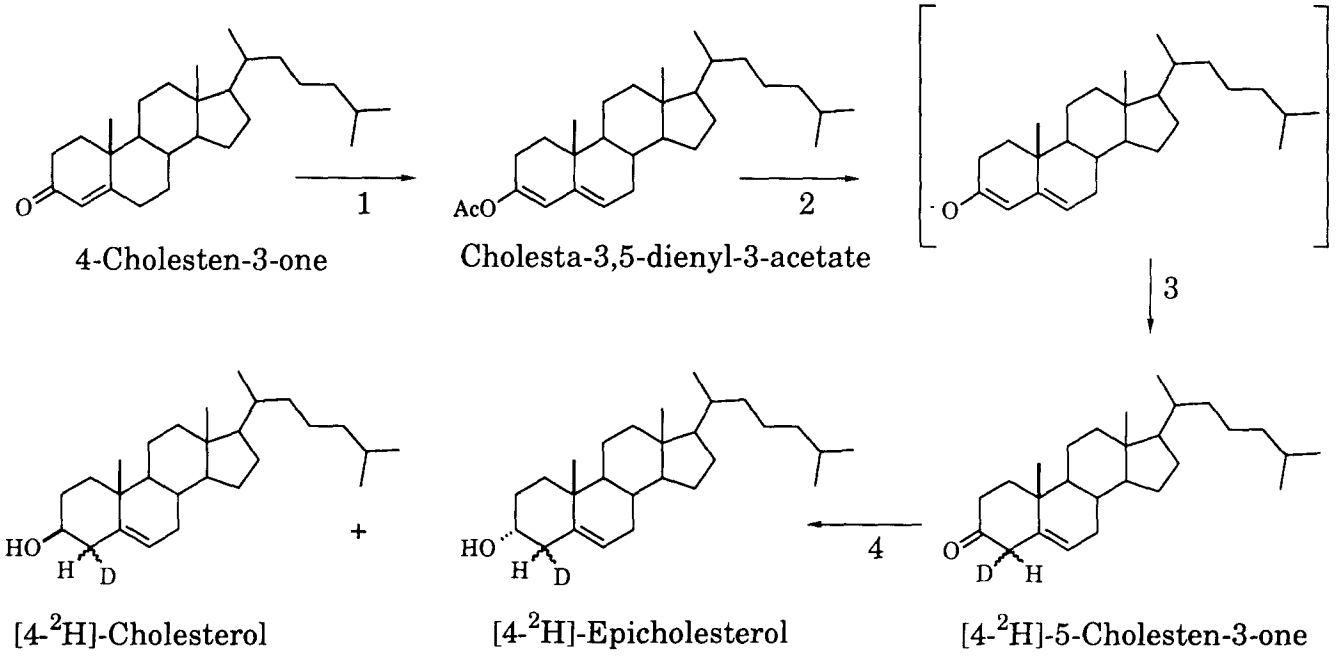

Figure 2 Synthesis of [4-^{2}H]cholesterol. 1, $CH_3CO_2C(CH_3)=CH_2$ and H_2SO_4; 2, NaOH, $NaBH_4$, D_2O, and $CH_3OCH_2CH_2OCH_3$; 3, D_2O; 4, $NaBH_4$.

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

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

^{1}H NMR spectra were recorded on a Varian 500 spectrometer (operating at 500 MHz for proton nuclei) by using $CDCl_3$ or pyridine-d_5 as the solvent and tetramethylsilane (TMS) as the internal standard. ^{13}C NMR spectra were recorded on a VXR-300 spectrometer (operating at 75 MHz for carbon nuclei) by using $CDCl_3$ as both the solvent and internal standard. ^{2}H NMR spectra were recorded on a VXR-300 spectrometer (operating at 46 MHz) using chloroform as the solvent.

Incubation of E. coprostanoligenes *with [4-^{3}H,4-^{14}C]cholesterol*

The BC medium contained the following components per liter: casitone, 10 g; yeast extract, 10 g; cholesterol, 2 g; lecithin, 1 g; $CaCl_2$ dihydrate, 1 g; sodium thioglycolate, 0.5 g; and resazurin, 1 mg.[5]

Both [4-^{3}H]cholesterol (0.3 μL, 2.6 μCi in 95% ethanol) and [4-^{14}C] cholesterol (0.3 μL, 2.5 μCi in 95% ethanol) were added to 1 mL of BC medium inoculated with a fresh culture of *E. coprostanoligenes*. After incubation of the labeled sterols with the bacterium for 5 days at 37°C under anaerobic conditions, the sterols were extracted twice with 2 mL of chloroform/methanol (2:1, v/v). The nonpolar phase was concentrated to about 100 μL, and chromatography was performed on silica gel thin-layer plates (type G, 0.75 mm thickness) with hexane:ethyl acetate (75:25, v/v) as the solvent. When stained with a 0.2% ethanolic solution of 2,7-dichlorofluorescein, two bands were observed. Bands corresponding to cholesterol and coprostanol were scraped into scintillation vials for isotopic measurements. ^{3}H and ^{14}C in the double-labeled sterols were determined by the two-channel ratio method in a Beckman LS-8000 liquid scintillation spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA, USA).

Determination of isotope in different positions in coprostanol

Determination of the position of tritium in isolated coprostanol was carried out by oxidation of coprostanol to coprostanone and base-catalyzed exchange of hydrogens at the 2- and 4-positions. This procedure was developed by using deuterium and subsequently applied to tritiated compounds. The methods of Parish and Chitrokorn[9] was adapted as follows: coprostanol (24 mg, 0.062 mmol) was dissolved in 2 mL CH_2Cl_2, anhydrous $CaCO_3$ powder (25 mg, 0.25 mmol) was added to the solution, pyridinium chlorochromate (45 mg, 0.209 mmol) was added, and the product was purified by flash chromatography with hexane/ethyl acetate (8:2, v/v) as the solvent. The identity and purity of the compound was checked further by NMR.

Coprostanone (4 mg, 0.010 mmol) was heated at 95°C for 24 h in a solution of dioxane (100 μL) and deuterated water (50 μL, 2.8 mmol, 99.9 atom %D) containing sodium methoxide (15 mg, 0.25 mmol). The reaction mixture was cooled and acidified with 0.1N HCl and extracted with diethyl ether. The ether extract was washed with water until neutral; then, the solvent was dried over $MgSO_4$ and evaporated. The residue was checked with NMR. These conditions caused complete exchange of all hydrogens from C-2 and C-4. This treatment was expected to remove all isotopes in the C-4 position after incubation with the cholesterol-reducing bacterium.

The procedure described in the previous paragraph was carried out on radioactive coprostanol in exactly the same manner, but NMR spectra were not determined. To confirm that the exchange was complete, samples were resubmitted to the exchange conditions until no further loss of tritium was observed.

Resting cell assays

Assays were conducted as described by Li et al.[7] Namely, bacteria were cultured in cholesterol-free media for 48 h at 37°C before being harvested by centrifugation. Then, the washed bacterial cells

were suspended in 25 mM sodium phosphate buffer at pH 7.5 with 45 mM sodium pyruvate and 5 mM sodium thioglycolate. Control assay mixtures contained the bacterial suspension that had been boiled for 10 min. Assays were started by addition of the [1α,2α-^{3}H] micellar cholesterol substrate. After incubation at 37°C for 1 h, assay mixtures were extracted, and organic extracts were concentrated under N_2 to about 100 μL. Sterols were separated by TLC with standards chromatographed on each plate. Corresponding bands were scraped into scintillation vials for determination of radioactivity.

Incubation of E. coprostanoligenes *with other steroids*

BC media were prepared with 5-cholesten-3-one, 4-cholesten-3-one, coprostanone, pregnenolone (4-pregnen-3β-ol-20-one), progesterone (4-pregnen-3,20-dione), androstenediol (5-androsten-3β,17β-diol), and etienic acid (5-androsten-3β-ol-17β-carboxylic acid) instead of cholesterol, respectively. Then, media were inoculated with 1% of a 2-day-old cholesterol-free culture of *E. coprostanoligenes* and incubated at 37°C for 5 days. The cultures were extracted, and sterols were separated on TLC plates.

Results and discussion

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

Structure determined by the mass spectrum. Mass spectra were used to establish structures of synthesized compounds. Two samples of undeuterated cholesterol and two samples of deuterated cholesterol were run by solid probe at 70 eV. Averaged results for the molecular ion region are shown in Table 1. To determine the amount of ^{2}H present in the isotopically labeled cholesterol mixture, it was necessary to correct the [4-^{2}H]cholesterol values for contributions from ^{13}C. The measured values for unlabeled cholesterol were very close to the theoretical values calculated by using natural abundance tables; we used the measured values for the following analysis. As can be seen in Table 1, the M-1 (1.01) and M-2 (2.20) peaks were small, so these ions were ignored. Expressed as fractional abundances, the results were: 30% undeuterated cholesterol, 56% D1, and 13% D2. Further examination was carried out to obtain evidence of where cholesterol was labeled.

Proton NMR assignments for cholesterol and deuterated cholesterol. The 500 MHz proton magnetic resonance spectra of cholesterol and deuterated cholesterol were recorded in both chloroform and pyridine. Table 2 shows chemicalshift assignments and integration for cholesterol and deuterated cholesterol in $CDCl_3$ and in pyridine-d_5. Integration of the resonance at 2.26 ppm in chloroform solvent and at 2.62 ppm in pyridine solvent showed one proton each as compared with two protons each in the undeuterated compound. From these spectra, it was concluded that one C-4 proton contributed to the resonance at 2.26 ppm in chloroform solvent and at 2.62 ppm in pyridine solvent. These spectra showed that deuterium was at C-4. Because the C-4α and C-4β resonances could not be resolved, however, the spectra provided no evidence of the stereochemistry of deuteration.

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

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

[a]Mass-to-charge ratio.

Table 2 Proton chemical shift assignments and integral for cholesterol and deuterated cholesterol in $CDCl_3$ and in pyridine-d_5

	In $CDCl_3$		In pyridine-d_5	
Proton location[a]	Chemical shift (ppm)[b]	Δ integral[c]	Chemical shift (ppm)[d]	Δ integral[c]
6	5.34	0.0	5.43	0.0
3	3.51	0.0	3.85	0.1
4a, 4e	2.26	0.9	2.62	1.1
2e			2.11	0.1
7a, 7e			2.03	0.0
1a, 1e, 2a			1.80	0.0

[a]Number refers to carbon number of cholesterol, a to axial, and e to equatorial.
[b]Assignments from Barry et al.[10]
[c]Difference of integral for the hydrogen between cholesterol and deuterated cholesterol.
[d]Assignments from Sawan et al.[11]

^{13}C Nuclear magnetic resonance spectra of cholesterol and deuterated cholesterol. We corroborated the ^{1}H NMR assignment of label position with ^{13}C NMR studies, which are more informative because of the better dispersal of ^{13}C signals. To provide strong evidence for the deuterium position, ^{13}C NMR spectra were recorded on a VXR-300 spectrometer (operating at 75 MHz for carbon nuclei) by using $CDCl_3$ as both the solvent and internal standard (77.06 ppm) with proton decoupling. The ^{13}C NMR spectrum of deuterated cholesterol matched that of cholesterol except that C-4 appeared at δ 42.0 (t, J = 18 Hz) rather than δ 42.4 (s).[12] Figure 3 shows an expansion of the C-4 region. When hydrogen was replaced by deuterium, the C-4 signal underwent a 0.25 ppm upfield shift, and became a 1:1:1 triplet with a coupling constant of 18 Hz, as expected.[13] The resonances of the C-3, C-5, and C-6 were broadened somewhat because of unresolved carbon-deuterium coupling (data not shown). The introduction of a second deuterium on C-4 usually results in a disappearance of the C-4 signal, or C-5 multiplicities; however, appreciable upfield shifts and line broadening were observed at the C-3, C-5, and maybe C-6.[14] In this experiment, line broadening was observed at C-3, C-5, and C-6.

Deuterium NMR spectrum. To confirm the previous evidence and to find whether deuterium was present at a po-

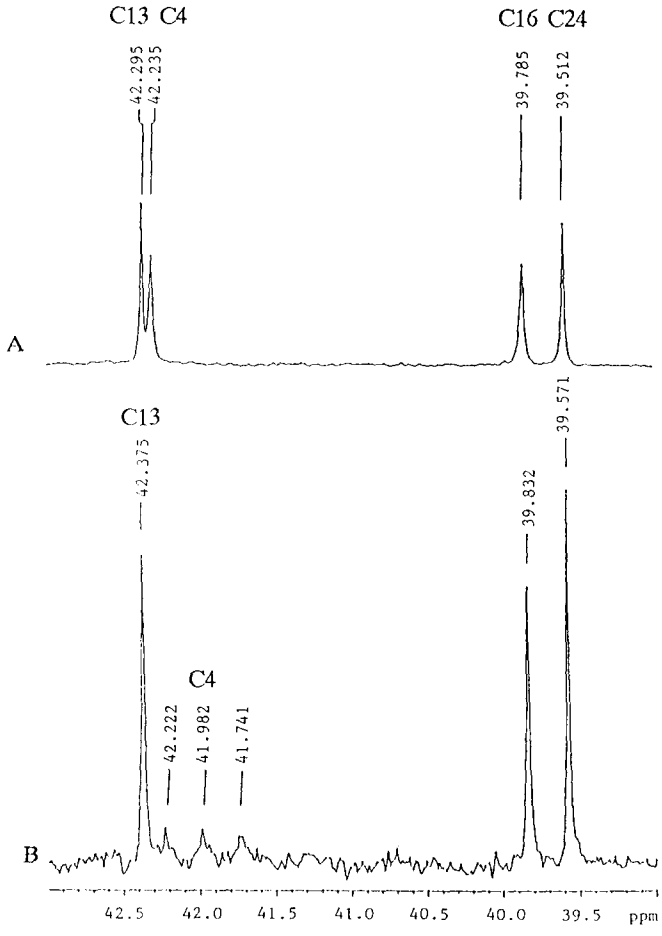

Figure 3 The expansion of the C-4 region of ^{13}C spectra of cholesterol (A) and deuterated cholesterol (B) in $CDCl_3$. Note that the C-4 carbon has been broadened, shifted, and split by the deuterium.

sition other than C-4, the ^2H NMR spectrum of deuterated cholesterol was recorded at 46 MHz in chloroform. The chemical shift (2.24 ppm) observed for deuterium was similar to that seen for the C-4 proton (2.26 ppm) in ^1H NMR spectra of cholesterol and deuterated cholesterol. From these spectra, it was concluded that only one proton was attached to the C-4 position. In other words, one deuterium was located on C-4. There was no strong evidence, however, to show whether the deuterium was axial or equatorial. This question was answered by the analysis described next.

Determination of fraction of label in the 4α- and 4β-position of [4-^{2}H]-5-cholesten-3-one. In contrast to the cholesterol spectra, the 4α and 4β hydrogen signals were resolved in the ^1H NMR spectrum of cholestenone. Doublets were seen at 2.79 ppm and 3.25 ppm, with a germinal coupling constant of 16.5 Hz. These peaks were assigned to 4α and 4β, respectively, by 1D nuclear Overhauser effect (NOE) spectroscopy on the basis of a strong NOE between the C-19 methyl at the 3.25 peak. Thus, the amount of deuterium in an axial and equatorial position was determined in this compound simply by integration. Table 3 shows chemical shift assignments and integration for 5-cholesten-3-one and [4-^{2}H]-5-cholesten-3-one. Integration of the 3.25 ppm doublet showed a 58% decrease because of deuterium being located at the axial position of C-4, and integration of the 2.79 ppm double showed a 28% decrease because of deuterium being located at the equatorial position of C-4. Therefore, 67% {0.58/(0.58 + 0.28)} of the deuterium was in the β-position. The measurement provided an estimate of 14% excess undeuterated cholesterol, which closely matches the value of 17% derived from mass spectral data (30% D0-13% D2).

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

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

[a]Number refers to carbon number in cholesterol
[b]With respect to internal TMS.
[c]Relative area under peak for 5-cholesten-3-one.
[d]Relative area under peak for [4-^{2}H]-5-cholesten-3-one.

Mechanism of cholesterol reduction to coprostanol by E. coprostanoligenes

Time required for maximal reduction of cholesterol by *E. coprostanoligenes*. The maximal yield of reduced sterol (coprostanol) was obtained in 5 days when almost 90% of cholesterol was converted into coprostanol (Figure 4). Isolated coprostanol retained 97% ± 2.5% of the tritium originally present in cholesterol.

Products of *E. coprostanoligenes* in cholesterol-containing culture. According to Björkhem and Gustafsson, 4-cholesten-3-one and coprostanone might be intermediates in the conversion of cholesterol into coprostanol[1]; therefore, we investigated whether *E. coprostanoligenes* produces these intermediates in the reduction of cholesterol

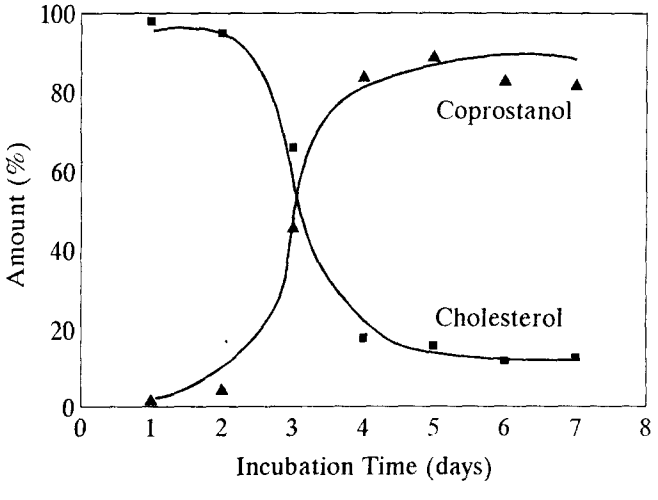

Figure 4 Change of cholesterol and coprostanol concentrations in the culture with *E. coprostanoligenes*.

to coprostanol. The TLC plates of the extracts of incubations described in the previous section only showed two bands—one for cholesterol and one for coprostanol (Figure 5). Eyssen et al. reported that production of coprostanol on a preparative scale by *Eubacterium* 21,408 always yielded small amounts of 4-cholesten-3-one and coprostanone.[15] Their data supported the indirect pathway for formation of coprostanol. In the current study, however, we demonstrated that coprostanone and 4-cholesten-3-one, if formed, were further transformed rapidly to coprostanol when *E. coprostanoligenes* was growing in the cholesterol-containing medium.

Isotope positions in coprostanol. To determine whether the label had been retained at C-4 or transferred to C-6, coprostanol was oxidized to coprostanone and the C-4 and C-2 hydrogens were exchanged with base. We worked out conditions, as before, by using deuterated materials. In the deuterium exchange experiment, the ^{13}C NMR spectra confirmed complete deuteration at the expected position(s), C-4 (nonradioactive coprostanone was used rather than radioactive coprostanone). Figure 6 shows expansion of chemical shifts for coprostanone and deuterated coprostanone in $CDCl_3$ solvent. The peaks corresponding to C-2 and C-4 were deuterated completely. We determined the location of ^3H in coprostanol isolated from incubations with *E. coprostanoligenes* as follows. Treatment of tritiated coprostanol ($^3H/^{14}C$ = 1.07) with pyridinium chlorochromate to form coprostanone did not remove any of the ^3H ($^3H/^{14}C$ = 1.06), whereas base-catalyzed conversion of coprostanone to deuterated coprostanone with sodium methoxide and deuterated water removed about 36% of the ^3H ($^3H/^{14}C$ = 0.67). Resubmission of the exchanged coprostanone to the sodium methoxide for a further 96 h caused no more change in the $^3H/^{14}C$ ratio (0.69), indicating that exchange was completed and not just slowed by a tritium isotope effect. Therefore, we concluded that 64% of the ^3H in coprostanone was located in the C-6 position.

This ratio of about 2:1 for C-6:C-4 matches the 2:1 ratio of β:α seen for deuterium at C-4. The data indicated that no solvent exchange occurred during proton transfer from C-4β to C-6 by the enzyme. Whereas an incomplete exchange of coprostanone also would explain such a high retention of label, we believe that the redeuteration of coprostanone ruled out that possibility. Our present work is consistent with a pathway from cholesterol to coprostanol involving 4-cholesten-3-one and coprostanone as intermediates.

From studies on biohydrogenation of [4β-^3H,4-^{14}C]cholesterol by *Eubacterium* ATCC 21,408, Parmentier and Eyssen also concluded that significant amounts of tritium could be removed from the C-4β position and that more than 70% of the tritium was transferred to the C-6 position of coprostanol.[3] The present investigation with a pure culture of *E. coprostanoligenes* suggests a similar pathway, with even less proton exchange during transfer. Isomerization of a 5–6 double bond to a 4–5 double bond by a mechanism involving transfer of the C-4 H to the C-6 position during conversion of cholesterol into coprostanol indicates that *E. coprostanoligenes* also follows the so-called indirect pathway with formation of 4-cholesten-3-one.

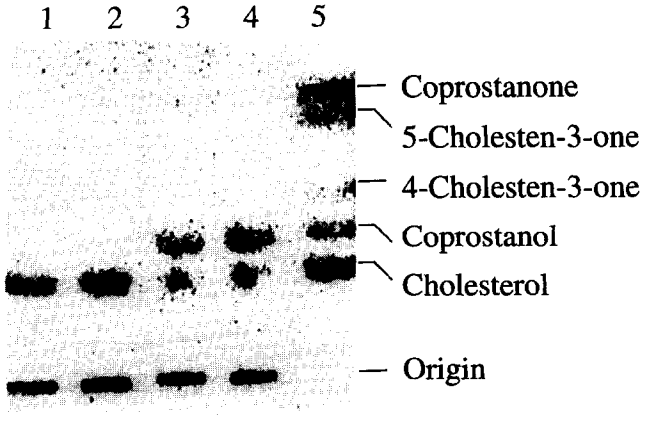

Figure 5 TLC analysis of *E. coprostanoligenes* culture in BC medium. Lane 1 and lane 2, uninoculated culture; lane 3 and lane 4, inoculated culture incubated for 5 days; lane 5, standards.

Products in resting cell assays. To verify further the pathway by which *E. coprostanoligenes* converts cholesterol to coprostanol, resting cell assays were conducted. The results show that bands corresponding to 4-cholesten-3-one and coprostanone were seen on TLC plates in addition to bands of cholesterol and coprostanol (Figure 7A). No 5-cholesten-3-one bands were observed. When these bands were scraped into the scintillation vials and counted for radioactivity, amounts of metabolites of cholesterol produced by *E. coprostanoligenes* were determined (Figure 7B). The data showed that, except for the final product coprostanol, a significant amount of 4-cholesten-3-one and a small amount of coprostanone were produced in the conversion of cholesterol to coprostanol by *E. coprostanoligenes*. Thus, a multistep (indirect) pathway for cholesterol reduction by *E. coprostanoligenes* again was supported by this test.

Reduction of other steroids by *E. coprostanoligenes*. Eyssen et al. reported that *E.* ATCC 21,408 was capable of converting cholesterol into coprostanol; it also could convert 4-cholesten-3-one and coprostanone into coprostanol.[14] We investigated, therefore, whether *E. coprostanoligenes* was able to reduce those intermediates to coprostanol in the growth medium described earlier. The results are shown in Figure 8. All the intermediates were reduced to coprostanol. Furthermore, radioactive coprostanone was incubated with *E. coprostanoligenes* for 3 days in basic cholesterol growth medium at 37°C. Coprostanone was reduced efficiently to coprostanol. Isolated coprostanol had the same $^3H/^{14}C$ ratio (1.05) as original coprostanone. *E. coprostanoligenes* also converted pregnenolone, progesterone, androstenediol, and etienic acid to their reduced products in the growth media (data not shown). Therefore, *E. coprostanoligenes* seems to have a broader substrate specificity than other steroid 5β-reductases,[16] which might have applications in the pharmaceutical industry.

In summary, we have shown that more than 60% of 4-^3H of cholesterol was transferred by an intramolecular shift to the C-6 position of coprostanol during the process of reduction by *E. coprostanoligenes* with [4-^3H,4-^{14}C]cholesterol as

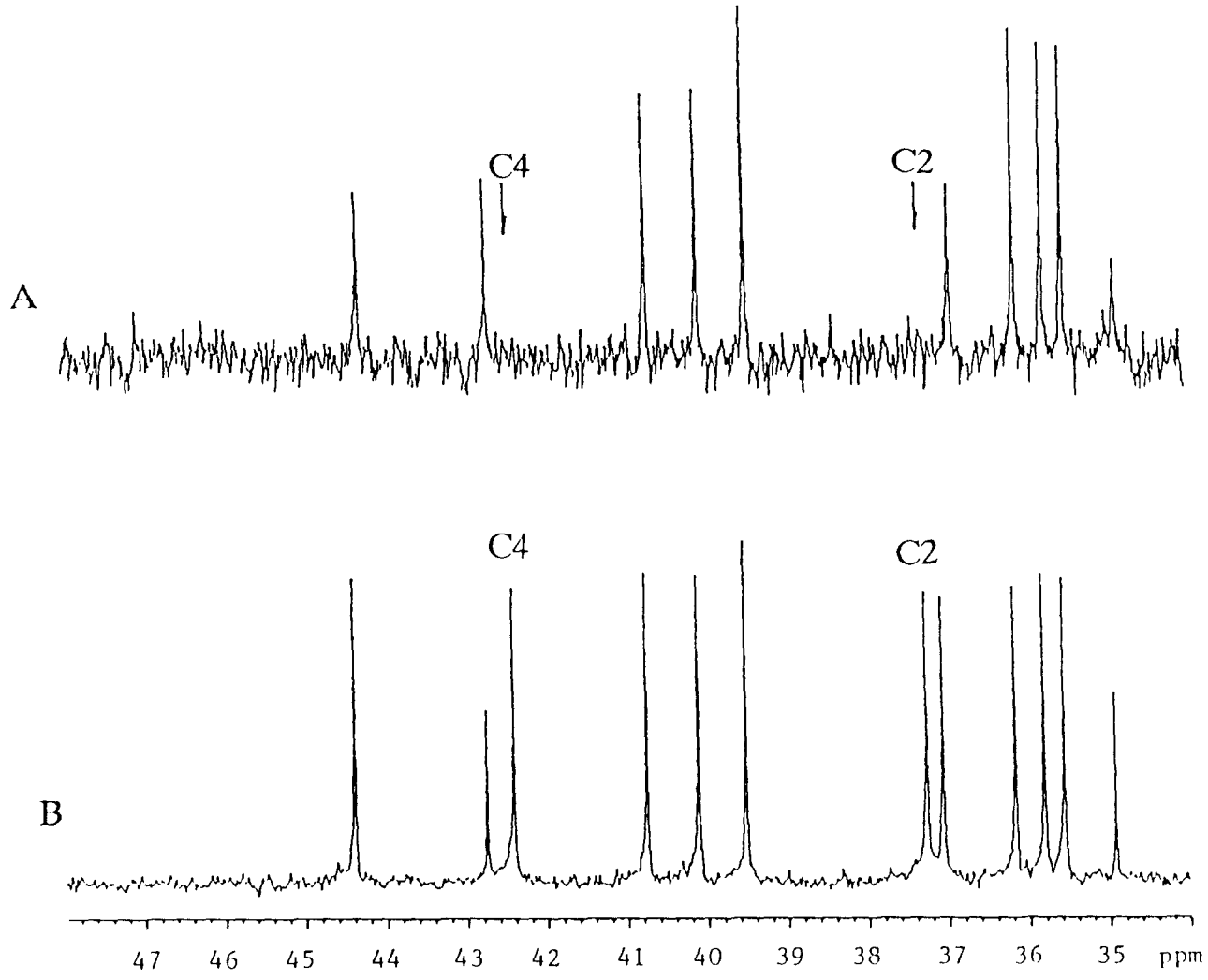

Figure 6 The expansion of chemical shifts for coprostanone (B) and deuterated coprostanone (A) showing that C-2 and C-4 disappeared because of deuterium substitution.

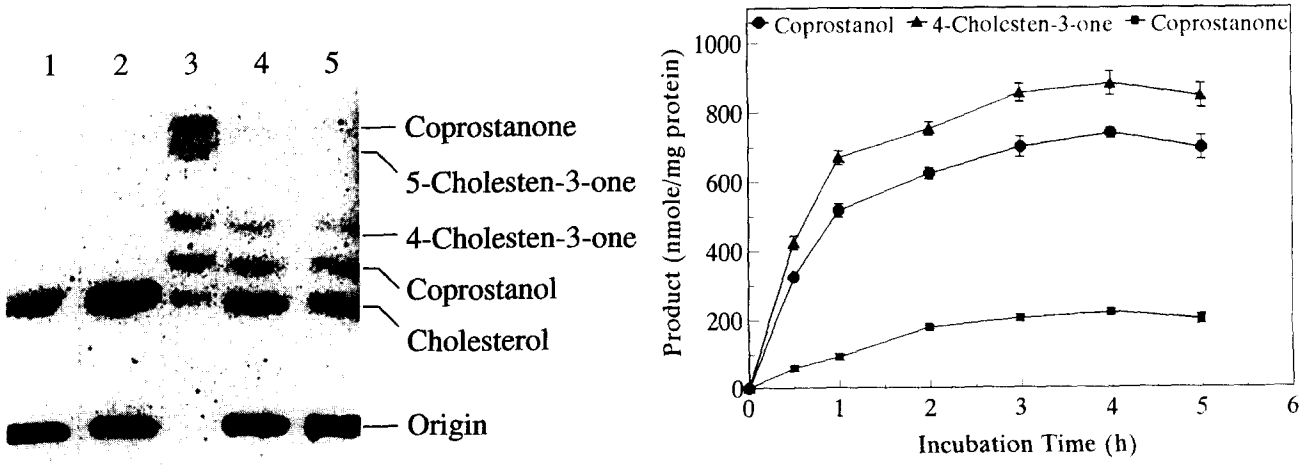

Figure 7 Products in the resting cell assays with *E. coprostanoligenes*. (A) TLC analysis. Lane 1 and lane 2, assays with killed (boiled) bacteria (controls); lane 3, standards; lane 4 and lane 5, assays with live bacteria. (B) Quantitation of metabolites. Data are expressed as the mean of triplicate assays ± standard error.

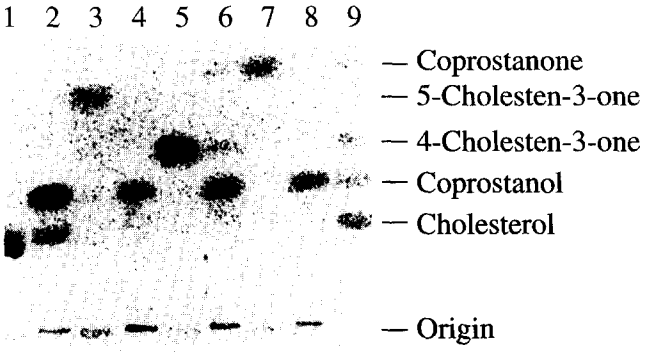

Figure 8 TLC analysis of *E. coprostanoligenes* cultures with cholesterol and other steroids in the growth medium. The incubation time was 5 days. Lane 1 and lane 2, uninoculated and inoculated cholesterol-containing culture, respectively; lane 3 and lane 4, uninoculated and inoculated 5-cholesten-3-one-containing culture, respectively; lane 5 and lane 6, uninoculated and inoculated 4-cholesten-3-one-containing culture, respectively; lane 7 and lane 8, uninoculated and inoculated coprostanone-containing culture; lane 9, standards.

the substrate in a growth medium. The data suggest that the indirect pathway was used for cholesterol reduction. In resting cell assays, both 4-cholesten-3-one and coprostanone were produced in addition to coprostanol; furthermore, *E. coprostanoligenes* efficiently converted 5-cholesten-3-one, 4-cholesten-3-one, and coprostanone to coprostanol. Therefore, our data prove that the major pathway for reduction of cholesterol by *E. coprostanoligenes* involves the intermediate formation of 4-cholesten-3-one followed by reduction of the latter to coprostanol through coprostanone.

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