Initial Steps in the Anoxic Metabolism of Cholesterol by the Denitrifying Sterolibacterium denitrificans*§*

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The anoxic metabolism of the ubiquitous triterpene cholesterol is challenging because of its complex chemical structure, low solubility in water, low number of active functional groups, and the presence of four alicyclic rings and two quaternary carbon atoms. Consequently, the aerobic metabolism depends on oxygenase catalyzed reactions requiring molecular oxygen as co-substrate. Sterolibacterium denitrificans is shown to metabolize cholesterol anoxically via the oxidation of ring A, followed by an oxygen-independent hydroxylation of the terminal C-25 of the side chain. The anaerobic hydroxylation of a tertiary carbon using water as oxygen donor is unprecedented and may be catalyzed by a novel molybdenum containing enzyme.

Isoprenoids constitute an extremely large and diverse group of natural compounds. They are built up from one or more five-carbon units, active derivatives of isoprene. The family includes triterpenes, such as cholesterol and diploptene; diterpenes, such as abietic acid, which is an important constituent of plant resins and constitutes the largest pool of diterpenes in nature; plant growth hormones; insect juvenile hormones; and many more compounds (1). Among them cholesterol is ubiquitous (Fig. 1). This sterol is present in the membranes of most eukaryotes and serves as precursor to steroid hormones, vitamin D, and the bile acids. Many bacterial membranes most tain hopanoids, similar pentacyclic sterol-like molecules.

The complete aerobic mineralization of cholesterol by various genera of bacteria, such as Arthrobacter, Corynebacterium, Mycobacterium, Nocardia, Pseudomonas, and Rhodococcus has been studied in some detail (2, 3) (Fig. 2A). This interest is due to the biotechnological applications of sterol transforming enzymes with high regio- and stereo-specificity in industrial sexual hormone synthesis (4). The aerobic metabolism of cholesterol 1 starts with oxidation to cholest-4-en-3-one 2 by NAD(P)⁺ or FAD⁺-dependent cholesterol dehydrogenase, with the reduction of molecular oxygen to hydrogen peroxide (5, 6). This enzyme was studied in detail because of its importance in clinical applications (7–10). The side chain is then degraded via hydroxylation, two turns of β-oxidation and one retroaldol-reaction. Following degradation of the side chain, the ring system is cleaved and degraded. Ring A is oxidized to a phenol unit which is split by a meta-cleavage dioxygenase. At least four transformations that require molecular oxygen as co-substrate reportedly occur during the mineralization of cholesterol.

In contrast very little is known about the mechanisms that operate under anoxic conditions. It is obvious that anoxic metabolism involves unprecedented, oxygen-independent steps. The best studied anoxic reactions so far involve the incomplete transformation of cholesterol. The double bond in cholesterol is reduced to form coprostanol by intestinal fermentative bacteria (11), either directly by cholesterol reductase or via an alternative indirect pathway (12). Many strictly anaerobic cholesterol-reducing bacteria have been isolated and characterized from the gut contents of mammals (13–15) and were found to belong to the genus Eubacterium. Furthermore, intestinal bacteria can transform cholic acid and chenodeoxycholic acid into 15–20 different bile acid metabolites (16). Well-known transformations include deconjugation of bile acids, dehydrogenation of hydroxyl groups at the C-7 position, reduction of oxo bile acids, and dehydroxylation of bile acids. In all cases studied so far, the cyclic carbon rings remain intact.

Some nitrate-reducing β-Proteobacteria were isolated on oxygen containing or unsaturated monoterpenes as sole carbon source and electron donor, including Thauera spp. (17) and Alcaligenes spp. (18). The biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic ecosystems was recently reviewed (19). In all cases studied, denitrifiers completely oxidize their isoprenoid substrates to CO2. So far, only two denitrifying bacterial strains related to the genera Thauera and Azotococcus (β-subclass of the Proteobacteria) (20, 21) were obtained that can grow under anoxic andoxic conditions with cholesterol as sole carbon and energy source.

Anoxic metabolism of cholesterol is mechanistically interesting since this triterpene is intrinsically difficult to attack due to its complex chemical structure, low solubility in water, low number of active functional groups, and the presence of four alicyclic rings and two quaternary carbon atoms (Fig. 1). Cholesterol and related sterols are thus important biological markers in studying the biological origin and geological fate of organic compounds (22, 23). In this study we propose the initial steps in anoxic cholesterol metabolic pathway using Sterolibacterium denitrificans as a model organism (Fig. 2B).

**EXPERIMENTAL PROCEDURES**

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Materials and Bacterial Strain

[4C-14C]Cholesterol was obtained from American Radiolabeled Chemicals Inc./Biotrend Chemikalien GmbH (Köln, Ger-
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**Bacterial Cultures**

*S. denitrificans* was grown anaerobically at 30 °C under nitrogen atmosphere. The gas phase (headspace) consisted of nitrogen gas. Large scale cultivation was performed in a 200-liter fermentor. The medium contained in 1 liter of distilled water: 0.5 g of NH₄Cl, 0.5 g of MgSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, and 0.85 g of NaNO₃ (10 mM). After autoclaving sterile 50 ml of KH₂PO₄ solution (10 g l⁻¹), 50 ml of NaHCO₃ solution (84 g l⁻¹), vitamins (1 ml l⁻¹) (24), EDTA-chelated mixture of trace elements (1 ml l⁻¹) (25), and selenite and tungstate solution (1 ml l⁻¹) (26) were added. The pH of the medium was adjusted to 7.0 with 2 M HCl. NaNO₃ was added continuously to 10 mM when the terminal electron acceptor added initially (10 mM NaNO₃) was consumed. 2.5 mM cholesterol was added to the growth medium as sole carbon and energy source. Cells were harvested by centrifugation in the exponential growth phase at A₅₇₈ nm of 0.8 to 1.0 (optical path 1 cm) and then stored at −70 °C. In a small scale fed-batch culture (100 ml), *S. denitrificans* cells were incubated with 2.5 mM cholesterol under denitrifying conditions, to which [4C-14C]-cholesterol (2×10⁷ dpm) was added as tracer. After different time intervals of incubation (0, 11, 26, 33, 50, 59, and 75 h) samples were withdrawn from the culture, and the following factors were quantified: the growth of bacterial cells (A₅₇₈ nm), the residual amount of nitrate and cholesterol in the medium, the amount of 14C remained in the growth medium, and 14C assimilated in the biomass. The separation of *S. denitrificans* cells from residual cholesterol by centrifugation (10,000 x g for 15 min) was not successful. Therefore, the culture samples (1 ml) were extracted with the same volume of ethyl acetate three times to isolate the remained [4C-14C]cholesterol from the water fraction containing the biomass. After centrifugation (10,000 x g for 10 min) the biomass including cell debris and lipid remained in the water phase and interface, whereas...
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[4C-14C]cholesterol remained in the ethyl acetate phase. The collected ethyl acetate fraction was evaporated, and the residue was re-dissolved in 1 ml of ethanol. The amount of 14C remained in 1 ml water fraction (mainly the assimilated biomass) and 14C extracted by ethyl acetate and re-dissolved in 1 ml ethanol (mainly the remained [4C-14C]cholesterol) was determined as described later.

Preparation of Cell Extracts

All steps used for preparation of cell extracts were performed at 4 °C under anoxic conditions. Frozen cells were suspended twice the volume of 20 mM MOPS-K+ buffer (pH 7.9) containing 0.1 mg of DNase I ml⁻¹. Cells were broken by passing the cell suspension through a French pressure cell (American Instruments, Silver Spring, MD) twice at 137 megapascals. The cell lysate was fractionated by two steps of centrifugation: the first step involved a centrifugation for 30 min at 20,000 × g to get rid of the cell debris, unbroken cells, and residual cholesterol. The supernatant (crude cell extract) was then centrifuged at 100,000 × g for 1.5 h to separate soluble proteins from membrane proteins.

In Vitro Assays

Assays were routinely performed under anoxic conditions at 30 °C for 16 h under a nitrogen gas phase. The assay mixtures (0.3 ml) for producing intermediates S1, S2, and S3 contained soluble proteins (5 mg) extracted from S. denitrificans cells grown anoxically on cholesterol, 20 mM MOPS-K⁺ buffer (pH 7.9), 5 mM NAD⁺, and 0.2 ml 14C-cholesterol (120,000 dpm). For producing intermediates S1, S2, and S3 in large scale, the reaction mixture was enlarged to 300 ml. Electron acceptor specificity was tested by using the following electron acceptors (5 mM) at 0.2 ml 14C-cholesterol (120,000 dpm): NaNO₃, K₃[Fe(CN)₆], NAD⁺, NADP⁺, phenazine methosulfate, 2,6-dichlorophenolindophenol (DCPIP), methylene blue, and duroquinone. The large scale assay for the production of intermediates S4 and S5 was performed as follows: 300 ml of soluble proteins extracted from S. denitrificans cells were precipitated at 25% ammonium sulfate saturation, and 5 mM K₃[Fe(CN)₆] was prepared in an anaerobic glove box before the introduction of 18O₂ gas. After the addition of 18O₂ gas, 2.5 ml of a 10 mM cholest-4-en-3-one solution in 2-propanol was added to the reaction mixture (final concentration 0.5 mM).

1) 18O₂-treated Assay—17.5 ml of 18O₂ gas was introduced into an anaerobic glass bottle containing 70 ml of headspace (95% N₂ and 5% H₂, 1 atm) and 50 ml of reaction mixture. The final 18O₂ concentration in the headspace was ~20%. The 50-ml reaction mixture containing 20 mM MOPS-K⁺ buffer (pH 7.9), the soluble protein fraction (60 mg of total protein) precipitated at 25% ammonium sulfate saturation, and 5 mM K₃[Fe(CN)₆] was prepared in an anaerobic glove box before the introduction of 18O₂ gas. After the addition of 18O₂ gas, 2.5 ml of a 10 mM cholest-4-en-3-one solution in 2-propanol was added to the reaction mixture (final concentration 0.5 mM).

2) 18O-Labeled Water-treated Assay—3 ml of 66 mM MOPS-K⁺ buffer (pH 7.9) and 7 ml of 18O-labeled water were mixed and used to re-suspend the pellet obtained after ammonium sulfate precipitation (25% saturation) of cell extract (soluble fraction containing 30 mg of protein). The reaction was started by adding 5 mM K₃[Fe(CN)₆] and 0.5 mM cholest-4-en-3-one to the anoxic assay. The final 18O-water content was ~66%.

3) Control Experiment—50 ml of reaction mixture containing the same components as those of 18O₂-treated assay was used to which no 18O₂ gas was introduced into the headspace.

Measurement of Cholesterol and Nitrate

Quantification of cholesterol was carried out by using the α-phthalaldehyde method (27). Culture samples of 1 ml were extracted twice with an equal volume of ethyl acetate. After evaporation of ethyl acetate under vacuum, the residue was dissolved in 2 ml of glacial acetic acid containing 1 mg of α-phthalaldehyde. After 10 min at room temperature, 1 ml of concentrated sulfuric acid was added and immediately mixed. After further incubation at room temperature for 10 min, the absorbance was measured photometrically at 590 nm. Nitrate was determined by the 2,6-dimethylphenol photometric method. 0.2-ml samples (containing 0.02–0.08 μmol of nitrate) were mixed with 0.2 ml of solution A (10 mg ml⁻¹ sulfamic acid in water) and 1.6 ml of solution B (85% H₃PO₄-98% H₂SO₄ (1:1, v/v)). After 10 min, 0.2 ml of solution C (12 mg ml⁻¹ 2,6-dimethylphenol in glacial acetic acid) was added to the mixtures. After incubation at room temperature for 1 h, the concentration of nitrate was measured photometrically at 334 nm.

Measurement of the Amount of 14C

The amount of 14C in liquid samples (1 ml) was determined by liquid scintillation counting using 3 ml of Rotiszint 2200 scintillation mixture (Roth). The counting efficiency was determined via the channel ratio method.
**TLC**

Products were first extracted twice by equal volume of ethyl acetate, and the ethyl acetate-soluble fraction was concentrated under vacuum. The standards and extracted products were separated on silica gel aluminum TLC plates (Silica gel 60 F_{254}, thickness, 0.2 mm, 20 by 20 cm; Merck). The following solvent system was used: n-hexane-ethyl acetate (65:35, v/v). The compounds were visualized under UV light at 254 nm or by spraying the TLC plates with concentrated H$_2$SO$_4$. The $^{14}$C-labeled products were localized by autoradiography with a phosphorimaging plate (Fuji Photo Film Co., Ltd., Kanagawa, Japan).

**High Performance Liquid Chromatography (HPLC)**

The following systems were used for separation, isolation, and identification of standards and products formed from cholesterol.

(i) System 1—For isolation and identification of products S1, S2, and S3, an analytical RP-C$_{18}$ column (LiChrospher 100, end capped, 5 μm, 120 by 4 mm; Merck) was used with a flow rate of 0.8 ml min$^{-1}$ at room temperature. The mobile phase comprised a mixture of two solvents: A (30% (v/v) acetonitrile) and B (80% (v/v) 2-propanol). The separation was performed with a linear gradient of solvent B from 80 to 90% within 30 min. Standards and $^{14}$C-labeled products were detected by using two detectors (UV at 210 or 240 nm and a flow-through radioactivity detector with a solid scintillation cell connected to the HPLC system) in series.

(ii) System 2—The column and detectors used for isolation of product M1 was the same as described in system 1. The separation was performed isocratically with 90% (v/v) 2-propanol as an eluent and a flow rate of 0.8 ml min$^{-1}$.

(iii) System 3—An analytical RP-C$_{18}$ column (Luna 18(2), 5 μm, 150 by 4.6 mm; Phenomenex, Aschaffenburg, Germany) was used for isolation of products S4 and S5. Solvents A and B in the mobile phase and the detectors were the same as described for system 1. The separation was performed with a linear gradient of solvent B from 40% to 70% within 30 min and a flow rate of 0.8 ml min$^{-1}$ at room temperature.

**UV-visible Spectroscopy**

Standards and HPLC-purified products were dissolved in acetonitrile in the range of 10–100 μg ml$^{-1}$. UV absorption spectra of these compounds were obtained by using a lambda 2S UV/VIS spectrometer (PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany).

**Mass spectrometry**

Chemical ionization (CI)-mass and electron impact (EI)-mass spectra were recorded with a Thermo Finnigan TSQ700 triple-quadrupole mass spectrometer equipped with a direct inlet probe (Thermo Electron, Dreieich, Germany). The ionizing energy and source temperature of EI-mass spectrometric measurements were 70 eV and 180 °C, respectively. The measurements of CI-mass spectra were operating in positive ion mode at 130 eV ionizing energy and ammonia was used as the reagent gas. ESI-mass spectra were recorded with a Applied Biosystems API 2000 triple quadrupole instrument running in positive ion mode. Experiments were performed using an Agilent 1100 LC binary pump connected directly to the interface. The flow rate was 0.2 ml min$^{-1}$ (methanol-10 mM ammonium acetate (90:10, v/v)). The source temperature was maintained at 375 °C. Positive ion ESI was carried out at 5,500 V, with declustering potential of 100 V, focusing potential of 300 V, and entrance potential of 10 V. Unit resolution was used with a step size of 0.1 amu in the range of 80–500 atomic mass unit. Acquisition methods were performed using the Analyst software, version 1.4.1. (Applied Biosystems).

**NMR Spectroscopy**

NMR spectra were recorded with a Bruker DRX-spectrometer at 27 °C. Chemical shifts δ were recorded and reported in ppm relative to CHCl$_3$ (1H: δ = 7.26) and CDCl$_3$ (13C: δ = 77.0) as internal standard.

**RESULTS**

**Denitrifying Growth of S. denitrificans with $^{[4C,14C]}$-Cholesterol**—It is well known that some fermentative bacteria can transform cholesterol under anaerobic conditions to derivatives such as coprostanol (11, 12). Such transformation reactions are limited to changes of functional groups but the ring system of the substrate is not attacked. To test whether S. denitrificans oxidizes cholesterol completely to CO$_2$, we incubated this strain in chemically defined medium with cholesterol as the carbon source and nitrate as an electron acceptor. After different intervals of incubation, the residual amount of cholesterol that remained in the medium and the amount that was assimilated into the biomass was measured. To facilitate the measurements, [$^{4C,14C}$]cholesterol (200,000 dpm ml$^{-1}$) was added to the growth medium (100 ml) as tracer.

As shown in Fig. 3, bacterial growth (measured as the increase of the optical density) was accompanied by a decrease in the concentration of cholesterol and in the total radioactivity. Furthermore, bacterial growth was accompanied by accumulation of radioactive $^{14}$C in the biomass. The results clearly indicate the ability of S. denitrificans to degrade $^{14}$C-labeled cholesterol to volatile CO$_2$ and to assimilate the carbon atoms from the ring system of cholesterol.

Actually, the results conform well with the theoretical stoichiometry for anoxic cholesterol mineralization under denitriifying conditions (21), which follows the equation (for explanation, see legend to Fig. 3): C$_{27}$H$_{46}$O + 30.4 NO$_3$ + 30.4 H$^+$ → 27 CO$_2$ + 15.2 N$_2$ + 38.2 H$_2$O.

**Transformation of $^{[4C,14C]}$Cholesterol by Cell Extracts of S. denitrificans**—To identify potential intermediates of anaerobic cholesterol metabolism, high density cell suspensions of S. denitrificans were incubated with [$^{4C,14C}$]cholesterol and sodium nitrate. After different time intervals the mixture was analyzed by TLC and autoradiography. At least one $^{14}$C-labeled product (S3) was detected, and the amount of this product increased with time. The $R_f$ value and retention time of this product matched exactly with those of the authentic cholesta-1,4-dien-3-one sample (data not shown).

To obtain more information, in vitro transformation of [$^{4C,14C}$]cholesterol under anoxic conditions was studied. Crude cell extract was fractionated by ultracentrifugation into
soluble protein and membrane-bound protein fraction, and all three fractions were incubated with 0.2 mM [4C-14C]cholesterol and 5 mM NAD$^+$ in 100 mM MOPS-K$^+$ buffer. Samples were analyzed by TLC and autoradiography. The maximal synthesis of labeled products by the soluble protein fraction occurred at pH 7.9, whereas the membrane protein fraction produced different compound(s) only at pH 6.5 (data not shown).

At pH 7.9, at least six radioactive spots were observed some of which were very weak (Fig. 4). Crude extract produced mainly a compound designated S3, which was also observed with whole cells. When the soluble protein fraction was incubated with NAD$^+$, next to product S3 two other dominant products designated S1 and S2 appeared. The membrane-bound protein fraction alone did not produce products S1-S3. However, one fast migrating protein designated M1 was formed in large amount at pH 6.5.

Formation of products from [4C-14C]cholesterol by the soluble protein fraction also occurred when other electron acceptors were added instead of NAD$^+$ (Fig. 5). K$_3$[Fe(CN)$_6$] and phenazine methosulfate yielded two slowly migrating products, S4 and S5, besides small amounts of S3, whereas DCPIP produced mostly S4. The soluble protein fraction was then fractionated by ammonium sulfate precipitation. Maximal amounts of S4 and S5 were produced with the 25% ammonium sulfate saturation fraction (pH 7.9, 5 mM K$_3$[Fe(CN)$_6$]).

According to Kieslich’s report (2), in oxic cholesterol degradation pathway the side chain of cholesterol is degraded via oxygen-dependent side chain hydroxylation, β-oxidation, and retroaldol reaction, and some intermediates with a carboxylic group in the side chain were observed (Fig. 2A). We acidified the reaction mixtures of in vitro assays to pH 2 before the products were extracted by water-ethyl acetate partition, but no acidic products were found. In addition, we also added Mg-
ATP and coenzyme A to reaction mixtures of *in vitro* assays to enable CoA-thioester formation, but no new products were observed (data not shown).

Products S1–S5 and M1 were also observed when the *in vitro* assays were performed under oxic conditions (data not shown), indicating that the initial enzymes of the anaerobic process are not inhibited by oxygen.

**Identification and Structure Elucidation of Products Derived from Cholesterol**—Products (M1, S1–S5) resulting from cholesterol transformation were extracted with ethyl acetate and purified by TLC and subsequent HPLC (see "Experimental Procedures"). The products S1–S3 were identified by reference to TLC, HPLC mobility, and UV absorption of authentic standards. The identification of S4 and S5 relies mainly on mass and NMR spectra. The CI-mass spectrum of S1 revealed a molecular mass of 384 Da. The maximal ultraviolet absorption near 200 nm suggested the absence of a conjugated double bond structure. The UV absorption pattern, retention time, and *Rf* value of this compound matched exactly with those of authentic cholest-5-en-3-one (Table 1).

According to CI-mass spectrum of S2, its molecular weight was also 384 Da. Yet, the maximal ultraviolet absorption at 238 nm indicated the presence of a conjugated double bond system. In addition, retention time and *Rf* value of this compound matched exactly with those of authentic cholest-4-en-3-one (Table 1).

For product S3, the maximal ultraviolet absorption at 243 nm is likely due to the presence of a conjugated double bond structure. The CI-mass spectrum suggested that its molecular mass was 382 Da (Table 1). The EI-mass spectrum of S3 (see supplemental Fig. S1) with a dominant fragment ion peak at *m/z* 122 matched exactly with that of cholesta-1,4-dien-3-one in a spectral data base for organic compounds (SDBS).

The CI-mass spectrum of S4 indicated its molecular weight was 400 Da (Table 1). The absence of molecular ion peak and the presence of a dominant M-18 peak (loss of H2O) in the EI-mass spectrum (see supplemental Fig. S1) suggested the potential existence of a tertiary hydroxyl group. In addition, a characteristic fragment ion peak at *m/z* 124 in EI-mass spectrum and the identical UV absorption maximum at 238 nm (Table 1) suggested product S4 was closely similar to S2 in ring A system.

The CI-mass spectrum of S5 indicated its molecular weight was 398 Da. No molecular ion peak, but a dominant M-18 peak, appeared in EI-mass spectrum of S5 (see supplemental Fig. S1). The difference in EI-mass spectra is due to the difference in ring A system between S2 and S3 (S3 has an additional C=C structure in C-1 position).

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The exact structures of S4 and S5 were further elucidated by NMR spectroscopy (Fig. 6 and Table 2).
Evidence for Anaerobic Side Chain Hydroxylation—To look for the formation of the hydroxy groups at C-25 of S4 and S5, three in vitro transformation assays were carried out. 1) In an $^{18}$O$_2$-treated assay, $^{18}$O$_2$ gas (20%, v/v) was added to the oxygen-free headspace of one reaction mixture as the sole source of molecular oxygen (i.e., O$_2$ was 100% labeled). 2) A $^{18}$O-labeled water-treated assay contained ~66% of $^{18}$O-labeled water (mol/mol) in the anoxic reaction mixture (10 ml). 3) A control assay (50 ml) was incubated under anoxic conditions without the addition of $^{18}$O$_2$ gas or $^{18}$O-labeled water. Products S4 and S5 were purified from these three assays and their molecular weights were determined by ESI-mass spectrometry (Fig. 7). The two S4 samples purified from $^{18}$O$_2$-treated assay and control assay had the same molecular weight (400 Da) (Fig. 7, A and B), and this held true also for the two S5 samples (398 Da) (Fig. 7, D and E). In contrast, around two third of product S4 purified from $^{18}$O-labeled water-treated assay showed an increase in the molecular weight from 400 Da to 402 Da (Fig. 7C). The increase in the molecular weight from 398 to 400 Da was also observed in product S5 purified from $^{18}$O-labeled water-treated assay (Fig. 7F). The proportion of products S4 and S5 with increased molecular weight (around 66%) matched exactly with the proportion of $^{18}$O-labeled water (~66%) in the in vitro assay. In addition, in some other experiments, 2 mM sodium dithionite, 2 mM 1,4-dithiothreitol, 5 mM 2-mercaptoethanol, or 10 mM sodium ascorbate was added to remove residual O$_2$ still present in the anaerobic assay. In all cases, both S4 and S5 were still produced at a similar rate as in the presence of oxygen (data not shown). These results demonstrate that the oxygen atom of the incorporated hydroxyl groups at C-25 of the products S4 and S5 originated from water, not from molecular oxygen.

Possible Side Product Formed Nonspecifically by Membrane Protein Fraction—One product (M1) synthesized by the membrane protein fraction of S. denitrificans was also purified. Its CI-mass spectrum revealed that the molecular mass of M1 was 368 Da. No strong ultraviolet absorption around 240 nm was observed which indicated the absence of conjugated double bond structure in M1. TLC and HPLC behavior of M1 was similar to that of cholesta-3,5-diene (Table 1).

**DISCUSSION**

This work represents the first study of the complete anaerobic metabolism of a sterolic compound. We propose an outline for the initial reactions (Fig. 2B), which use some common, but also some fundamentally different, intermediates as compared with the aerobic pathway. The anaerobic hydroxylation of the cholesterol side chain implies a novel type of dehydrogenase acting on a tertiary carbon that is not only unique in terms of enzyme catalysis, but this kind of reaction may be biotechnologically useful. The synthesis of heteroatom-substituted quaternary carbon centers is of great importance for the production of natural products and pharmaceuticals. Thus, numerous different, mostly nonenzymatic methods have been established, proceeding usually via a nucleophilic or electrophilic addition to a double bond (28). The oxidative activation of a tertiary sp$^3$ C–H bond using oxygen-independent enzyme catalysis introduces an unprecedented method with possible applications far beyond the example described herein.

**Proposed Initial Reactions of the Anaerobic Pathway**—First the hydroxyl group at C-3 is oxidized to the keto group leading to cholesta-5-en-3-one (S1). A subsequent isomerization yields cholesta-4-en-3-one (S2). These two reactions may be catalyzed by one bi-functional enzyme, as in the aerobic pathway (10), with the difference that cholesterol dehydrogenases and cholesterol oxidases from the aerobic pathway require molecular oxygen as co-substrate. In the indirect anaerobic incomplete cholesterol reduction pathway cholesterol is transformed via cholesta-3,5-diene (S2) to cholesta-3,5-dien-3-one (S3). These two reactions may be catalyzed by one bi-functional enzyme, as in the aerobic pathway (10), with the difference that cholesterol dehydrogenases and cholesterol oxidases from the aerobic pathway require molecular oxygen as co-substrate. In the indirect anaerobic incomplete cholesterol reduction pathway cholesterol is transformed via cholesta-3,5-diene (S2) to cholesta-3,5-dien-3-one (S3).

**TABLE 2**

| $^{13}$C NMR spectral data for S2, S4, and S5 in CDCl$_3$ | $\delta_c$ (S2) | $\delta_c$ (S4) | $\delta_c$ (S5) |
|---|---|---|---|
| ppm | | | |
| 199.52 | 199.62 | 186.43 |
| 171.57 | 171.63 | 169.44 |
| 123.70 | 123.71 | 156.00 |
| 56.05 | 71.04 | 127.39 |
| 55.82 | 55.99 | 123.73 |
| 53.76 | 55.82 | 71.04 |
| 42.33 | 53.75 | 55.95 |
| 39.58 | 44.34 | 55.41 |
| 39.44 | 42.36 | 52.33 |
| 38.54 | 39.58 | 44.31 |
| 36.06 | 38.55 | 43.60 |
| 35.69 | 36.33 | 42.63 |
| 35.64 | 35.67 | 39.45 |
| 35.56 | 35.64 | 36.28 |
| 33.94 | 35.57 | 35.65 |
| 32.90 | 33.94 | 35.49 |
| 31.00 | 32.90 | 33.64 |
| 28.13 | 31.99 | 32.89 |
| 27.95 | 29.31 | 29.31 |
| 24.13 | 29.18 | 29.19 |
| 23.76 | 28.14 | 28.09 |
| 22.77 | 24.12 | 24.33 |
| 22.51 | 20.98 | 22.82 |
| 20.98 | 20.72 | 20.72 |
| 18.59 | 18.56 | 18.65 |
| 17.33 | 17.34 | 18.52 |
| 11.90 | 11.91 | 12.00 |

**Figure 7**

**Proposed Initial Reactions of the Anaerobic Pathway**

1. In an $^{18}$O$_2$-treated assay, $^{18}$O$_2$ gas (20%, v/v) was added to the oxygen-free headspace of one reaction mixture as the sole source of molecular oxygen (i.e., O$_2$ was 100% labeled). 2) A $^{18}$O-labeled water-treated assay contained ~66% of $^{18}$O-labeled water (mol/mol) in the anoxic reaction mixture (10 ml). 3) A control assay (50 ml) was incubated under anoxic conditions without the addition of $^{18}$O$_2$ gas or $^{18}$O-labeled water. Products S4 and S5 were purified from these three assays and their molecular weights were determined by ESI-mass spectrometry (Fig. 7). The two S4 samples purified from $^{18}$O$_2$-treated assay and control assay had the same molecular weight (400 Da) (Fig. 7, A and B), and this held true also for the two S5 samples (398 Da) (Fig. 7, D and E).
FIGURE 7. ESI-mass spectrum (positive mode) of S4 and S5. A, S4 purified from the anoxic control assay. B, S4 purified from the $^{18}$O$_2$-treated assay. C, S4 purified from the $^{18}$O-labeled H$_2$O-treated assay. D, S5 purified from the anoxic control assay. E, S5 purified from the $^{18}$O$_2$-treated assay. F, S5 purified from the $^{18}$O-labeled H$_2$O-treated assay.
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carbon source under anoxic conditions corroborates the conclusion that S2 is a true intermediate of the cholesterol catabolic pathway.

The third step involves a dehydrogenation reaction producing cholesta-1,4-dien-3-one (S3) with the characteristic conjugated double bond system at ring A. This compound has not been reported previously to be included in anoxic cholesterol transformation or degradation. 3-Ketosteroid-Δ1-dehydrogenase from Comamonas testosteroni TA441 (31) catalyzes the conversion of androst-4-ene-3,17-dione to 1,4-diene derivatives. The 1,4-dien-3-one system in ring A is transformed in this aerobic pathway to an aromatic structure which is attacked by meta-cleavage enzymes. However, this reaction requires a dioxygenase and molecular oxygen and thus cannot work under anoxic conditions. So far it is not obvious what could be the sense of producing the 1,4-dien-3-one structure in the anoxic cholesterol degradation.

Unprecedented Anaerobic Hydroxylation of the Side Chain—
The following intriguing reactions, which are postulated because of the formation of S4 and S5, are proposed to differ fundamentally from those described for the aerobic pathway. Thus, hydroxylation of S2 at C-25 produces the intermediate S4. A similar hydroxylation reaction of S3 yields the intermediate S5. Alternatively, S5 can be also produced via 1,2-dehydrogenation of S4. Further metabolism may proceed via cleavage of the ring A or the degradation of the side chain.

It has been known that monoxygenases catalyze many hydroxylation reactions in steroid hormone metabolism of vertebrates and use NADPH as the usual electron donor (1). However, the hydroxylation leading to S4 and S5 was not catalyzed by a monoxygenase, because an electron acceptor rather than an electron donor was required. We presented evidence as to the origin of the oxygen atom introduced at C-25 position of products S4 and S5. The presence of 20% 18O2 did not result in 18O incorporation, but the introduction of 18O-labeled H2O did. Remarkably, this enzyme appears not to be oxygen sensitive. Enzymes catalyzing such a type of reaction belong to the molybdenum-containing hydroxylases enzyme family and they use water as the source of the oxygen atom incorporated into their substrates (32). One of these enzymes that was recently characterized is the molybdenum-iron-sulfur containing ethylbenzene dehydrogenase from Azotobacter strains EB1 and EbN1 (33, 34). This enzyme catalyzes the oxidation of ethylbenzene to (S)-1-phenylethanol, thus hydroxylation of the C-2 (a secondary carbon) of the side chain. We postulate that a novel enzyme may catalyze the unprecedented hydroxylation reaction at the tertiary carbon leading to S4 and S5.

Possible Dead-end Product Produced by Membrane Fraction at Acidic pH—Membrane-bound proteins produced under acidic conditions (pH 6.5) another 368-Da product M1 similar to cholesta-3,5-diene, that is, it has lost oxygen at C3 position. This compound may not be a true intermediate in anoxic cholesterol metabolism, (i) because M1 was produced by membrane-bound proteins at pH 6.5 whereas the other products were produced by soluble proteins at pH 7.9, and (ii) the supposed structure of product M1 is very different from other purified products in which the hydroxyl group is oxidized to the carbonyl group. In contrast, in product M1 the hydroxyl group is lost and the modification of ring A makes an enzymatic attack even more difficult. Therefore, the production of M1 from cholesterol may be attributed to an unspecific membrane-bound dehydrogenase/dehydratase activity.

Comparison with Aerobic Cholesterol Pathway—It appears that the anaerobic pathway differs from the described aerobic pathway in important aspects. Underoxic conditions, the aromatization of ring A of S3 facilitates the oxygenolytic ring fission via the meta-cleavage pathway. Obviously, ring cleavage under anoxic conditions must be mediated by an oxygen-independent strategy. In the aerobic pathway, cholesta-4-ene-3-one (S2) is hydroxylated by a monoxygenase at the terminal 26-methyl group to produce the corresponding primary alcohol, which is subsequently oxidized to the corresponding acid, followed by β-oxidation of the side chain. In the anoxic pathway hydroxylation occurs at the tertiary C-25 with water, and the tertiary alcohol cannot be oxidized further.

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