The degradation of cholesterol by *Pseudomonas* sp. NCIB 10590 under aerobic conditions

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Abstract The metabolic pathway of cholesterol degradation by bacteria has not been completely established. Several possible intermediates have not been identified and many pathway delineations have not involved the use of the cholesterol molecule per se and just one bacterial species. The bacterial degradation of cholesterol by *Pseudomonas* sp. NCIB has been studied. Major biotransformation products included cholest-5-en-3-one, cholest-4-en-3-one, 26-hydroxycholest-4-en-3-one, androsta-1,4-dien-3-17-dione, cholest-4-en-3-one-26-oic acid, chol-4-en-3-one-24-oic acid, pregn-4-en-3-one-20-carboxylic acid, and pregna-1,4-dien-3-one-20-carboxylic acid. Studies with selected intermediates have enabled the elucidation of a comprehensive pathway of cholesterol degradation by bacteria.—**Owen, R. W., A. N. Mason, and R. F. Bilton.** The degradation of cholesterol by *Pseudomonas* sp. NCIB 10590 under aerobic conditions. *J. Lipid Res.* 1983. **24:** 1500–1511.

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The search for microorganisms that can decompose cholesterol began almost 40 years ago. Tak (1) observed in 1942 that several species of *Mycobacteria* could utilize cholesterol as the sole carbon source. Turfitt (2) revealed in 1947 that many strains of *Proactinomyces* were capable of up to 35% cholesterol decomposition. Later studies by Turfitt (3) revealed that it was possible to isolate metabolites of cholestenone from growing cultures of *Proactinomycetes erythropolis.* Products identified were 3-oxoetiocholest-4-enoic acid, A-nor-3,5-secocholest-5-en-3-oic acid, and isocaproic acid. In 1947 Horvath and Kramli (4) showed that a species of *Azotobacter* could transform cholesterol to cholestenone, 7-dehydrocholesterol and presented evidence for side-chain cleavage by also detecting methyl heptanone in the culture medium.

Stronger evidence for cholesterol side-chain cleavage was provided by Whitmarsh (5) who showed that a *Nocardia* soil isolate could convert cholesterol to pregn-4-en-3-oxo-20-carboxylic acid, androst-4-en-3,17-dione (AD), and androsta-1,4-dien-3,17-dione (ADD) in the presence of 7-hydroxyquinoline. The use of inhibitors to enable detection of intermediary metabolites was utilized by Nagasawa et al. (6–8) In one study (6) they noted that complete decomposition of cholesterol could be achieved by many species of the genera *Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protoaminobacter, Serrattia,* and *Streptomyces.* Further work by this group (7) revealed that using the chelating agent $\alpha,\alpha,'$-dipyridyl (α,α'-D), cholesterol side-chain cleavage could be detected (as evinced by the detection of ADD) in all the above-mentioned genera. Parallel studies (8) revealed that a range of inhibitors, α,α'-D, orthophenanthroline, 8-hydroxyquinoline, $CuSo_4$, and $NiSO_4$, were the most efficient at causing cholesterol metabolite accumulation. They concluded that these agents exerted their effects by inhibiting 9α-hydroxylation of ADD.

In the same decade a different approach was followed by Sih, Wang, and Tai (9). Using *Nocardia restrictus* they demonstrated the production of 3-hydroxy-19-bisnorchola-1,3,5(10)-trien-22-oic acid and 3-hydroxy-19-bisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid from 19-hydroxy-cholest-4-en-3-one (9) which supported the observation of Whitmarsh (5) in that the degradation of the hydrocarbon side-chain of cholesterol proceeds via C_{22} acidic intermediates. However, this study failed to show that the actual C_{22} acids isolated could be converted to ADD. In parallel studies (10) using radiolabeled cholesterol they also detected and identified 3-oxochol-4-en-24-oic acid. However, radiolabeled lithocholic acid was used to show that a C_{24} steroid could be converted to a C_{22} steroid with concomitant release of acetic acid. Sih, Wang, and Tai (11) confirmed their original work by eventually isolating four C_{22} acidic ste-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; α,α'-D, α,α'-dipyridyl; AD, androst-4-en-3,17-dione; ADD, androsta-1,4-dien-3,17-dione; NaDCA, sodium deoxycholate; 4CTO, cholest-4-en-3-one; PMR, proton magnetic resonance; *Pseudomonas* sp. 10590, *Pseudomonas* sp. NCIB 10590.

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roids from 19-hydroxy-cholest-4-en-3-one. In this study they also demonstrated the production of radiolabeled propionic acid during the conversion of 3-oxo-19-bis-norchol-4-en-22-oic acid to a 17-oxo-steroid. Subsequently, Sih and co-workers (12) using labeling studies established that *Nocardia restrictus* could convert cholesterol to 3-oxochola-1,4-dien-24-oic acid with the release of radioactive propionic acid. Again, labeled lithocholic acid was used to demonstrate acetic acid production. On the basis of these studies Sih et al. (9–12) have proposed that cholesterol side-chain cleavage by bacteria proceeds via C_{24} and C_{22} acidic intermediates during transformation to 17-oxo-steroids with the concomitant release of 2 moles of propionic acid and 1 mole of acetic acid. Support for the work of Sih et al. (9–12) comes from the studies of Arima, Nakamatsu, and Beppu (13) who have shown that a species of *Nocardia* produces pregna-1,4-dien-3-one-20-carboxylic acid and ADD during the fermentation of cholesterol in the presence of α,α'-D and O-phenanthroline.

If the mechanism of sterol side-chain cleavage proposed by Sih et al. (9–12) is correct then it seems logical that the initial step in the sequential degradation of sterols involves functionalization of one of the terminal methyl groups. Zaretskaya et al. (14) have shown that such a reaction is carried out by a species of *Mycobacterium* when incubated with cholesterol as evinced by the production of 27-hydroxycholest-4-en-3-one.

Recently Wovcha et al. (15) have used mutants of *Mycobacterium fortuitum*, blocked at various stages in the degradation pathway of β-sitosterol, to accumulate intermediates including AD, ADD, ring A-degraded tricyclic compounds, and several 9α-hydroxysteroids.

Thus, the bacterial degradation of cholesterol and β-sitosterol is fairly well understood. However, a complete pathway has yet to be elucidated involving the unmodified cholesterol molecule and using just one bacterial strain.

Because *Pseudomonas* sp. NCIB 10590 (*Pseudomonas* sp. 10590) has proved to be highly efficient in the degradation of bile acids (16–20) and grows well on cholesterol as sole carbon source, we have studied the aerobic metabolism of cholesterol by this strain.

MATERIALS

5α-Cholestane and ADD were obtained from Koch-Light (Colnbrook, Bucks, England). Cholesterol, sodium deoxycholate (NaDCA) and α,α'-dipyridyl (α,α'-D) were obtained from BDH (Poole, Dorset, England). All reference steroids were 100% pure as judged by thin-layer chromatography (TLC) and gas–liquid chromatography (GLC). General reagents were of 'Analar grade' purchased from BDH, and all solvents (including n-propanol) were redistilled before use.

METHODS

Analytical

Infrared (IR) spectra, ultraviolet (UV) spectra, nuclear magnetic resonance (NMR) spectra, mass spectra (MS), and gas–liquid chromatographic–mass spectra (GLC–MS) were obtained as described previously (21). TLC and GLC methodology was also carried out as described previously (21). Isolated metabolites obtained by preparative TLC were crystallized from the elution solvent (ethyl acetate) and recrystallized from methanol–dichloromethane 1:9 unless otherwise stated. The biotransformation medium used, contained (g/l of distilled water): cholesterol, 0.5; K_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 2.0; $(NH_4)_2SO_4$, 1.0; KNO_3, 0.5; niacin, 0.01; biotin, 0.125; $MgSO_4 \cdot 7H_2O$, 0.10; $CaCl_2$ (dried), 0.01; and $FeSO_4 \cdot 7H_2O$, 0.005; final pH 7.0. The cholesterol carbon source (1% solution) was prepared as a thick creamy suspension, by first dissolving 10 g in diethyl ether (30 ml) followed by an equal volume of distilled water, plus 0.2 ml of detergent (Lipsol; LIP Equipment and Services Ltd., Shipley, England). The mixture was agitated with an Ultraturrax homogenizer causing dispersal of the sterol into fine particles and evaporation of the diethyl ether. The solution was made up to 1 liter with distilled water prior to sterilization at 15 lb per square inch for 20 min. Mineral salts, trace elements, and magnesium sulphate solutions were also sterilized separately prior to aseptic admixture.

The organism

Pseudomonas sp. 10590 was routinely maintained on slopes of the biotransformation medium solidified by the addition of 2% Agar No. 1. Additional stocks of the organism were also maintained on similar slopes in which cholesterol was substituted by NaDCA. Seed cultures for the cholesterol experiments were provided by growing *Pseudomonas* sp. 10590 to stationary phase on NaDCA as sole carbon source for 24 hr at which time the bile acid had been completely metabolized (as judged by TLC and GLC). The organism was grown in Erhlenmeyer shake-flask cultures on an LH Engineering orbital shaker (200 rev/min) at 28°C.

Cholesterol metabolism in the absence of inhibitors

A 1-liter aerobic fermentation of cholesterol (A) by *Pseudomonas* sp. 10590 was terminated at 48 hr by direct solvent extraction of the culture with an equal volume of ethyl acetate (×3). The pooled ethyl acetate extracts

were dried over anhydrous magnesium sulfate and evaporated to dryness in vacuo to yield 350 mg of residue. The dry residue was dissolved in 10 ml of dichloromethane and was separated by preparative TLC (20 plates) in the solvent system methanol–dichloromethane 1:19 (by vol) yielding residual cholesterol (110 mg) and one neutral metabolite (B).

Cholest-4-en-3-one (B). Recrystallization of metabolite B gave white crystals (180) mg) mp 80°C. IR 1684 (3-ketone) and 1618 cm^{-1} (C_4-C_5 double bond); UV 241 nm (ξ 15990), NMR (δ) 0.70, 1.18 (6H, s, 18-CH_3 and 19-CH_3), 0.84, 0.87, 0.92 (9H, s, 21-CH_3, 26-CH_3, and 27-CH_3), 5.70 (1H, s, 4-H); MS, M^+ 384 (38%) ($C_{27}H_{44}O$ requires M^+ 384), base peak at m/e 124 (4-en-3-one, 100%) and m/e 271 (M^+-8C-side chain, 18%). GLC, R_f 3.70; TLC, R_f 1.33; after oxidation, R_f 1.33; after acetylation, R_f 1.33; and after reduction, R_f 1.33.

Cholesterol metabolism in the presence of inhibitors

The metabolism of cholesterol by *Pseudomonas* sp. 10590 in the presence of inhibitors was initially carried out in 250-ml Erhlenmeyer shake flasks containing 100 ml of biotransformation medium. Duplicate experiments were set up containing either α,α'-D (0–10 mM) or n-propanol (0–10%) to ascertain the optimum concentration and per cent of inhibition, respectively, of inhibitor required to cause maximum metabolite accumulation. The cultures were seeded with *Pseudomonas* sp. 10590 and were incubated for 48 hr at 28°C. The inhibitors were added and incubation was continued for a further 24 hr. Analysis of ethyl acetate extracts (methylated with ethereal diazomethane (22)) was carried out by GLC. Neutral steroids were quantitated with reference to the flame ionization response of 5α-cholestane and acidic steroids by reference to methyl DCA. Both reference steroids were added to the cultures prior to extraction.

Cholesterol metabolism in the presence of α,α'-D (0.6 mM)

A 1-liter fermentation of cholesterol (0.5 mg/ml) by *Pseudomonas* sp. 10590 was carried out for 48 hr at 28°C. Analysis of a culture aliquot (10 ml) by TLC and GLC revealed the presence of cholesterol and metabolite B only. α,α'-D (0.6 mM) was added to the medium and incubation was continued for a further 24 hr. At the end of the fermentation the medium was extracted with ethyl acetate to yield 270 mg of dried residue. The residue was dissolved in 50 ml of ethyl acetate, washed twice with distilled water (75°C) to remove α,α'-D, and was dried again to give 254 mg of residue. The dried residue was separated by preparative TLC (15 plates) into a series of fractions from which steroids B–H were crystallized.

Cholest-4-en-3-one (B). Recrystallization of metabolite B gave white crystals (87 mg).

Androst-4-en-3,17-dione (C). Recrystallization of metabolite C gave white crystals (8 mg), mp 164°C. IR 1736 (17-ketone), 1666 (3-ketone), and 1616 cm^{-1} (C_4-C_5 double bond); UV 242 nm (ξ 16020); NMR (δ) 0.91, 1.21 (6H, s, 18-CH_3 and 19-CH_3), 2.25–2.55 (2H, m, 16-CH_2) and 5.74 (1H, s, 4-H); MS, M^+ 286 (100%) ($C_{19}H_{24}O_2$ requires M^+ 286) and m/e 124 (4-en-3-one, 73%). GLC, R_f 1.39; TLC, R_f 1.10; after oxidation, R_f 1.10; after acetylation, R_f 1.10; and after reduction, R_f 0.70.

Androsta-1,4-dien-3,17-dione (D). Recrystallization of metabolite D gave white prisms (32 mg), mp 136–137°C. IR 1732 (17-ketone), 1650 (3-ketone), 1616 and 1598 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds); UV 245 nm (ξ 16360); NMR (δ) 1.02, 1.33 (6H, s, 18-CH_3 and 19-CH_3), 2.40–2.60 (2H, m, 16-CH_2), 6.12 (1H, s, 4-H), 6.26 (1H, showing further splitting, J = 10 Hz, 2-H), 7.06 (1H, d, J = 10 Hz, 1-H); MS, M^+ 284 (34%) ($C_{19}H_{24}O_2$ requires M^+ 284), m/e 122 (1H, 1,4-dien-3-one, 100%). GLC, R_f 1.60; TLC, R_f 1.00; after oxidation, R_f 1.00; after acetylation, R_f 1.00; and after reduction, R_f 0.68.

Chol-4-en-3-one-24-oic acid (E). Recrystallization of metabolite E gave white crystals (16 mg), mp 141–142°C. Methyl ester analysis: IR 1746 (24-$COOCH_3$), 1676 (3-ketone), and 1620 cm^{-1} (C_4-C_5 double bond); UV 241 nm (ξ 12700); NMR (δ) 0.60, 1.06 (6H, s, 18-CH_3 and 19-CH_3), 0.85 (3H, s, 21-CH_3), 3.56 (3H, s, 24-OCH_3) and 5.65 (1H, s, 4-H); MS, M^+ 386 (76%) ($C_{25}H_{38}O_3$ requires M^+ 386) m/e 124 (4-en-one, 85%) and m/e 271 (M^+ −115, 6C-side chain, 29%). GLC, R_f 6.20; TLC, R_f 1.21; after oxidation, R_f 1.21; after acetylation, R_f 1.21; and after reduction, R_f 1.21.

Chola-1,4-dien-3-one-24-oic acid (F). Recrystallization of metabolite F gave white crystals (5 mg). Methyl ester analysis: IR 1745 (24-$COOCH_3$), 1665 (3-ketone) 1610, and 1598 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds), UV 245 nm; NMR (δ) 0.79, 1.21 (6H, s, 18-CH_3 and 19-CH_3), 0.93 (3H, d, J = 6 Hz, 21-CH_3), 3.67 (3H, s, 24-OCH_3), 6.24 (1H, d, J = 10 Hz, 2-H), 6.50 (1H, s, 4-H), 7.01 (1H, d, J = 10 Hz, 1-H), MS, M^+ 384 (11%) ($C_{25}H_{36}O_3$ requires M^+ 284) m/e 122 (1,4-dien-3-one, 100%) and m/e 269 (M^+ −115, 6C-side-chain, 14%). GLC, R_f 8.70; TLC, R_f 1.18; after oxidation, R_f 1.18; after acetylation, R_f 1.18; and after reduction, R_f 1.18.

Pregn-4-en-3-one-20-carboxylic acid (G). Recrystallization of metabolite G gave white crystalline needles (12 mg), mp 160°C. Methyl ester analysis: IR 1743 (22-$COOCH_3$), 1670 (3-ketone), and 1616 cm^{-1} (C_4-C_5 double bond); UV 241 nm; NMR (δ) 0.72, 1.17 (6H, s, 18-CH_3 and 19-CH_3), 1.19 (3H, s, 21-CH_3), 3.64 (3H, s, 22-OCH_3) and 5.71 (1H, s, 4-H); MS, M^+ 358 (68%) ($C_{22}H_{34}O_3$ requires M^+ 358), m/e 124 (4-en-3-one, 100%) and m/e 271 (M^+

−87, 4C-side chain, 10%). GLC, R_f 3.09; TLC, R_f 1.19; after oxidation, R_f 1.19; after acetylation, R_f 1.19; and after reduction, R_f 1.19.

Pregna-1,4-dien-3-one-20-carboxylic acid (H). Recrystallization of metabolite H gave white spiky needles (28 mg), mp 212–214°C. IR 1738 (22-$COOCH_3$), 1672 (3-ketone), 1628 and 1608 cm^{-1} (C_1-C_2 and C_4 and C_5 double bonds); NMR (δ) 0.75, 1.22 (6H, s, 18-CH_3 and 19-CH_3), 1.17 (3H, d, J = 6Hz, 21-CH_3), 3.64 (3H, s, 22-OCH_3), 6.05 (1H, s, 4-H), 6.21 (1H, d, showing further splitting, J = 10 Hz, 2-H), 7.01 (1H, d, J = 10 Hz, 1-H); MS, M^+ 356 (30%) ($C_{23}H_{32}O_3$ requires M^+ 356), m/e 122 (1,4-dien-3-one, 100%) and m/e 269 (M^+ −87, 4C-side chain, 7%). GLC, R_f 3.48; TLC, R_f 1.10; after oxidation, R_f 1.10; after acetylation, R_f 1.10; and after reduction, R_f 1.10.

Cholesterol metabolism in the presence of 2% n-propanol

A 1-liter fermentation of cholesterol (0.5 mg/ml) by *Pseudomonas* sp. 10590 was carried out for 48 hr at 28°C. The initial stage of the fermentation was identical to the α,α′-D experiment. After the addition of 2% n-propanol and incubation for a further 24-hr, the culture was extracted with ethyl acetate yielding 341 mg of dried residue. This residue was separated by preparative TLC (20 plates) in the solvent system methanol–dichloromethane 1:19 (v/v) into a series of fractions from which steroids B–P were crystallized.

Metabolites B (234 mg), D (3 mg), E (4 mg), and H (20 mg) were found to be identical to cholest-4-en-3-one, androsta-1,4-dien-3,17-dione, chol-4-en-3-one-24-oic acid, and pregna-1,4-dien-3-one-20-carboxylic acid, respectively.

Cholest-5-en-3-one (I). Recrystallization of metabolite I gave white crystals (20 mg). IR 1726 (3-ketone) and 1670 cm^{-1} (C_5-C_6 double bond). GLC, R_f 3.60; TLC, R_f 1.35; after oxidation, R_f 1.35; after acetylation, R_f 1.35; and after reduction, R_f 0.76.

26-Hydroxycholest-4-en-3-one (J). Recrystallization of metabolite J gave white crystals (10 mg), mp 139–140°C. IR 3490 (C_{26}-hydroxyl), 1660 (3-ketone) and 1610 cm^{-1} (C_4-C_5 double bond); UV 241 nm; NMR (δ) 0.70, 1.18 (6H, s, 18-CH_3 and 19-CH_3), 0.89, 0.93 (6H, s, 21-CH_3 and 26-CH_3), 3.46 (2H, s, C-26 protons neighboring on oxygen atom) and 5.7 (1H, s, 4-H); MS, M^+ 400 (80%) ($C_{26}H_{44}O_2$ requires M^+ 400), m/e 124 (4-en-3-one, 100%), m/e 385 (M^+ −15, CH_3, 10%), m/e 358 (M^+ −92, CH_2CO, 34%) and m/e 229 (M^+ −171, 75%). GLC, R_f 10.04; TLC, R_f 0.80; after oxidation, R_f 0.65; after acetylation, R_f 1.30; and after reduction, R_f 0.80.

26-Hydroxycholesta-1,4-dien-3-one (K). Recrystallization of metabolite K gave orange crystals (2 mg). IR 3465 (C_{26}-hydroxyl), 1666 (3-ketone), 1620 and 1606 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds). M^+ 398 (60%) ($C_{26}H_{42}O_2$ requires M^+ 398), m/e 121 (1,4-dien-3-one 100%), m/e 269 (M^+ −129, 8C-side chain, 11%) and m/e 227 (M^+ −171, 21%). GLC, R_f 12.17; TLC, R_f 0.70; after oxidation, R_f 0.55; after acetylation, R_f 1.21; and after reduction, R_f 0.70.

9α-Hydroxyandrost-4-en-3,17-dione (L). Recrystallization of metabolite L gave white needle-like crystals (3 mg) mp 212–214°C. IR 3480 (hydroxy), 1740 (17-ketone), 1660 (3-ketone) and 1610 cm^{-1} (C_4-C_5 double bond); UV 242 nm (ξ 15,400); MS, M^+ 302 (100%) ($C_{19}H_{26}O_3$ requires M^+ 302), m/e 124 (4-en-3-one, 64%), m/e 137 (cleavage between C_9 and C_{10} followed by C_7/C_8 cleavage, 29%). GLC, R_f 1.96; TLC, R_f 0.64; after oxidation, R_f 0.64; after acetylation, R_f 1.02; and after reduction, R_f 0.27.

9α-Hydroxyandrosta-1,4-dien-3,17-dione (M). Recrystallization of metabolite M gave white crystals (2 mg), IR 3450 (hydroxy), 1735 (17-ketone), 1665 (3-ketone), 1610 and 1602 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds); UV 244 (ξ 15,000); MS, M^+ 300 (100%) ($C_{19}H_{24}O_3$ requires M^+ 300), m/e 122 (1,4-dien-3-one, 95%) and and m/e 135 (cleavage between C_9 and C_{10} followed by cleavage at C_7/C_8, 31%). GLC, R_f 2.30; TLC, R_f 0.61; after oxidation, R_f 0.61; after acetylation, R_f 1.00; and after reduction, R_f 0.25.

3-Hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (N). Recrystallization of metabolite N gave yellow crystals (1 mg) mp 122°C. IR 3360 (hydroxy), 1740 (17-ketone), 1705 (9-ketone), 1608, 1585 and 1500 (aromatic ring), 1280 and 1250 (phenol) and at 855 and 800 (o,m-substituted phenol ring); UV 218, 283 nm; MS, M^+ 300 (39%) ($C_{19}H_{24}O_3$ requires M^+ 300), m/e 134 (phenolic A-ring + 2C fragments, 100%) and m/e 122 (phenolic A-ring, 63%). GLC, R_f 1.24; TLC, R_f 0.86; after oxidation, R_f 0.86; after acetylation, R_f 1.22; and after reduction, R_f 0.44.

Cholest-4-en-3-one-26-oic acid (O). Metabolite O could not be crystallized, but was obtained as a dry gummy residue (10 mg). Analysis of this compound however showed it to be identical to cholest-4-en-3-one-26-oic acid synthesized by Jones' chromic oxidation of 26-hydroxycholesterol. Analysis of the methyl ester derivative revealed IR 1740 (26-$COOCH_3$), 1700 (3-ketone) and 1610 cm^{-1} (C_4-C_5 double bond); UV 243 nm. GLC, R_f 15-20; TLC, R_f 1.31; after oxidation, R_f 1.31; after acetylation, R_f 1.31; and after reduction, R_f 1.31.

Cholesta-1,4-dien-3-one-26-oic acid (P). Metabolite P also could not be crystallized but formed a yellow gummy residue (2 mg). This compound was identical in its properties to the Jones' chromic oxidation product of 26-hydroxycholesta-1,4-dien-3-one. Analysis of the methyl ester of P revealed IR 1750 (26-$COOCH_3$), 1660 (3-ketone), 1610 and 1600 cm^{-1} (C_1-C_2 and C_4-

C_5 double bonds); UV 246 nm (ξ 14,000). GLC, R_f 16.0; TLC, R_f 1.18; after oxidation, R_f 1.18; after acetylation, R_f 1.18; and after reduction, R_f 1.18.

Cholesterol intermediary metabolism

To elucidate more clearly the sequential nature of cholesterol side-chain cleavage, the major presumptive intermediates in the pathway were incubated with *Pseudomonas* sp. 10590 in the presence of the relevant inhibitors. The experiments were carried out in duplicate with culture volumes in the range 10–250 ml depending upon the amount of available substrate. Neutral steroid substrates (0.02%) were prepared in the same way as cholesterol whilst acidic substrates (0.02%) were prepared as sodium salts. Procedures were identical to the large scale experiments and major metabolites of intermediary metabolism were identified by TLC and GLC.

RESULTS

Pseudomonas sp. 10590 gave confluent growth on agar plates containing cholesterol and in aqueous media showed the capacity to degrade the sterol completely after 72 hr incubation. During the biodegradation period a transient metabolite appeared which reached a maximum after 48 hr growth. Analysis of this metabolite (B) revealed it to be identical to authentic cholest-4-en-3-one (4CTO) in its chromatographic and spectroscopic properties. Side-chain cleavage products were not detected under these conditions. This is in complete contrast to bile acid degradation by the *Pseudomonas* sp. under similar conditions (16–20). Typically during the metabolism of chenodeoxycholic acid (17) after an initial lag phase, C_{19} and C_{22} side-chain cleavage products reached a maximum at 12 hr and 14.5 hr, respectively.

In the presence of selective enzyme inhibitors such as α,α'-D and n-propanol, a number of metabolites built up in the medium during the fermentation of cholesterol by *Pseudomonas* sp. 10590. Maximum metabolite accumulation occurred with 0.6 mM α,α'-D and 2% n-propanol, respectively.

Cholesterol fermentation in the presence of α,α'-D

Pseudomonas sp. 10590 grew fairly rapidly on cholesterol mineral salts medium of pH 7.0 at 28°C. The course of the fermentation was followed by TLC analysis of ethyl acetate extracts of culture aliquots; the extent of UV absorbance at 242 nm of the dried extracts (taken up in methanol) indicated the amount of ring-A $\alpha\beta$-unsaturated ketone present. The absorbance of 4CTO reached a maximum after 48 hr and then rapidly decreased. However, after addition (48 hr) of 0.6 mM α,α'-D, the decrease was very slight at 72 hr. A 1-liter culture was extracted after 72 hr. Separation of the extract by preparative TLC yielded a major neutral metabolite (B) and two minor metabolites (C and D) (**Fig. 1**), with one major acidic metabolite (H), and several minor acidic metabolites (E–G) (**Fig. 2**).

Compound B was identical to authentic 4CTO in its chromatographic and spectroscopic properties. The minor neutral metabolites C and D were identical to authentic androst-4-en-3,17-dione and androsta-1,4-dien-3,17-dione, respectively, in their chromatographic and spectroscopic properties.

The major acidic metabolite (H) was isolated as a crystalline solid. The methyl ester of H gave a mass spectrum showing a low intensity molecular ion at m/e 356 and an intense (base peak) ion at m/e 122, suggesting a steroidal 1,4-dienone structure (23). Confirmation of the structure of ring A was provided by the IR spectrum (1672, 1628, and 1608 cm^{-1}, $\alpha\beta$-unsaturated ketone), the UV spectrum (λ max 244 nm, di-β-substituted $\alpha\beta$-unsaturated ketone in a six-membered ring, double bond exocyclic) (24) and by the PMR spectrum (three vinylic protons in the range δ 6.05–7.01 ppm). Compound H could not be oxidized, acetylated, or reduced, inferring the absence of hydroxyl groups and unconjugated ketone groups. The mass spectrum of the methyl ester of H also showed a low intensity ion at m/e 269 corresponding to the loss of a 4-carbon side chain from C_{17}. Compound H was therefore assigned the structure 3-oxo-pregna-1,4-dien-20-carboxylic acid. Of the minor acidic compounds, metabolite G gave a mass spectrum showing a molecular ion at m/e 358 and an intense (base peak) ion at m/e 124, suggesting a steroidal 4-en-3-one structure (23). Confirmation of the structure of ring A was provided by the IR spectrum (1670, 1616 cm^{-1}, $\alpha\beta$-unsaturated ketone) the UV spectrum (λ max 241 nm, di-β-substituted $\alpha\beta$-unsaturated ketone, double bond exocyclic) (24) and by the proton magnetic resonance (PMR) spectrum (one vinylic proton at δ 5.71 ppm). Compound G could not be oxidized, acetylated, or reduced, inferring the absence of hydroxyl groups and unconjugated ketone groups. The mass spectrum of methyl G also showed a low intensity ion at m/e 271 corresponding to the loss of the side chain from C_{17}. Compound G was therefore assigned the structure 3-oxo-pregn-4-en-20-carboxylic acid.

Another minor acidic metabolite (E) showed an intense molecular ion at m/e 386 and an intense ion at m/e 124, inferring a steroidal 4-en-3-one structure. Confirmation of this structure was obtained from the IR, UV, and PMR spectra. The mass spectrum of methyl E also showed a low intensity ion at m/e 271

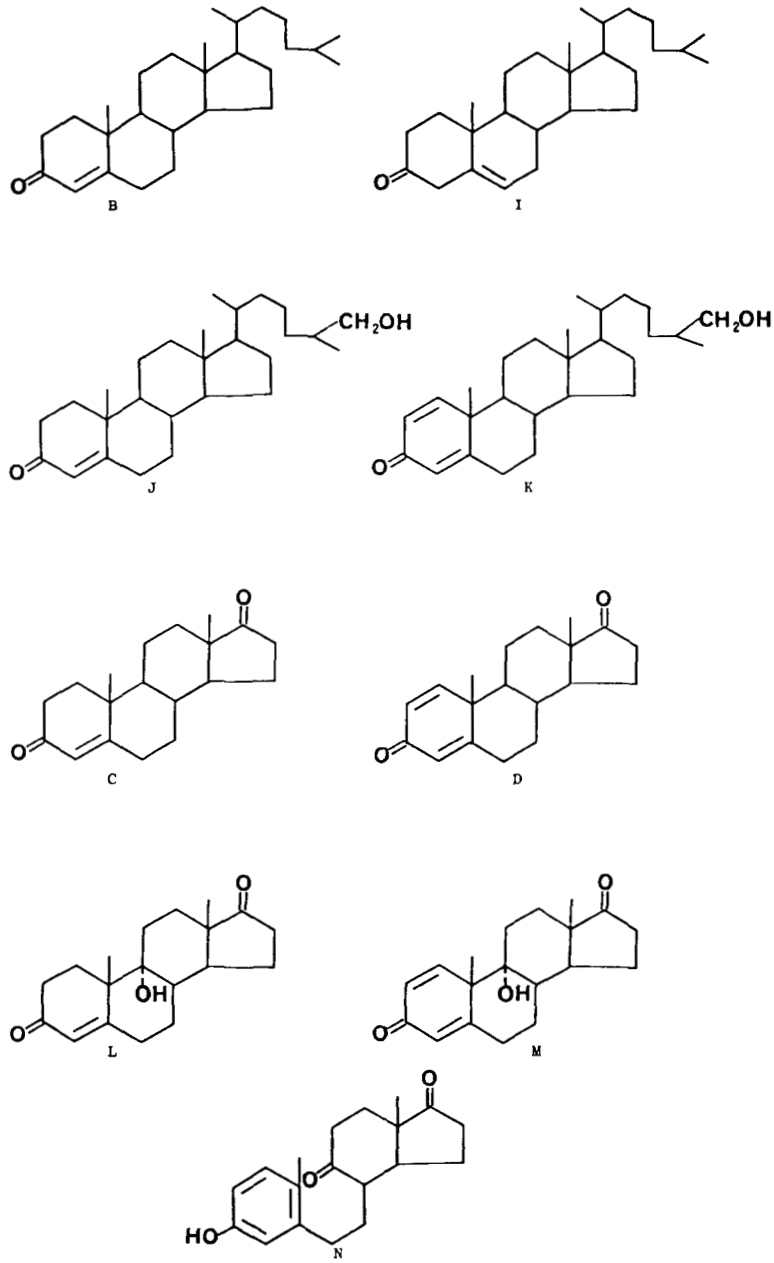


Fig. 1. Neutral metabolites isolated after aerobic catabolism of cholesterol by *Pseudomonas* sp. NCIB 10590.

corresponding to the loss of the full bile acid side chain (6-carbon) from C_{17}. Compound E was therefore assigned the structure chol-4-en-3-one-24-oic acid.

The remaining acidic metabolite (F) showed a low intensity molecular ion at m/e 384 and an intense (base peak) ion at m/e 122 suggesting a steroidal 1,4-dienone structure. This was confirmed by the UV, IR, and PMR spectra. The mass spectrum of methyl F also showed a low intensity ion at m/e 269 corresponding to the loss of the full bile acid side chain. Compound F was therefore assigned the structure chola-1,4-dien-3-one-24-oic acid.

Cholesterol fermentation in the presence of 2% n-propanol

When 2% n-propanol was added during the log phase of growth (48 hr) of *Pseudomonas* sp. 10590, a build-up of metabolites occurred (some of which had not been observed previously). A 1-liter culture was extracted after 72 hr. Separation of the extract gave a major neutral component (B) and seven minor neutral components (compounds D, I–N) (Fig. 1), with one major acidic component (O) and three minor acidic components (compounds E, H, and P) (Fig. 2).

Compound B and one of the minor neutral metabolites

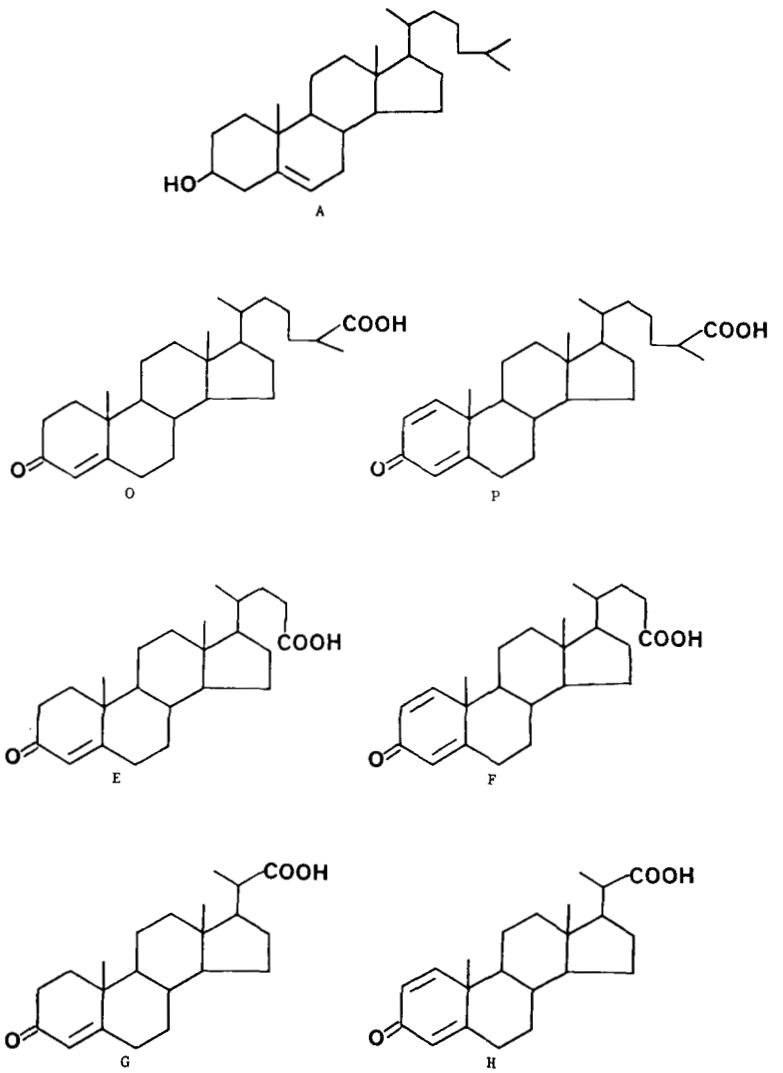


Fig. 2. Acidic metabolites isolated after aerobic catabolism of cholesterol (A) by *Pseudomonas* sp. NCIB 10590.

(D) were identical to authentic 4CTO and androsta-1,4-dien-3,17-dione, respectively. One of the minor neutral compounds (I) was identical in its chromatographic and spectroscopic properties to cholest-5-en-3-one. Of the five remaining neutral metabolites, compound J revealed an intense molecular ion at m/e 400 and an intense ion at m/e 124 suggesting a steroidal 4-en-3-one structure. Confirmation of the structure of ring A was provided by the IR spectrum (1660 and 1610 cm^{-1}, $\alpha\beta$-unsaturated ketone) and UV spectrum (λ max 241 nm, di-β-substituted $\alpha\beta$-unsaturated ketone, double bond exocyclic) and by the PMR spectrum (one vinylic proton at δ 5.70 ppm). The PMR spectrum also revealed singlet signals due to the protons of the angular methyl groups at C_{18} (δ 0.70 ppm) and at C_{19} (δ 1.18 ppm) as well as two partially overlapping doublets (J = 7Hz) at δ 0.89 ppm and at 0.93 ppm belonging to two methyl groups coupled with a neighboring methine proton. The spectrum also contained a doublet signal (5 Hz) at δ 3.46 ppm equal to a 2-proton signal. The chemical shift of this signal indicated that the corresponding protons were located close to an oxygen atom and thus interaction was occurring with the hydroxyl proton according to the spin-spin mechanism. This indicated that the molecule contained a primary hydroxyl group. Compound J could be acetylated with acetyl chloride further substantiating the presence of a hydroxyl group, but could not be reduced indicating the absence of an unconjugated ketone group. This was confirmed by the IR spectrum which displayed a significant peak (3490 cm^{-1}) in the hydroxyl group region. The primary nature of the hydroxyl group and its location was also proved when compound J was oxidized with Jones' chromic reagent (25). This produced a metabolite which after methylation was identical to methyl 3-oxo-cholest-4-en-3-one-26-oic acid synthesized by the same method from 26-hydroxycholesterol. Compound J was

therefore assigned the structure 26-hydroxycholest-4-en-3-one.

Another of the neutral components, compound (K), was isolated as pale orange crystals. The mass spectrum of K revealed an intense molecular ion at m/e 398 and an intense ion at m/e 122 indicative of a steroidal 1,4-dienone structure. Confirmation of the A-ring structure was provided by the UV and IR spectra. Compound K was easily acetylated but could not be reduced, suggesting the presence of a hydroxyl group. This was confirmed by the IR spectrum. The location of the hydroxyl group was again proved by oxidation of K with Jones' reagent. This produced a metabolite which after methylation was identical to methyl 3-oxo-cholest-1,4-dien-3-one-26-oate synthesized from methyl 3-oxo-cholest-4-en-26-oate by DDQ oxidation (26). Compound K was therefore assigned the structure 26-hydroxycholesta-1,4-dien-3-one.

Two (L and M) of the three remaining metabolites appeared to be structurally related. The mass spectrum of L showed a base peak molecular ion at m/e 302 and an intense ion at m/e 124 suggestive of a steroidal 4-en-3-one structure. Confirmation of this structure was provided by the UV and IR spectra. Compound L could not be oxidized but was readily acetylated and reduced indicating the presence of a hydroxyl group and an unconjugated ketone group. The fact that the hydroxyl group could not be oxidized inferred that it was in a tertiary position. The position of the hydroxyl group was ascertained from the mass spectrum which showed an ion of low intensity at m/e 137. This is characteristic of 9α-hydroxy-4-en-3-oxo steroids and the fragment is produced by cleavage between C_9 and C_{10} followed by cleavage at C_7 and C_8 (27). Compound L was therefore assigned the structure 9α-hydroxyandrost-4-en-3,17-dione.

The mass spectrum of compound M showed a base peak molecular ion at m/e 300 and an intense ion at m/e 122 indicative of a steroidal 1,4-dienone structure. Compound M could not be oxidized, but was readily acetylated and reduced indicating the presence of a hydroxyl group and an unconjugated ketone group. The fragment ion at m/e 135 was again suggestive of a 9α-hydroxy A-ring unsaturated steroid. Compound M was therefore assigned the structure 9α-hydroxyandrosta-1,4-dien-3,17-dione.

The final neutral metabolite (N) isolated was recognized as a phenolic compound initially by its UV spectrum λ max 283 nm, which underwent a bathochronic shift on addition of base to λ max 292 nm. The compound, when sprayed with Folin and Ciocalteau reagent on TLC and developed in an atmosphere of ammonia, gave a characteristic blue color which is specific for phenolic hydroxyl groups. The mass spectrum of N shows a molecular ion at m/e 300 and an intense ion at m/e 134 which is typical of 9,10-secosteroids. The compound formed a monotrimethyl silyl-ether with a molecular ion at m/e 372 indicating the presence of one hydroxyl group. The base peak of the spectrum was at m/e 206 suggesting that the hydroxyl group was attached to ring A. Compound N could not be oxidized, but was readily acetylated and reduced, thus inferring the presence of a hydroxyl group and unconjugated ketone groups. This was confirmed by the IR spectrum which displayed two peaks in the unconjugated ketone group region at 1740 and 1705 cm^{-1} and one peak in the hydroxyl group region at 3360 cm^{-1}. The IR spectrum also showed the following peaks at 1608, 1585, and 1500 (aromatic ring), 1280 and 1250 (phenol) and at 856 and 800 cm^{-1} (o, m-substituted phenol ring) which are typical of phenolic steroids. Compound N was therefore assigned the structure 3-hydroxy-9,10-seco-1,3,5(10)-androstatrien-9,17-dione.

The major acidic metabolite (O) was isolated as a dry gummy residue. The methyl ester was identical in its TLC, GLC, and spectroscopic properties to methyl-3-oxo-cholest-4-en-26-oate synthesized from 26-hydroxycholesterol. The unsaturated nature of the A ring was provided by the UV spectrum (λ max, 243 nm, di-β-substituted $\alpha\beta$-unsaturated ketone, double bond exocyclic) and by the IR spectrum (1700 and 1610 cm^{-1}, $\alpha\beta$-unsaturated ketone). Compound O could not be oxidized, acetylated, or reduced indicating the lack of hydroxyl and free ketonic groups. Because insufficient material was available for mass spectra and PMR analysis, this metabolite was tentatively assigned the structure cholest-4-en-3-one-26-oic acid.

One of the minor acidic metabolites (P) was also isolated as a gummy residue. The methyl ester was identical in its TLC, GLC, and spectroscopic properties to methyl 3-oxo-cholesta-1,4-dien-26-oate synthesized from 26-hydroxycholesta-1,4-diene-3-one. The unsaturated nature of ring-A was confirmed by the UV spectrum (λ max 246 nm) and by the IR spectrum (1660, 1610, and 1600 cm^{-1}). Compound P could not be oxidized, acetylated, or reduced indicating the absence of hydroxyl groups and unconjugated ketone groups. Again lack of material for MS and PMR analyses enabled only a tentative assignation of structure, namely cholesta-1,4-dien-3-one-26 oic acid.

The remaining minor metabolites (E and H) were found to be identical to chol-4-en-3-one-24-oic acid and pregna-1,4-dien-3-20-carboxylic acid, respectively.

Cholesterol intermediary metabolism

The results (**Table 1**) of cholesterol intermediary metabolism indicate that each metabolite possessing a side chain is an intermediate in sequential side-chain degradation. Each metabolite gave rise to a sequential intermediate, thereby indicating that they were not by-

TABLE 1. Products of cholesterol intermediate metabolism by *Pseudomonas* sp. NCIB 10590

Substrate	Inhibitor	Major Products	
		Neutral	Acidic
Cholest-5-en-3-one	α,α′-dipyridyl	cholest-4-en-3-one androst-4-en-3,17-dione androsta-1,4-dien-3-17-dione	chol-4-en-3-one-24-oic acid chola-1,4-dien-3-one-24-oic acid pregn-4-en-3-one-20-carboxylic acid pregna-1,4,-dien-3-one-20-carboxylic acid
	n-propanol	26-hydroxycholest-4-en-3-one 26-hydroxycholesta-1,4-dien-3-one cholest-4-en-3-one androsta-1,4-dien-3,17-dione 9α-hydroxyandrosta-1,4-dien-3,17-dione	cholest-4-en-3-one-26-oic acid cholesta-1,4-dien-3-one-26-oic acid chol-4-en-3-one-24-oic acid pregn-1,4-dien-3-one-20-carboxylic acid
Cholest-4-en-3-one	α,α′-dipyridyl	androst-4-en-3,17-dione androsta-1,4-dien-3,17-dione	chol-4-en-3-one-24-oic acid chola-1,4-dien-3-one-24-oic acid pregn-4-en-3-one-20-carboxylic acid pregna-1,4-dien-3-one-20-carboxylic acid
	n-propanol	26-hydroxycholest-4-en-3-one 26-hydroxycholesta-1,4-dien-3-one androsta-1,4-dien-3,17-dione	cholest-4-en-3-one-26-oic acid cholesta-1,4-dien-3-one-26-oic acid pregn-1,4-dien-3-one-20-carboxylic acid
26-Hydroxycholest-4-en-3-one	α,α′-dipyridyl	androst-4-en-3,17-dione androsta-1,4-dien-3,17-dione	chol-4-en-3-one-24-oic acid pregna-1,4-dien-3-one-20-carboxylic acid
	n-propanol	26-hydroxycholesta-1,4-dien-3-one androsta-1,4-dien-3-one	cholest-4-en-3-one-2-oic acid chol-4-en-3-one-24-oic acid pregna-1,4-dien-3-one-20-carboxylic acid
Cholest-4-en-3-one-2-oic acid	α,α′-dipyridyl	androst-4-en-3,17-dione androsta-1,4,-dien-3,17-dione	chol-4-en-3-one-24-oic acid chola-1,4-dien-3-one-24-oic acid pregna-1,4-dien-3-one-20-carboxylic acid
	n-propanol	androsta-1,4-dien-3,17-dione	cholesta-1,4-dien-3-one-27-oic acid chol-4-en-3-one-24-oic acid pregna-1,4-dien-3-one-20-carboxylic acid
Chol-4-en-3-one-24-oic acid	α,α′-dipyridyl	androst-4-en-3,17-dione androsta-1,4-dien-3,17-dione	chola-1,4-dien-3-one-24-oic acid pregn-4-en-3-one-20-carboxylic acid pregna-1,4-dien-3-one-20-carboxylic acid
Cholesta-1,4-dien-3-one-24-oic acid	α,α′-dipyridyl	androsta-1,4-dien-3,17-dione	pregna-1,4-dien-3-one-20-carboxylic acid
Pregn-4-en-3-one-20-carboxylic acid	α,α′-dipyridyl	androst-4-en-3,17-dione androsta-1,4-dien-3,17-dione	pregna-1,4-dien-3-one-20-carboxylic acid
Pregna-1,4-dien-3-one-20-carboxylic acid	α,α′-dipyridyl	androsta-1,4-dien-3,17-dione	

products in the large scale experiments with cholesterol as substrate.

DISCUSSION

Past studies on the bacterial catabolism of cholesterol have been limited by the lack of accumulated intermediates. In the absence of specific enzyme inhibitors, the only compounds to accumulate in substantial yield are 4CTO (6) and ADD (7). The use of α,α′-D and n-propanol-inhibited cultures of *Pseudomonas* sp. 10590 has enabled us to accumulate the pathway intermediates postulated by Sih et al. (9–12) as well as several additional derivatives.

In *Pseudomonas* sp. 10590, the first reaction of cholesterol catabolism is 3β-hydroxysteroid dehydrogenation which is catalyzed by an NAD requiring 3β(17β)-hydroxysteroid dehydrogenase rather than the cholesterol oxidase found in *Arthrobacter* (6) and *Nocardia* spp.

(28). In the above organism, this enzyme is located in the periplasmic space (29) and thus may be involved in sterol functionalization necessary for binding to transport proteins (30) prior to further degradative reactions mediated in the cytosol.

We isolated the first product, cholest-5-en-3-one (4% yield at 27 hr) using n-propanol-inhibited cholesterol cultures. This product does not normally accumulate and is rapidly isomerized to 4CTO. 4CTO, the sole accumulation product at 48 hr in uninhibited aerobic cholesterol cultures, does not appear to be a good substrate for the Δ′ dehydrogenase because the 1,4-dienone does not accumulate in either normal or inhibited fermentations.

In non-inhibited cultures, 4CTO reaches a concentration of 360 mg/liter (36%) after 48 hr. No steroidal material was detectable at 72 hr. In contrast, 4CTO persisted at 170 mg/liter (17%) after 72 hr in α,α′-D-treated cultures and at 463 mg/liter (46% yield) when n-propanol was used. Thus 4CTO is always the major accumulation product indicating in this organism and perhaps many others that the rate-limiting step in cholesterol degradation is functionalization at C_{26}.

The accumulation of intermediates with oxygen functions at C_{26} occurred only in the n-propanol-inhibited fermentations. Evidence for 26-hydroxycholest-4-en-3-one and the Δ′ homologue indicates that side-chain functionalization is probably via a cytochrome P_{450}-mediated hydroxylation of the C_{26} methyl group and sequential oxidation via the C_{26} aldehyde to the C_{26} carboxylic acid involving alcohol and aldehyde dehydrogenases. Evidence from our earlier work on steroid dehydrogenases (29) leads us to suggest that these reactions also may occur in the periplasmic space. In the absence of any alternative evidence, it is presumed that steroid side-chain removal is mediated by sequential β-oxidation. The next reaction, namely thiokinase-mediated activation of the carboxylic acid group at C_{26} to the acyl CoA derivative, must therefore occur in the cytoplasm. The first products of side-chain cleavage, namely chol-4-en-3-one-24-oic acid (metabolite E) and its Δ′ homologue (metabolite F) (very low concentrations) accumulate in both α,α′-D and n-propanol-treated cultures, and are identical in structure to the early products of lithocholate metabolism by *Pseudomonas* sp. 10590 (31). These intermediates are degraded to C_{22} derivatives (bisnor acids) which accumulate mainly in α,α′-D cultures. Interestingly, the Δ^{14} bisnor acid predominates over its Δ^4 homologue (5%:2.2%) and indicates that partial side-chain removal results in the formation of a more effective substrate for the Δ′-nuclear dehydrogenase.

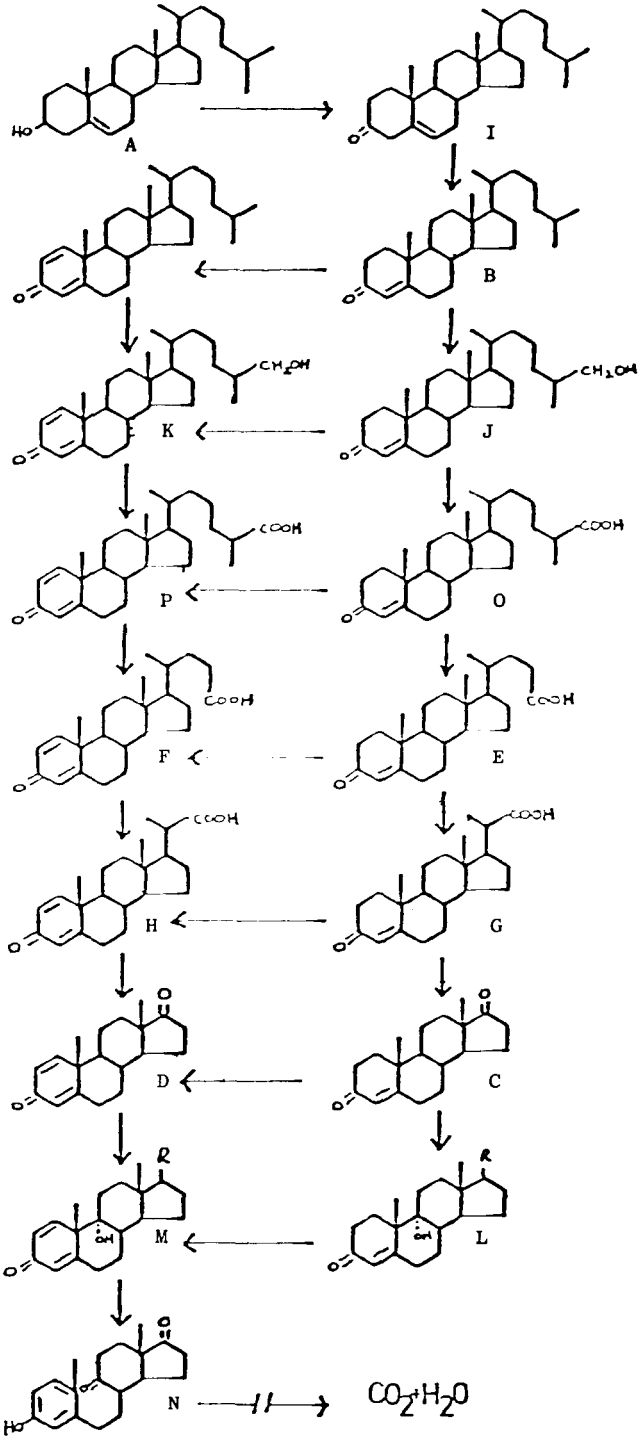
The final reaction of side-chain cleavage results in the formation of C_{19} ketosteroids with ADD predominating. In a study of androstane degradation, Dodson and Muir (32) demonstrated 9α-hydroxylation of AD and ADD. More recently, Wovcha et al. (15) have isolated 9α-OH ADD as a product of β-sitosterol metabolism in *Mycobacterium fortuitum*. We have been able to demonstrate the accumulation of small amounts of the 9α-hydroxy derivatives of AD and ADD in n-propanol cultures. Loss of steroid structure is seen with the accumulation of trace quantities of the 9,10-secosteroid (metabolite N). The 9α-hydroxy steroids probably give rise to the secophenol by a reverse aldol type reaction as proposed by Dodson

TABLE 2. Yield of cholesterol metabolites with respect to starting material

Inhibitor	Time	Metabolite	Yield
	hr		%
None	48	cholest-4-en-3-one (B)	36
α,α′-dipyridyl	72	cholest-4-en-3-one (B)	17.0
		androst-4-en-3,17-dione (C)	1.0
		androsta-1,4-dien-3,17-dione (D)	5.0
		chol-4-en-3-one-24-oic acid (E)	3.0
		chola-1,4-dien-3-one-24-oic acid (F)	0.7
		pregn-4-en-3-one-20-carboxylic acid (G)	2.2
		pregna-1,4-dien-3-one-20-carboxylic acid (H)	
n-propanol	72	cholest-4-en-3-one (B)	46.3
		cholest-5-en-3-one (I)	3.9
		26-hydroxycholest-4-en-3-one (J)	2.1
		26-hydroxycholesta-1,4-dien-3-one (K)	0.4
		androsta-1,4-dien-3,17-dione (D)	0.4
		9α-hydroxyandrost-4-en-3,17-dione (L)	0.5
		9α-hydroxyandrosta-1,4-dien-3,17-dione (M)	0.3
		3α-hydroxy-9,10-seco-1,3,5(10)androstatrien-9,17-dione (N)	0.15
		cholest-4-en-3-one-26-oic acid (O)	2.1
		cholesta-1,4-dien-3-one-26-oic acid (P)	0.4
		chol-4-en-3-one-24-oic acid (E)	0.8
		pregna-1,4-dien-3-one-20-carboxylic acid (H)	0.4

and Muir (32). Further degradation of secophenols has been described in detail by Miclo and Germain (33). It has also been demonstrated that loss of the steroid nucleus can occur via oxidative attack at C_4 to yield cholest-4-en-3-one-4-ol and ultimately Windaus oxo-acids (34). We have been unable to detect such intermediates but, in the absence of studies with [4-^{14}C] and [26-^{14}C]labeled cholesterol, we cannot fully discount this pathway.

On the basis of our initial experiments with cholesterol as substrate (**Table 2**) and subsequent feeding ex-



Scheme 1. Proposed pathway of cholesterol (A) degradation by *Pseudomonas* sp. NCIB 10590 under aerobic conditions. Compounds B–N were isolated during this study.

periments with selected intermediates in the presence of α,α'-D and n-propanol (Table 1), we wish to propose the following pathway (Scheme 1) for the biodegradation of cholesterol by *Pseudomonas* sp. 10590.

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