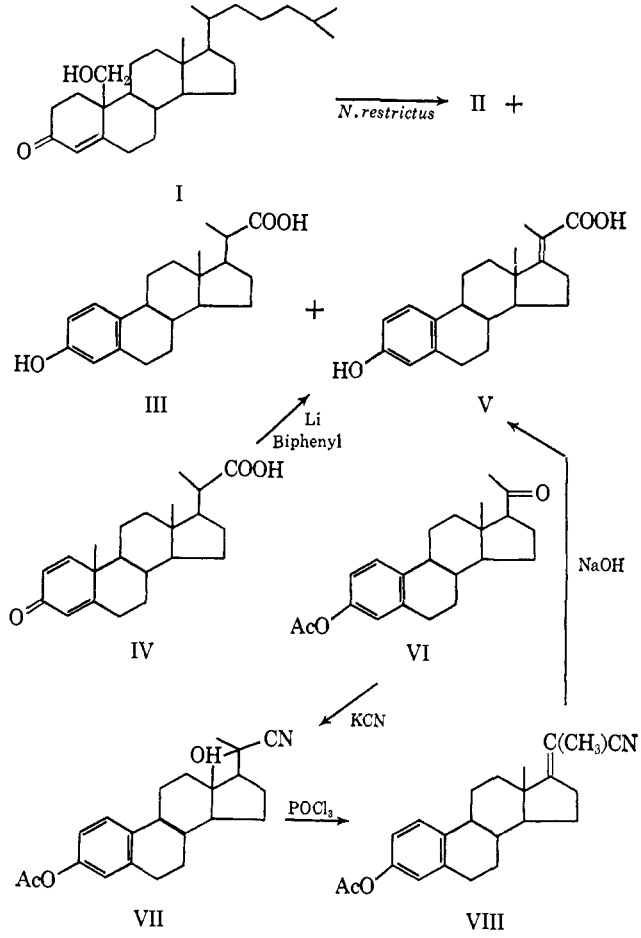
C_{22} Acid Intermediates in the Microbiological Cleavage of the Cholesterol Side Chain

Sir:

As early as 1913, Sohngen[1] disclosed that species of *Mycobacteria* were capable of growing in a medium containing cholesterol as the only carbon source. Since then, several conflicting reports have appeared on the mode of microbial degradation of the cholesterol side chain. Horvath and Kramli[2] claimed to have obtained methylheptanone after exposure of cholesterol to *Azotobacter sp.;* this indicated that a fission of the C-17–C-20 bond had occurred. Turfitt[3] reported the isolation of 3-oxoetiochol-4-enoic acid, isocaproic acid, and A-nor-3,5-secocholestan-5-on-3-oic acid from a prolonged large-scale fermentation of cholest-4-en-3-one with *Proactinomyces erythropolis*, which suggested that the mode of side-chain cleavage proceeded *via* a fission of the C-20–C-22 bond, in a manner analogous to that of the mammalian system.[4] On the other hand, Whitmarsh[5] found that a *Nocardia* soil isolate in the presence of 8-hydroxyquinoline converted cholesterol in low yields to 3-oxobisnorchol-4-en-22-oic acid, androst-4-ene-3,17-dione, and androsta-1,4-diene-3,17-dione. From these reports, one cannot ascertain the correct mechanism of microbial degradation of the cholesterol side chain. This communication is concerned with the identification of C_{22} acids as key intermediates in the breakdown of the hydrocarbon side chain of cholesterol and the mode of their conversion into 17-keto steroids.

Previous publications have shown that 19-oxygenated derivatives of cholesterol could be efficiently transformed by microorganisms into 17-keto steroids.[6,7] By shortening the incubation period to 48 hr, 19-hydroxycholest-4-en-3-one (I) was converted by *Nocardia restrictus* (ATCC 14887) into two new acidic products, besides estrone (II). The first product, mp 216–218°, was assigned the structure, 3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid (III) on the basis of the following: mol wt (mass spectrum) 328; λ_{max}^{alc} 274 mμ (ε 2560); λ_{max}^{Nujol} 3.04, 5.87, 6.21, and 6.67 μ; its nmr[8] spectrum exhibited bands at τ 9.33 (3 H, singlet, CH_3 at C-18), 8.87 (3 H, doublet, J = 7 cps, CH_3 at C-21), and 3.58 (singlet), 3.51 (doublet, J = 9 cps), and 2.99 (doublet, J = 9 cps) (3 H, aromatic protons). The proposed structure was further confirmed by synthesis. Reaction of bisnorchola-1,4-dien-3-on-22-oic acid (IV) by Dryden's method[9] afforded III, mp 216–218°, identical with a sample obtained by fermentation.

The second product was V, mp 241–243°; mol wt (mass spectrum), 326; $[\alpha]^{26}D$ +28° (dioxane); λ_{max}^{alc} 280 mμ (ε 2300) and 225 mμ (ε 19,500); λ_{max}^{Nujol} 2.98, 5.99, 6.21, 6.30, and 6.67 μ; the nmr spectrum showed bands at τ 9.12 (3 H, singlet, CH_3 at C-18), 8.11 (3 H, singlet, CH_3 at C-21), and 3.57 (singlet), 3.44 (doublet, J = 9 cps), and 2.97 (doublet, J = 9 cps) (3 H, aromatic protons). These physical data are consistent with the structure 3-hydroxy-19-norbisnorchola-1,3,5(10),17-(20)-tetraen-22-oic acid (V). This assignment was confirmed by synthesis. Treatment of 3-acetoxy-19-norpregna-1,3,5(10)-trien-20-one (VI) with KCN afforded the cyanohydrin VII, which on dehydration with $POCl_3$ gave 3-acetoxy-19-norpregna-1,3,5(10),17-(20)-tetraen-20-carbonitrile (VIII), mp 180–182°; $[\alpha]^{26}D$ +30° ($CHCl_3$); λ_{max}^{alc} 219 mμ (ε 26,000); $\lambda_{max}^{CHCl_3}$ 4.52, 5.72, 6.13, 6.21, and 6.70 μ. Alkaline hydrolysis of VIII yielded V,[10] mp 240.5–242° (identical with the sample from fermentation with respect to mixture melting point and infrared spectrum).



Although III was very poorly metabolized into estrone[11] even after prolonged incubation with *N. restrictus* and *Nocardia sp.* (ATCC 19170),[12] 3-oxobisnorchol-4-en-22-oic acid and 3-oxo-6,19-oxidobisnorchol-4-en-22-oic acid were efficiently converted into androst-4-ene-3,17-dione and 6,19-oxidoandrost-4-ene-3,17-dione, respectively, by these organisms.

In order to determine the metabolic fate of the three-carbon side chain, 3-hydroxybisnorchol-17(20)-en-22-

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(2) J. Horvath and A. Kramli, *Nature*, 160, 639 (1947).
(3) G. E. Turfitt, *Biochem. J.*, 42, 376 (1948).
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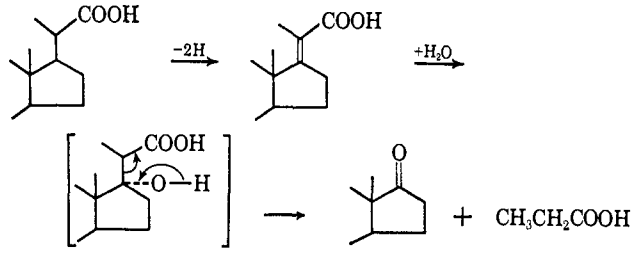
(5) J. M. Whitmarsh, *Biochem. J.*, 90, 23P (1964).
(6) C. J. Sih and K. C. Wang, *J. Am. Chem. Soc.*, 87, 1387 (1965).
(7) C. J. Sih, S. S. Lee, Y. Y. Tsong, K. C. Wang, and F. N. Chang, *ibid.*, 87, 2765 (1965).
(8) Nuclear magnetic resonance spectra were determined on a Varian Associates recording spectrometer (A-60) at 60 Mc in deuterated dimethyl sulfoxide. Chemical shifts are reported in τ values (parts per million) [G.V.D. Tiers, *J. Phys. Chem.*, 62, 1151 (1958)].
(9) H. L. Dryden, Jr., G. M. Webber, and J. J. Wieczorek, *J. Am. Chem. Soc.*, 86, 742 (1964).
(10) The designation of V in the diagram is preferred since the carboxyl group is larger than the methyl and the drastic hydrolysis conditions used (175° in 2-hydroxyethyl ether) would be expected to afford the most stable configuration of V.
(11) This anomaly could be reconciled by the fact that 3-hydroxycholesta-1,3,5(10)-triene was not metabolized by these organisms under these conditions (see ref 7).
(12) This organism was formerly named CSD-10.

Table I. Propionate-^{14}C from 3-Hydroxybisnorchol-17(20)-en-22-oic Acid-22-^{14}C

S-Benzylisothiuronium propionate, mp 154–155° No. of recrystallization	Spec act., counts/min mmole
First	17,750
Second	19,050
Third	18,620
Distribution of ^{14}C in propionate	**Radioactivity, counts/min**
Sodium propionate	6500
Carbon 1	6260
Carbons 2 and 3 (as ethylamine)	0

oic acid-22-^{14}C was synthesized *via* a similar sequence of reactions as outlined, except 3β-acetoxy-5α-pregnan-20-one was used as the starting material. When it was exposed to washed cells of *Nocardia sp.* (ATCC 19170), a radioactive volatile acid, whose chromatographic behavior on a Celite column[13] was identical with that of propionic acid, was obtained. It was isolated as its S-benzylisothiuronium salt, mp 154–155°; its specific activity remained constant after three recrystallizations. Degradation of the propionic acid molecule[14] revealed that all the radioactivity resided in the carboxyl carbon of propionic acid (Table I).

The data reported herein clearly show that the degradation of the hydrocarbon side chain of cholesterol proceeds *via* C_{22} acid intermediates, which confirms the finding of Whitmarsh. Since 3-oxobisnorchol-4-en-22-oic acid and 3-oxobisnorchol-17(20)-en-22-oic acid could be converted into androst-4-ene-3,17-dione by these microorganisms under *anaerobic* conditions, one may envisage the degradation of the three-carbon side chain involving dehydrogenation, hydration, and aldolytic fission.[15]



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The Mechanism of Microbial Conversion of Cholesterol into 17-Keto Steroids

Sir:

In the previous communication, we established the participation of C_{22} acid intermediates in the microbiological transformation of cholesterol into 17-keto steroids. We herein report the reactions leading to the formation of C_{22} acid intermediates from cholesterol (C_{27}), thus completing the degradative sequence of the hydrocarbon side chain.

Exposure of cholesterol-26,27-^{14}C to cells of *Nocardia restrictus* (ATCC 14887) resulted in the formation of a radioactive volatile acid. Its chromatographic behavior on a Celite column[2] was identical with that of propionic acid. The product was identified by admixture with nonisotopic propionic acid and crystallized as its S-benzylisothiuronium salt, mp 153–155°; the specific activity remained essentially constant after three recrystallizations. Degradation of the propionic acid[3] revealed that carbons 1 and 3 of the molecule contained all of the radioactivity in a ratio of 1:1 (Table I). This further substantiates that the radioactive propionic acid is derived from the terminal isopropyl portion of the hydrocarbon side chain.

Table I. Propionate-^{14}C from Cholesterol-26,27-^{14}C

S-Benzylisothiuronium propionate, mp 153–155° No. of recrystallization	Specific activity, counts/min mmole
First	33,450
Second	29,750
Third	29,800
Distribution of ^{14}C in propionate	**Radioactivity, counts/min**
Sodium propionate	2260
Carbon 1	1078
Carbon 2	24
Carbon 3	710

To demonstrate the formation of the steroidal counterpart (C-24 acid), radioactive cholesterol-4-^{14}C was incubated with cells of *N. restrictus*, in the presence of 10^{-3} *M* *o*-phenanthroline[4] and 3-oxochol-4-en-24-oic acid as carrier. After incubation for 4 hr, the radioactive products were isolated by paper chromatography.[5] This system gives a complete separation of cholesterol (R_f 0.9) from 3-oxochol-4-en-22-oic acid (R_f 0.44). The radioactive acid was combined with nonisotopic 3-oxochol-4-en-24-oic acid, and the specific activity after three crystallizations remained stable (90 counts/min mg). These results are all consistent with the formation of a C_{24} acid intermediate *via* fission of the C-24–C-25 bond, in a manner analogous to the conversion of cholesterol into bile acids in mammals.[6] To follow the metabolic fate of the C_{24} acid, lithocholic acid-24-^{14}C was exposed to cells of *N. restrictus*. In this case, a radioactive volatile acid having chromatographic behavior[2] similar to acetic acid was obtained. By admixture with nonisotopic acetic acid it was crystallized as its S-benzylisothiuronium salt, mp 138–141.5°, whose specific activity remained unchanged after several

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