Mechanisms of Steroid Oxidation by Microorganisms. XIV. Pathway of Cholesterol Side-Chain Degradation*

Charles J. Sih, H. H. Tai, Y. Y. Tsong, S. S. Lee,† and R. G. Coombe‡

ABSTRACT: Cells of *Nocardia restrictus* are capable of cleaving the side chain of cholesterol to yield propionic acid and C_{24} acid. Carrier experiments revealed the incorporation of 6% of the cholesterol-26,27-^{14}C isotope into propionic acid, which was characterized by preparation of *S*-benzylisothiuronium salt. Degradation of the isolated propionic acid demonstrated that only C-1 and -3 contained ^{14}C. Incubation of cholesterol-4-^{14}C in the presence of 3-oxochol-4-enic acid and *o*-phenanthroline permitted the recovery of labeled 3-oxochola-1,4-dienic acid. When lithocholic acid-24-^{14}C was incubated with *N. restrictus* with acetate as carrier, 10% of the substrate isotope was introduced into acetic acid. Exposure of 3-oxo-6,19-oxidochol-4-en-24-oic acid to microorganisms of the genus *Nocardia* yielded 3-oxo-9α-hydroxy-6,19-oxidochol-4-en-22-oic acid and 3-oxo-6,19-oxidoandrost-4-ene-3,17-dione. When 25ϵ-hydroxycholesterol (I), 25-keto-26-norcholesterol acetate (II), 3β,20ϵ-dihydroxy-5-cholenic acid γ-lactone (III), 24-keto-26-norcholesterol (IV), or 27-norcholesterol (V) was incubated with the microorganisms, the A ring of these compounds was degraded but there was no detectable metabolism of the side chains. The results of the present investigation favors the conclusion that the hydrocarbon side chain of cholesterol is degraded *via* conventional fatty acid oxidation to C_{22} acids, involving carbon–carbon bond fission at C-24–C-25 and C-22–C-23, resulting in the formation of 1 mole of propionic acid and 1 mole of acetic acid.

In studies reported in the previous paper (Sih *et al.*, 1968), it has been shown that enzyme systems from microorganisms of the genus *Nocardia* are capable of cleaving the side chain of cholesterol to yield C_{22} acids, which are in turn transformed into 17-keto steroids and propionic acid. This mode of side-chain cleavage appears to resemble the observation of Kautsky *et al.* (1958), who isolated isovaleric acid-1-^{14}C from the steam-volatile fatty acid fraction of liver after oral administration of 22-ketocholesterol-23-^{14}C to normal guinea pigs. In this paper, the reaction sequence leading to the formation of C_{22} acid intermediates from cholesterol (C_{27}) are reported, thus completing the degradative sequence of the hydrocarbon side chain.

A number of cholesterol analogs, derived from the chemical oxidation of cholesterol, are readily available. These include 25ϵ-hydroxycholesterol (I), 25-keto-26-norcholesterol acetate (II), 3β,20ϵ-dihydroxy-5-cholenic acid γ-lactone (III), 24-keto-26-norcholesterol (IV), and 27-norcholesterol (V). If the microbial and chemical degradation follow a common course, exposure of these compounds to suitable microorganisms should give important information toward defining the microbiological degradative sequence.

When 25ϵ-hydroxycholesterol (I) was incubated with *Nocardia restrictus*, *Nocardia opaca*, and *Mycobacterium sp.*, an acidic product accumulated. From a large-scale fermentation with *N. opaca*, this noncrystallizable acid (VI) was isolated. Its infrared spectrum showed bands at 2.95 (hydroxyl), 5.80 (carboxyl carbonyl), and 5.85 μ (six-membered ring ketone). The infrared spectrum of the potassium salt of VI exhibited peaks at 5.85 μ (ring carbonyl) and the band at 5.80 μ has now been shifted to 6.37 μ (COO^-). The nuclear magnetic resonance spectrum (Figure 1) of VI revealed bands at τ 9.06 (3 H, doublet, J = 6 cycles/sec, one secondary CH_3), 9.01 (3 H, singlet, one tertiary CH_3), and 8.80 (6 H, singlet, two tertiary CH_3's). These data suggest that ring A of I had been degraded since the signal corresponding to the angular methyl at C-10 was absent in the nuclear magnetic resonance spectrum of VI. Treatment of VI with acetic anhydride and sodium acetate afforded an enol lactone acetate (VII); its infrared spectrum showed peaks at 5.68 (lactone carbonyl), 5.77 (acetoxy carbonyl), 5.96 (double bond), and 8.00 μ (acetate, CO). Its nuclear magnetic resonance spectrum (Figure 2) exhibited bands at τ 9.22 (3 H, singlet, one tertiary CH_3), 9.06 (3 H, doublet, J = 6 cycles/sec, one secondary CH_3), 8.62 (6 H, singlet, two tertiary CH_3's), 8.11 (3 H, singlet CH_3 of acetate), and 4.88 (1 H, multiplet vinylic proton). The prolonged heating in acetic anhydride and sodium acetate apparently resulted in the acetylation of the tertiary hydroxyl group. This is substantiated by the

* From the School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706. *Received August 14, 1967.* This investigation was supported by Research Grants AM-04874 and AM-06110 of the National Institutes of Health and GB-1093 of the National Science Foundation.

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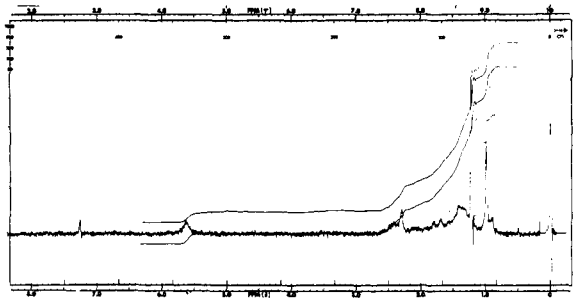


FIGURE 1: The nuclear magnetic resonance spectrum of 7aβ-methyl-1β-[1′-methyl-5′-hydroxyisohexyl]-5-oxo-3aα-hexahydro-4-indanpropionic acid (VI).

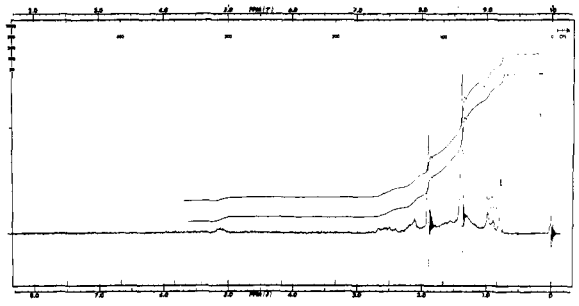
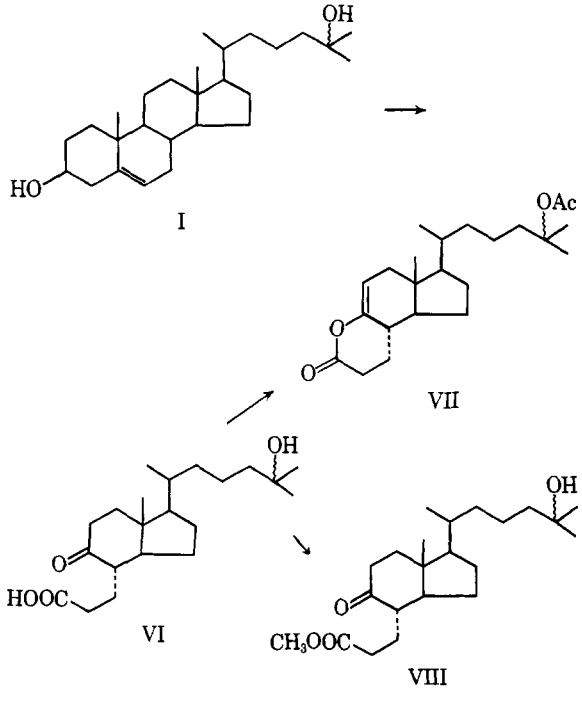


FIGURE 2: The nuclear magnetic resonance spectrum of 7aβ-methyl-1β-[1′-methyl-5′-acetoxyisohexyl]-5-hydroxy-$\Delta^{5(6)}$-3aα-tetrahydro-4-indanpropionic acid δ-lactone (VII).

disappearance of the band at 2.95 μ in the infrared spectrum of VII and the appearance of the band at τ 8.11 (3 H) in the nuclear magnetic resonance spectrum, corresponding to the acetate protons. Reaction of VI with diazomethane gave the methyl ester (VIII) which unfortunately was also noncrystallizable. Its infrared spectrum showed peaks at 2.80 (hydroxyl), 5.76 (ester carbonyl), 5.85 (ring carbonyl), 8.33 μ (ester, CO). Its nuclear magnetic resonance spectrum (Figure 3) showed peaks at τ 9.05 (3 H, doublet, J = 6 cycles/sec, one secondary methyl), 9.00 (3 H, singlet, one tertiary methyl), 8.84 (6 H, singlet, two tertiary methyls), and 6.42 (3 H, singlet, methyl of methoxy ester). On the basis of these data, products VI–VIII were assigned the structures shown in Scheme I.

SCHEME I



When 25-keto-26-norcholesterol acetate (II) was exposed to the aforementioned microorganisms, an acidic product (IX) accumulated, which again was not obtainable in a crystalline state. Its infrared spectrum showed absorption peaks at 5.80 (carboxyl carbonyl) and 5.85 μ (carbonyl); its nuclear magnetic resonance spectrum (Figure 4) showed bands at τ 9.03 (3 H, doublet, J = 6 cycles/sec, one secondary methyl), 9.00 (3 H, singlet, one tertiary methyl), 7.94 (3 H, singlet CH_3 of methyl ketone), and 0.15 (1 H, singlet COOH). Since the compound had no ultraviolet absorption and the signal corresponding to the tertiary methyl at C-10 was absent in its nuclear magnetic resonance spectrum, it was strongly suspected that the A ring again had been degraded. In a similar manner, IX was characterized by refluxing with acetic anhydride and sodium acetate to yield a crystalline enol lactone (X), mp 60.5–62°; its infrared spectrum showed absorption bands at 5.71 (lactone carbonyl), 5.85 (carbonyl), and 5.96 μ (double bond); its nuclear magnetic resonance spectrum (Figure 5) gave signals at τ 9.22 (3 H, singlet, one tertiary methyl), 9.05 (3 H, doublet, J = 6 cycles/sec, one secondary methyl), 7.94 (3 H, singlet, CH_3 of methyl ketone), and 4.88 (1 H,

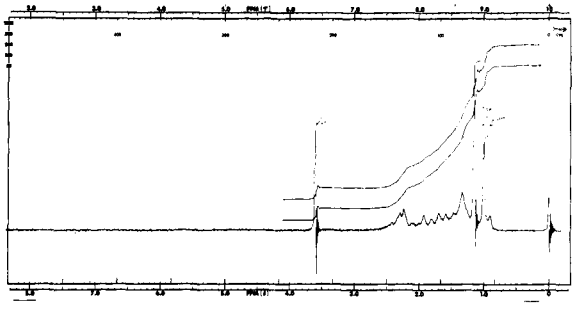


FIGURE 3: The nuclear magnetic resonance spectrum of 7aβ-methyl-1β-[1′-methyl-5′-hydroxyisohexyl]-5-oxo-3aα-hexahydro-4-indanpropionic acid methyl ester (VIII).

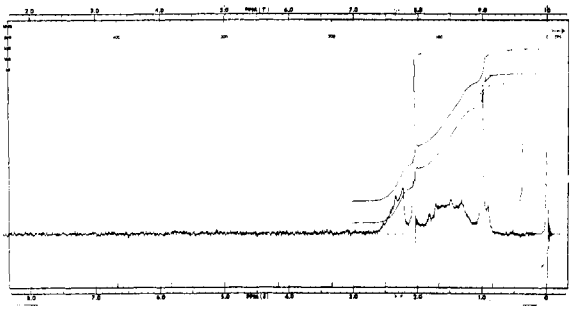


FIGURE 4: The nuclear magnetic resonance spectrum of 7aβ-methyl-1β-[1'-methyl-4'-oxopentyl]-5-oxo-3aα-hexahydro-4-indanpropionic acid (IX).

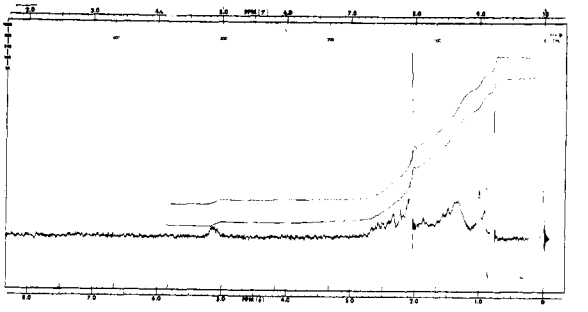
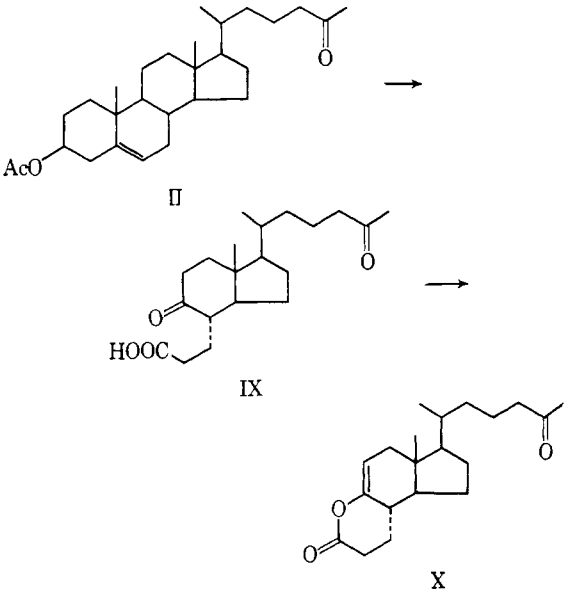


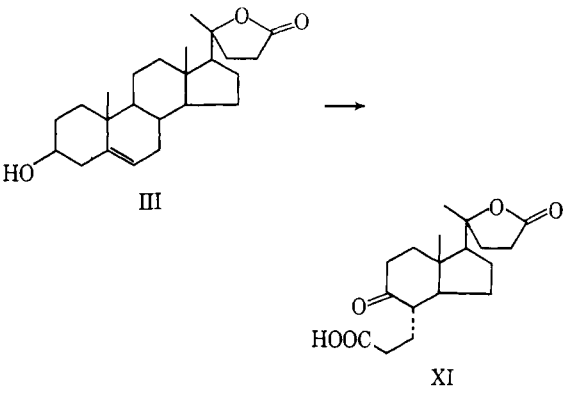
FIGURE 5: The nuclear magnetic resonance spectrum of 7aβ-methyl-1β-[1'-methyl-4'-oxopentyl]-5-hydroxy-$\Delta^{5(6)}$-3aα-tetrahydro-4-indanpropionic acid δ-lactone (X).

multiplet, vinylic proton). These results strongly support the following structures for IX and X.



Exposure of 3β,20ϵ-dihydroxy-5-cholenic acid γ-lactone (III) to *N. restrictus* resulted in the formation of a crystalline acid (XI), mp 142–143°; carbon–hydrogen analysis afforded values in good agreement with the empirical formula, $C_{18}H_{26}O_5$; its infrared spectrum showed absorption peaks at 5.68 (lactone carbonyl), 5.78 (carboxyl carbonyl), and 5.85 μ (six-membered ring carbonyl); its nuclear magnetic resonance spectrum (Figure 6) exhibited signals at τ 8.84 (3 H, singlet, one tertiary methyl) and 8.51 (3 H, singlet, one tertiary methyl). From these physical data, XI was assigned the chemical formula 7aβ-methyl-1β-[1'-methyl-1'-hydroxy-4'-butyric acid γ-lactone]-3aα-hexahydro-4-indanpropionic acid (XI).

It was found that both 24-keto-26-norcholesterol (IV) and 27-norcholesterol (V) were metabolized at an exceedingly slow rate when incubated with the aforementioned microorganisms and no apparent metabolic products were detectable. Since fermentative studies failed to provide pertinent information concerning the initial degradative reactions of the hydrocarbon side chain, it appeared that perhaps the problem could be best approached by radioactive tracer experiments.



Incubation of cholesterol-26,27-^{14}C with carrier propionic acid and *N. restrictus* resulted in a recovery of approximately 7% of the isotope in the steam-distillable fractions, which after column chromatography on Celite (535) (Swim and Utter, 1957) (Figure 7) or buffered silicic acid column (Moyle *et al.*, 1948) (Figure 8), yielded labeled propionic acid. Approximately 86% of the radioactivity present ini-

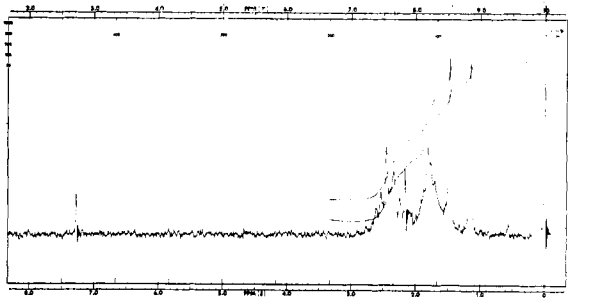


FIGURE 6: The nuclear magnetic resonance spectrum of 7aβ-methyl-1β-[1'-methyl-1'-hydroxy-4'-butyric acid γ-lactone]-5-oxo-3aα-hexahydro-4-indanpropionic acid (XI).

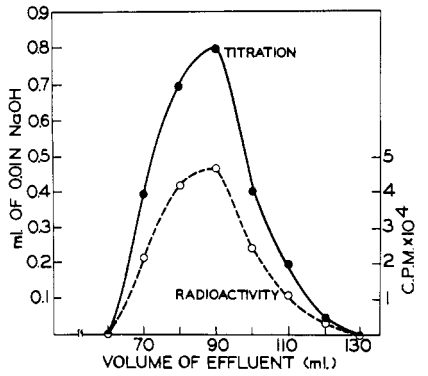


FIGURE 7: Elution profile of the steam-distillable fractions from incubations with carrier propionate and cholesterol-26,27-^{14}C on Celite (535) column (Swim and Utter, 1957).

tially in the steam distillate was recovered in the propionic acid peak. The product was identified by admixture with nonisotopic propionic acid and crystallized as its *S*-benzylisothiuronium salt (mp 153–155°), whose specific activity remained essentially constant after three recrystallizations. Degradation of the isolated propionic acid according to Phares (1951) yielded carbon dioxide and ethylamine; the amine was oxidized with permanganate, and the resulting acetic acid was submitted to the same degradative procedures. The results revealed that carbons 1 and 3 of the molecule contained all of the radioactivity in a ratio of 1:1 (Sih *et al.*, 1967). The small discrepancy in the values can be ascribed to experimental error. This further confirms that the radioactive propionic acid is indeed derived from the terminal isopropyl portion of the hydrocarbon side chain.

When either cholesterol-26-^{14}C or cholesterol-4-^{14}C was incubated with *N. restrictus*, it was found that the radioactivity disappeared readily without the apparent accumulation of any radioactive intermediates. In

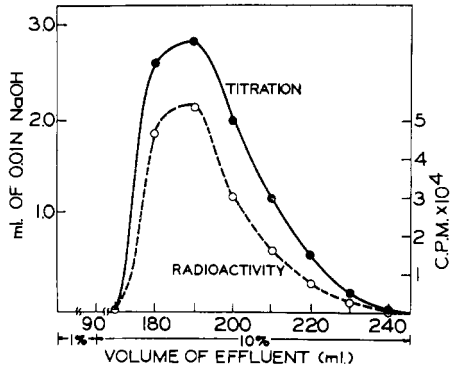


FIGURE 8: Partition chromatography of the steam-distillable fractions from incubations with carrier propionate and cholesterol-26,27-^{14}C on buffered silica gel column (Moyle *et al.*, 1948).

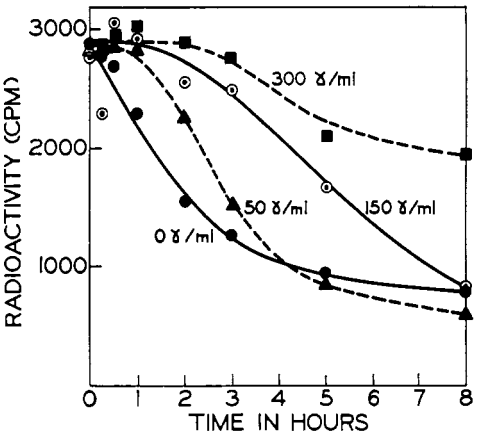


FIGURE 9: Effect of 3-oxochol-4-enic acid on the rate of metabolism of cholesterol-26,27-^{14}C. *N. restrictus* was grown in the mineral salts medium for 4 days. A 10% transfer was used to inoculate 25 ml of Difco nutrient broth. After 22 hr, 0.3 μc of cholesterol-26,27-^{14}C was added to all the flasks and 3-oxochol-4-enic acid was introduced as follows: (●—●) none, (▲—▲—▲) 50μg/ml, (⊙—⊙—⊙) 150 μg/ml, and (■—■—■) 300 μg/ml. At the specified intervals, 1 ml of reaction mixture was extracted with 2 ml of ethyl acetate. The organic phase (0.3 ml) was assayed in the scintillation counter.

order to trap the initial degradative intermediates, an experiment was conducted using cholesterol-26-^{14}C in the presence of varying concentrations of 3-oxochol-4-enic acid as carrier. Figure 9 shows that the rate of metabolism of cholesterol-26-^{14}C by *N. restrictus* can be substantially reduced by the addition of the presumed intermediate, 3-oxochol-4-enic acid, which is preferentially metabolized at a considerably faster rate. The Japanese workers (Noda Institute, 1965) reported that cholesterol could be efficiently transformed into androsta-1,4-diene-3,17-dione by microorganisms when inhibitors such as α,α′-dipyridyl or *o*-phenanthroline were added to the medium. Their observation suggested that these metal chelators apparently selectively interfered with ring cleavage enzyme systems but they have no effect on side-chain cleavage enzymes. Thus, *a priori*, one would predict that *o*-phenanthroline should inhibit the oxidation of cholesterol-4-^{14}C but should have no effect on the rate of disappearance of cholesterol-26-^{14}C. This proposition was indeed proved to be correct by the results which are shown in Figure 10. Being cognizant of the optimal conditions for the metabolism of the radioactive cholesterol substrates, a larger scale experiment was devised to demonstrate the formation of the steroidal counterpart (C_{24} acid). When *N. restrictus* was incubated with cholesterol-4-^{14}C in the presence of 3-oxochol-4-enic acid (50 μg/ml) and *o*-phenanthroline (10^{-3} M), four radioactive peaks were detected on the paper chromatographic strip (Figure 11). Peaks I and II were eluted from the paper, admixed with presumed intermediates, and recrystallized. It was observed that with peak I, the

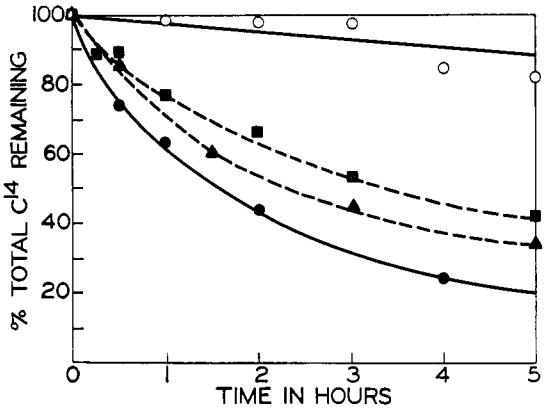


FIGURE 10: Effect of *o*-phenanthroline on the metabolism of cholesterol-4-^{14}C and cholesterol-26,27-^{14}C. The conditions used were the same as those described in Figure 10. *o*-Phenanthroline concentration was 10^{-3} M. (○) Cholesterol-4-^{14}C with *o*-phenanthroline added, (■) cholesterol-26-^{14}C with *o*-phenanthroline added, (●) cholesterol-4-^{14}C, and (▲) cholesterol-26-^{14}C.

specific activity did not remain constant with the following compounds: 3-oxobisnorchol-4-en-22-oic acid, 3-oxobisnorchola-1,4-dien-22-oic acid, 3-oxochol-4-enic acid, and 3-oxochola-1,4-dienic acid. Since the mobility of peak I corresponds very closely to 3-oxobisnorchola-1,4-dien-22-oic acid, it is likely that peak I is probably 3-oxobisnorchola-1,4,17(20)-trien-22-oic acid. Since the mobility of peak II corresponds to 3-oxochola-1,4-dienic acid, it was admixed with it and recrystallized. Table I shows that the specific activity remained essentially the same after three recrystallizations. Since radioactive peaks III and IV are less polar products, it would not be unreasonable to assume that peak III may correspond to cholesta-1,4-dien-3-on-26-oic acid and peak IV to cholesta-1,4-dien-3-on-26-ol. These results are all consistent with the formation of a C_{24} acid intermediate *via* fission of the C-24–C-25 bond.

In order to follow the metabolic fate of the C_{24} acid, lithocholic acid-24-^{14}C was incubated with carrier acetic acid and cells of *N. restrictus*. Approximately 11% of the isotope was recovered in the steam-distillable fractions, which after column chromatography on Celite 535 (Figure 12) (Swim and Utter, 1957) yielded labeled acetic acid. Approximately 98% of the radioactivity, present initially in the steam distillate, was recovered in the acetic acid peak. 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 recrystallizations. Schmidt degradation (Phares, 1951) of the molecule showed that the radioactivity resided exclusively in the carboxyl carbon (Sih *et al.*, 1967). This result indicates that the side chain of the C_{24} acid is cleaved between carbons 22 and 23, yielding acetic acid and a C_{22} acid.

When chol-5-enic acid was exposed to either *N. restrictus* or *Nocardia sp.* (ATCC 19170) under the conventional conditions, no significant quantities of products accumulated although both organisms were capable of metabolizing it readily. Thus, 3-oxo-6,19-oxidochol-4-en-24-oic acid (XVI) (Scheme II) was prepared with the view to block 1,2-dehydrogenation, a key reaction in the rupture of the steroid skeleton,

SCHEME II

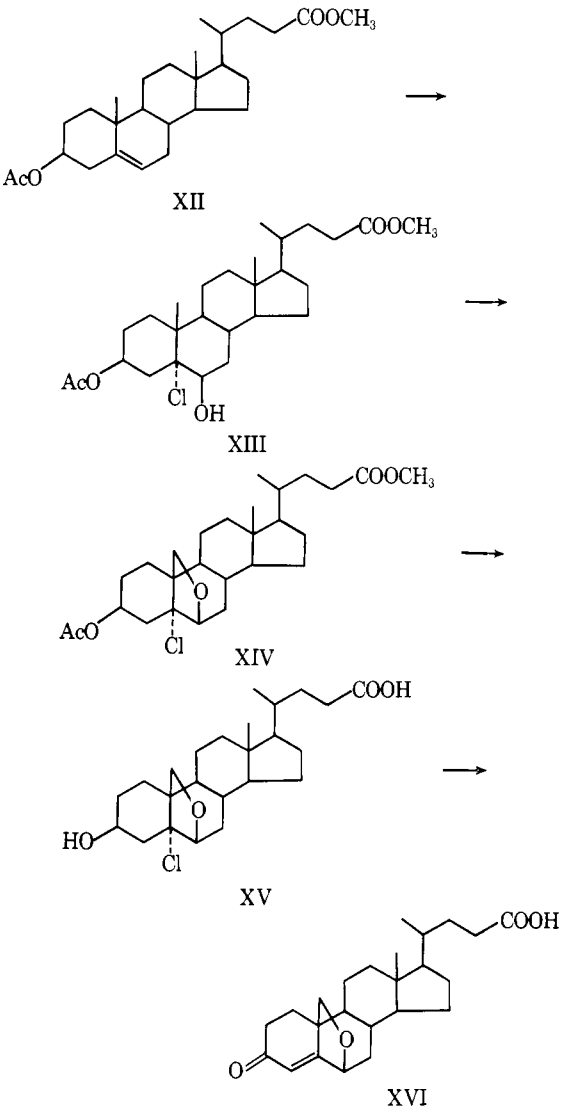


TABLE I: 3-Oxochola-1,4-dienic Acid-^{14}C from Cholesterol-4-^{14}C.

Recrystzn	Sp Act. (cpm/mg)
First	845
Second	801
Third	875

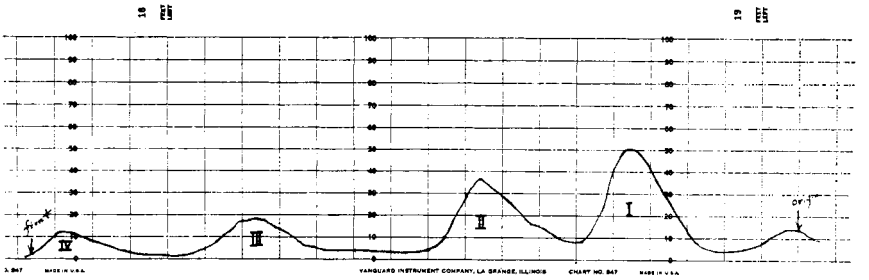
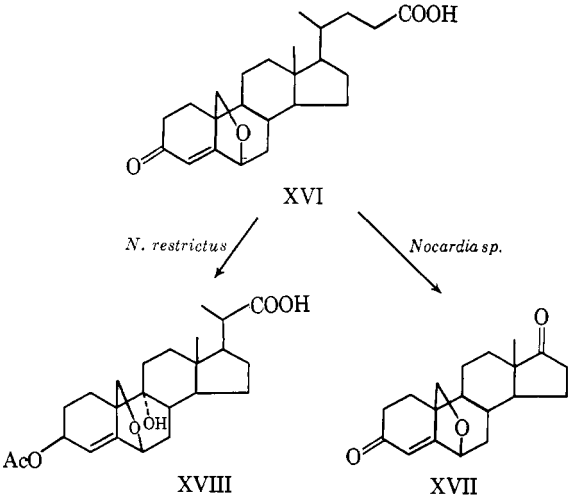


FIGURE 11: Isotope distribution on paper chromatograms of ethyl acetate soluble materials from 5.5-hr incubations with cholesterol-26,27-^{14}C.

so that the side chain would be selectively attacked, which should result in the accumulation of 17-keto steroids and possibly other intermediates. The following sequence of reactions was used for the synthesis of 6,19-oxidochol-4-en-24-oic acid (XVI). Reaction of 3β-acetoxychol-5-en-24-oic acid methyl ester (XII) with bleaching powder afforded the chlorohydrin (XIII) which was converted into 3β-acetoxy-5α-chloro-6,19-oxidocholan-24-oic acid methyl ester (XIV), *via* the hypoiodite reaction (Kalvoda *et al.*, 1963). Basic hydrolysis, followed by oxidation of the 3β-hydroxyl group with pyridine–chromic acid complex, afforded the desired 6,19-oxidochol-4-en-24-oic acid (XVI) directly. The over-all yield from XII was approximately 20%.

When 3-oxo-6,19-oxidochol-4-en-24-oic acid (XVI) (200 mg) was incubated with *Nocardia sp.* (ATCC 19170), 6,19-oxidoandrost-4-ene-3,17-dione (XVII) (30 mg) was obtained. On the other hand, when XVI was exposed to *N. restrictus*, an acidic product (XVIII) was isolated (mp 231–234°); its infrared spectrum showed bands at 3.00 (hydroxyl), 5.82 (carboxyl), 5.90, and 6.06 μ (conjugated carbonyl); its ultraviolet spectrum showed a maximum at 241 mμ (ϵ 11,000); and its nmr spectrum gave signals at τ 4.11 (1 H, singlet, vinylic H at C-4), 5.39 (1 H, doublet, J = 5 cycles/sec, H at C-6), 5.93 (doublet), 6.60 (doublet) (2 H, J = 10 cycles/sec, CH_2O at C-19), 8.90 (3 H, doublet, J = 8 cycles/sec, 21-CH_3), and 9.26 (3 H, singlet, 18-CH_3). Molecular weight analysis (mass spectrum) gave 374 and carbon–hydrogen analysis afforded value in good agreement with $C_{22}H_{30}O_5$. Since XVIII remained unchanged after treatment with pyridine and acetic anhydride or chromic trioxide in acetic acid, the newly introduced hydroxyl group must occupy a tertiary position. On the basis of the deshielding effect, caused by this newly introduced hydroxyl group on the vinylic proton peak at C-4 (−0.16 ppm) (Tori and Kondo, 1964) and the fact that *N. restrictus* is a known 9α-hydroxylator (Sih, 1962), XVIII was tentatively assigned the structure 9α-hydroxy-6,19-oxidobisnorchol-4-en-3-on-22-oic acid.



Discussion

The formation of hexahydroindanepropionic acid derivatives from 25ϵ-hydroxycholesterol (I), 25-keto-26-norcholesterol acetate (II), and 3β,20ϵ-dihydroxychol-5-enic acid γ-lactone (III) by microorganisms can readily be envisaged *via* the same reaction sequence in the formation of 7aβ-methyl-1,5-dioxo-3aα-hexahydro-4-indanpropionic acid from androst-4-ene-3,17-

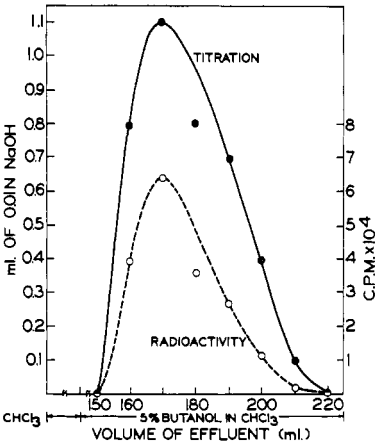
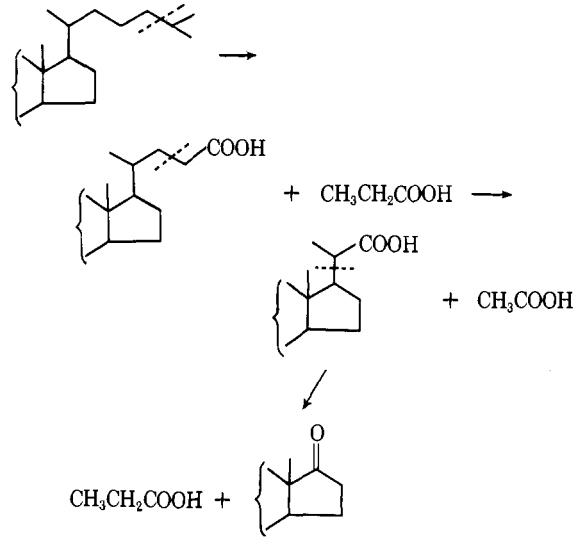


FIGURE 12: Partition chromatography of steam-distillable fractions from incubation with carrier acetate and lithocholic acid-24-^{14}C on Celite 535 column.

dione (Dodson and Muir, 1961; Sih *et al.*, 1966; Gibson *et al.*, 1966). Although this result gave no insight as to how the side chain of cholesterol is degraded initially, it strongly suggests that the enzymes responsible for side-chain degradation and ring cleavage do not follow a compulsory order but rather occur simultaneously and independently. Thus, introduction of blocking groups, which interfere with enzymatic reactions leading to ring cleavage, result in a selective attack on the hydrocarbon side chain with the eventual accumulation of 17-keto steroids. For example, 3-oxo-6,19-oxidocholest-4-ene could be efficiently transformed into 6,19-oxidoandrost-4-ene-3,17-dione by microorganisms (Sih *et al.*, 1965); apparently, the 6,19-oxido function interferes with $\Delta^{1,2}$ dehydrogenation, a reaction required for the rupture of steroid ring B. Conversely, compounds with modified hydrocarbon side chains, which cannot be handled by the side-chain cleavage enzymes, result in the accumulation of hexahydroindanpropionic acid derivatives by a selective attack on the ring. This is the case for substrates like 25ϵ-hydroxycholesterol (I), 25-keto-26-norcholesterol (II), and 3β,20ϵ-dihydroxychol-5-enic acid γ-lactone (III).

Intact cells of a *Mycobacterium* when exposed to cholesterol-4-^{14}C or cholesterol-26-^{14}C oxidized carbon 4 of the ring to carbon dioxide about four times as rapidly as carbon 26 of the side chain (Stadtman *et al.*, 1954). In other instances, it has been shown that radioactive $^{14}CO_2$ appeared more rapidly from cholesterol-26-^{14}C than cholesterol-4-^{14}C (Peterson and Davis, 1964). As the processes of side-chain breakdown and ring cleavage can proceed simultaneously and independently, these divergent reports can be easily attributed to a difference in experimental conditions, where the rates of metabolism of the side chain may be favored over those of ring cleavage or *vice versa*.

In the presence of chelating agents, such as *o*-phenanthroline and α,α'-dipyridyl, a wide variety of microorganisms were regarded to have the capacity for transforming cholesterol into androsta-1,4-diene-3,17-dione in high yields (Noda Institute, 1965). This finding clearly shows that these iron chelating agents inhibit ring cleavage enzyme or (s) without affecting enzymes responsible for side-chain metabolism. Since 9α-hydroxylation is the first reaction, involved in the further metabolism of androsta-1,4-diene-3,17-dione, it is reasonable to assume the functional role of these metal-binding agents as inhibitors of 9α-hydroxylase activity. The question, as to whether these inhibitors act directly upon the 9α-hydroxylase *per se* or whether they inhibit the biosynthesis of the enzyme, was resolved by the observation that induced cells of *N. restrictus* failed to convert androsta-1,4-diene-3,17-dione into 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione in the presence of 10^{-3} M *o*-phenanthroline. This observation indicates that *o*-phenanthroline inhibits 9α-hydroxylase activity directly. Horvath and Kramli (1947) claimed the isolation of methylheptanone after reaction of cholesterol with *Azotobacter sp.*, whereas Turfitt (1948) reported the isolation of isocaproic acid after exposure of cholesterol to *Proactinomyces erythropolis*. These findings would suggest that the cholesterol side chain is cleaved between C-17 and C-20 or C-20 and C-22, respectively. However, our results are completely inconsistent with either of these two modes of fission of the side chain but they appear to be consonant with the findings of Whitmarsh (1963). It was found that when cholesterol-26,27-^{14}C was incubated with *N. restrictus*, radioactive propionic acid was obtained; the isotope being equally distributed between carbons 1 and 3. Isotopically labeled 3-oxochola-1,4-dienic acid was isolated after exposure of cholesterol-4-^{14}C to the same microorganism. Furthermore, acetic acid-1-^{14}C was readily obtained after exposure of lithocholic acid-24-^{14}C to *N. restrictus* and 3-oxo-6,19-oxidochol-4-en-24-oic acid (XVI) was converted into 9α-hydroxy-6,19-oxidobisnorchol-4-en-3-on-22-oic acid (XVIII). Since the mechanism for the conversion of C_{22} acids into 17-keto steroids has been established in the previous paper (Sih *et al.*, 1968), the pathway of cholesterol side-chain degradation is completed. All the data support the view that the hydrocarbon side chain of cholesterol is degraded *via* conventional fatty acid oxidation, involving carbon–carbon bond fission at C-24–C-25, C-22–C-23, and C-17 and C-20, resulting in the formation of 2 moles of propionic acid and 1 mole of acetic acid.



This mode of formation of 17-keto steroids from cholesterol differs from that of the mammalian system (Shimizu *et al.*, 1962) which cleaves the C-20–C-22 bond, yielding isocaproic aldehyde and pregnenolone; subsequent breakage of the C-17–C-20 bond gives 17-keto steroids. However, the removal of the terminal isopropyl portion of the side chain appears to be analogous to the conversion of cholesterol into bile acids in mammals (Suld *et al.*, 1962).

Experimental Section

Materials. The following microorganisms were used in these studies: *N. restrictus* (ATCC 14887),

Nocardia sp. (ATCC 19170), *N. opaca*, *Mycobacterium sp.* (RTMP), and *Nocardia corallina*. The latter three organisms were kindly supplied by Dr. R. E. Kallio, University of Illinois, Urbana, Ill. All these microorganisms were maintained on nutrient agar slants, supplemented with 1% yeast extract and 1% glucose with the exception of *Nocardia sp.* (ATCC 19170) which was maintained as described previously (Sih *et al.*, 1968). All of the solvents and organic and inorganic chemicals were reagent grade. Petroleum ether refers to the fraction with a boiling point of 40–70°. Silicic acid (Mallinckrodt 2847) was used for column chromatography, and silica gel HF (E. Merck 7741) was used for thin-layer chromatography. Lithocholic acid-24-^{14}C (3 mc/mmole) was obtained from Tracerlab. Cholesterol-4-^{14}C (58 mc/mmole) and cholesterol-26-^{14}C (26.7 mc/mmole) were products of New England Nuclear Corp. 25-Oxo-26-norcholesterol acetate, 24-oxo-26-norcholesterol, 3β,20ϵ-dihydroxychol-5-enic acid γ-lactone, and 27-norcholesterol were kindly supplied by Dr. H. Herzog of the Schering Corporation. 25ϵ-Hydroxycholesterol was synthesized by the method of Ryer *et al.* (1950). Cholenic acid was kindly supplied by Dr. K. Heusler of Ciba Ltd., Basel.

Methods. Melting points, determined on a Thomas-Hoover melting point apparatus, are corrected. Ultraviolet absorption spectra were determined on a Cary Model 11 MS recording spectrophotometer and 95% ethanol was used as solvent. Infrared spectra were recorded on a Beckman IR5A double-beam infrared recording spectrophotometer. Microanalyses were carried out by Mr. J. Alicino of Metuchen, N. J. The nuclear magnetic resonance spectra were determined on a Varian Associates recording spectrometer (A60) at 60 Mcycles either in carbon tetrachloride, deuterated chloroform, or deuterated dimethyl sulfoxide with tetramethylsilane as an internal standard. Chemical shifts are reported in τ values (parts per million) (Tier, 1958). Values of $[\alpha]_D$ have been approximated to the nearest degree. Unless otherwise stated, small-scale fermentations were carried out in 250-ml erlenmeyer flasks, containing 50 ml of Difco nutrient broth with a 5% inoculum on rotary shakers (250 rpm, 1-in. stroke); large-scale fermentations were carried out in 2-l. erlenmeyer flasks, containing 400 ml of Difco nutrient broth. Incubation temperature was kept at 25°. Radioactivity was determined on a Packard scintillation counter (Model 524) which had a counting efficiency of 72% for ^{14}C.

Growth of N. restrictus Cells. N. restrictus was grown for 4 days in the mineral salts medium (Bushnell and Haas, 1941) containing 300 μg/ml of cholesterol. A 10% transfer was made into Difco nutrient broth containing 10 μg/ml of cholesterol. After 24 hr, the cells were harvested by centrifugation and used in the radioactive tracer experiments.

7aβ-Methyl-1β-[1'-methyl-5'-hydroxyisohexyl]-5-oxo-3aα-hexahydro-4-indanpropionic Acid (VI) from 25ϵ-Hydroxycholesterol (I). N. opaca, grown in Difco nutrient broth (720 ml) for 24 hr, was used as the inoculum for 36 2-l. erlenmeyer flasks, containing 14.4 l. of Difco nutrient broth. After 24-hr incubation on rotary shakers, 4.32 g of I dissolved in 144 ml of dimethylformamide was distributed equally among the flasks and the fermentation was continued for 96 hr. The culture broth was then acidified with glacial acetic acid to pH 2.0, and the cell debris was removed by filtration. The filtrate was extracted three times with three, 3-l. portions of chloroform. The combined chloroform extract was dried over sodium sulfate and evaporated to dryness under reduced pressure. The total residue (4.58 g) was dissolved in ml of an ethyl ether–benzene (1:1) mixture and extracted three times with three, 500-ml portions of 6% sodium bicarbonate. The sodium bicarbonate layer was acidified with 10% hydrochloric acid and extracted with chloroform. The chloroform layer (400 ml) was washed with water, dried over sodium sulfate, and concentrated to dryness to give 2.63 g of residue. The residue was applied over a silicic acid–Celite (95:5) column (3.5 × 40 cm), and the column was eluted with chloroform, followed by a mixture consisting of chloroform–methanol (98:2). Fractions containing VI were detected by thin-layer chromatography on silicic acid (dioxane–acetic acid–benzene, 12.5:2.5:45) and by partition paper chromatography (benzene–ethanol–water, 3:1:3). Fractions containing VI were pooled to yield 2.3 g of an oily residue. The infrared spectrum (liquid film) showed λ_{max} 2.95, 5.80, and 5.85 μ. The nuclear magnetic resonance spectrum in $CHCl_3$ is shown in Figure 1.

The neutral fraction was washed with water, dried over sodium sulfate, and the solvent was removed under reduced pressure. The residue was crystallized from acetone–petroleum ether (bp 40–70°) to yield 0.97 g of 25ϵ-hydroxycholesterol (mp 179–181°).

7aβ-Methyl-1β-[1'-methyl-5'-acetoxyisohexyl-5-hydroxy-$\Delta^{5(6)}$-3aα-tetrahydro-4-indanpropionic Acid δ-Lactone (VII). A mixture of VI (1.5 g), anhydrous sodium acetate (1.0 g), and acetic anhydride (50 ml) was heated on an oil bath (bp 90–95°) under an atmosphere of nitrogen. After 48 hr, the reaction mixture was dried under reduced pressure. The residue was extracted repeatedly with small portions of ethyl ether; the combined ethereal solution (150 ml) was washed twice with small portions of 6% sodium bicarbonate and then water. The ethereal layer was dried over sodium sulfate and evaporated to dryness to give an oily residue. The oily residue was applied over a small silicic acid column and eluted with chloroform. Fractions containing the enol lactone were collected and their homogeneity was established by silica gel thin-layer chromatography using chloroform as the mobile phase. The infrared spectrum (liquid film) showed λ_{max} 5.68, 5.77, and 5.96 μ. Its nuclear magnetic resonance spectrum (carbon tetrachloride) is shown in Figure 2.

Methylation of VI. An excess amount of diazomethane, prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, was mixed with 160 mg of VI in 20 ml of ethyl ether. The reaction mixture was left standing

at room temperature for 3 hr in the dark. The solvent was then removed by evaporation, and the residue was applied over a small silicic acid column, and the column was eluted with chloroform. Fractions containing the methyl ester (VIII) were collected, and its homogeneity was checked by silica gel thin-layer chromatography (solvent: acetone–chloroform, 15:85). Single spot fractions were pooled and used for infrared and nuclear magnetic resonance spectra analysis after removal of the solvent. The infrared spectrum (liquid film) showed λ_{max} 2.90, 5.76, 5.85, 8.33, and 8.97 μ. Its nuclear magnetic resonance spectrum (carbon tetrachloride) is shown in Figure 3.

7aβ-Methyl-1β-[1'-methyl-4'-oxopentyl]-5-oxo-3aα-hexahydro-4-indanpropionic Acid (IX) from 25-Keto-26-norcholesterol Acetate (II). *Mycobacterium sp.* (RTMP) was grown in Difco nutrient broth (1200 ml) for 24 hr. This served as the inoculum for 24 l. of Difco nutrient broth (62, 2-l. erlenmeyer flasks). After 24 hr on rotary shakers, 7.2 g of II in 210 ml of dimethylformamide was distributed equally to the flasks. After an additional 96 hr, the fermentation products were extracted and worked up in the usual manner (same as in the fermentation of I). The total residue obtained was 7.3 g. The residue was dissolved in 800 ml of an ethyl ether–benzene (1:1) mixture and extracted with 2% sodium carbonate solution three times. The aqueous layer (1200 ml) was neutralized with 10% hydrochloric acid and then adjusted to pH 2.0 with glacial acetic acid. After extraction of the aqueous layer with chloroform three times, the combined chloroform extract (750 ml) was washed with water, dried over sodium sulfate, and evaporated to yield an oily residue. The oil (820 mg) was passed through a small silicic acid column, using chloroform as the eluent. Fractions containing IX were detected by silica gel thin-layer chromatography (solvent: acetone–chloroform, 10:90). A total of 580 mg of homogeneous material was obtained. The infrared spectrum (liquid film) showed λ_{max} 5.80 and 5.85 μ. The nuclear magnetic resonance spectrum (carbon tetrachloride) is illustrated in Figure 4. From the neutral fraction, 5.8 g of the starting material (II) was recovered.

Enol Lactone of IX. A mixture of IX (150 mg), anhydrous sodium acetate (300 mg), and acetic anhydride (20 ml) was heated on an oil bath (bp 90–95°) under a nitrogen atmosphere. After 48 hr, the enol lactone (X) was isolated by the same procedure described previously. After crystallization from acetone–petroleum ether, 110 mg of X was obtained: mp 60.5–62°; λ_{max}^{Nujol} 5.71, 5.85, and 5.96 μ. The nuclear magnetic resonance spectrum in carbon tetrachloride is shown in Figure 5.

7aβ-Methyl-1β-[1'-methyl-1'-hydroxy-4'-butyric Acid γ-Lactone]-5-oxo-3aα-hexahydro-4-indanpropionic Acid (XI) from 3β,20ε-Dihydroxychol-5-enic Acid δ-Lactone (III). *N. restrictus* was grown in Difco nutrient broth (300 ml) for 24 hr which served as the inoculum for 6 l. of Difco nutrient broth (15, 2-l. erlenmeyer flasks). After incubation for 24 hr on a rotary shaker, 3.0 g of III, in 75 ml of dimethylformamide, was distributed equally among the flasks and the incubation was continued for another 82 hr. The culture broth was worked up in the usual fashion. The crystallizable portion of the residue consisted mainly of III (2.51 g).

The residue was dissolved in 300 ml of ethyl ether–benzene (1:1) and the nonsoluble crystalline substrate (III) was removed by filtration. The ethyl ether–benzene layer was extracted three times with three, 130-ml portions of 6% sodium bicarbonate. The aqueous layer was then acidified with acetic acid and extracted with chloroform. The chloroform extract (300 ml) was washed with water and dried over sodium sulfate. After removal of the solvent, 550 mg of XI was obtained. Several recrystallizations from acetone–ethyl ether gave an analytical sample: mp 142–143°; $[\alpha]_D^{25}$ +47° (*c* 0.79, in $CHCl_3$); $\lambda_{max}^{CHCl_3}$ 5.68, 5.78, and 5.86 μ; nuclear magnetic resonance spectrum in deuterated dimethyl sulfoxide is shown in Figure 6.

Anal. Calcd for $C_{18}H_{26}O_5$ (322.39): C, 67.06; H, 8.13. Found: C, 66.71; H, 8.06.

Propionic Acid-^{14}C from Cholesterol-26,17-^{14}C. To 2 g of wet cell paste of freshly grown *N. restrictus* cells in 25 ml of 0.05 M phosphate buffer (pH 7.8) was added 10 μc of cholesterol-26,27-^{14}C and 25 mg of sodium propionate. After the mixture was incubated for 2.5 hr, the contents were extracted three times with 50-ml portions of chloroform. The aqueous phase was then acidified and steam distilled until the distillate showed no significant quantities of radioactivity. The distillate (1.1×10^6 cpm) was neutralized by the addition of sodium hydroxide and concentrated to a small volume. An aliquot (one-sixth of the total) was chromatographed on a Celite column (Figure 7) and another similar aliquot was chromatographed on a buffered silica gel column (Figure 8). Fractions containing radioactivity were combined and concentrated to a specified volume and a known quantity of nonisotopic sodium propionate was added to prepare the *S*-benzylisothiuronium salt (Vogel, 1962), mp 153–155° (reported value 152°), and recrystallized to constant specific activity. The isolated propionic acid-^{14}C was degraded by the method of Phares (1951) and the results were reported previously (Sih *et al.*, 1967).

Conversion of Cholesterol-4-^{14}C into Radioactive 3-Oxochola-1,4-dienic Acid. To *N. restrictus* cells (2 g wet wt) suspended in 25 ml of 0.05 M phosphate buffer (pH 7.8) was added 10 μc of cholesterol-4-^{14}C, 1.25 mg of 3-oxochol-4-en-24-oic acid, and 4.95 mg of *o*-phenanthroline. At each time interval, 4-ml samples were taken from the reaction mixture, acidified with 3 drops of 2 N HCl, and extracted two times with 5 ml of ethyl acetate. The ethyl acetate layer (0.02 ml) was counted in the scintillation counter. The results are 0, 1, 2.5, 4, and 5.5 hr, respectively, for radioactivities of 5314, 5408, 5250, 5261, and 4954 cpm. The ethyl acetate extract (6 ml) was concentrated to a small volume and spotted on Whatman No. 1 paper. The paper was developed for 8.5 hr in the

toluene–propylene glycol system (Zaffaroni *et al.*, 1950). After drying the paper chromatogram, the ultraviolet absorption zones were marked under an ultraviolet scanner. The radioactive zones were detected by scanning the paper strips through a Vanguard Autoscanner Model 880 ADS with the automatic data system. A profile of the radioactive peaks is shown in Figure 9. The radioactive zones (I, II, III, and IV) were cut into small pieces and then extracted with 20% methanol in chloroform for 2 hr. The eluate was filtered and kept for recrystallization with nonisotopic compounds.

Since peak II had a mobility similar to 3-oxochola-1,4-dien-24-oic acid, it was admixed with 50 mg of nonisotopic 3-oxochola-1,4-dien-24-oic acid and recrystallized three times in methanol. The specific activity remained essentially constant as shown in Table I. When peak I was admixed with either 3-oxobisnorchol-4-en-22-oic acid or 3-oxobisnorchola-1,4-dien-22-oic acid, the specific activities did not remain constant after recrystallizations.

Formation of Acetic Acid-1-^{14}C from Lithocholic Acid-24-^{14}C. Lithocholic acid-24-^{14}C (5 μc) and sodium acetate (25 mg) were incubated with 2 g of washed cells of *N. restrictus* under the same conditions as the cholesterol-26,27-^{14}C case. The isolation procedures were conducted in the same manner as before. The steam-distillable fractions contained 8.26×10^5 cpm. An aliquot of the concentrated steam distillate (one-third of total) was chromatographed on a 15-g Celite partition column (Figure 12). The radioactive sodium acetate was used to prepare the *S*-benzylisothiuronium salt and the derivative was recrystallized to constant specific activity. The distribution of the isotope in the isolated acetic acid was degraded (Phares, 1951) and the results were reported previously (Sih *et al.*, 1967).

3β-Acetoxy-6β-hydroxy-5α-chlorocholanic Acid Methyl Ester (XIII). 3β-Acetoxychol-5-enic acid methyl ester (26 g) (XII) was dissolved in 500 ml of ethyl ether. After the addition of 42 g of bleaching powder and 1570 ml of water, the mixture was stirred vigorously for 5 min and 27 ml of glacial acetic acid was then added. After the reaction mixture was stirred for 30 min, it was diluted with methylene chloride–ether (1:1). After separation from the aqueous layer, the organic phase was washed with water several times, dried over sodium sulfate, and evaporated to dryness to yield 17.4 g (66.9%) of the crude product. A small sample was recrystallized from acetone–petroleum ether to give an analytical specimen: mp 154–155°; $[\alpha]_D^{26}$ −30.8° (*c* 1.0, in $CHCl_3$); λ_{max}^{Nujol} 2.91, 5.80, 5.88, and 7.9 μ.

Anal. Calcd for $C_{27}H_{43}ClO_5$: C, 67.10; H, 9.00. Found: C, 66.93; H, 8.74.

3β-Acetoxy-5α-chloro-6,19-oxidocholanic Acid Methyl Ester (XIV). A suspension of 108 g of lead tetraacetate and 36 g of calcium carbonate in 4.8 l. of cyclohexane was heated for 10 min at 80°. To this mixture was added 24 g of iodine and 17 g of the crude chlorohydrin (XIII) and the mixture was stirred and irradiated with a 500-w lamp for 60 min while the content was under reflux. The reaction mixture was cooled and filtered through Celite. The residue was exhaustively washed with ether and the combined filtrate was washed with 600 ml of 10% sodium thiosulfate solution twice, followed by three, 500-ml portions of water. The organic layer was dried over sodium sulfate and concentrated to dryness to give 17 g of residue. The yellow residue was chromatographed over a silicic acid–Celite (9:1) column (5.6 × 58 cm). The column was eluted with benzene and successively with 10, 20, and 30% chloroform in benzene; 23-ml fractions were collected. Fractions 716–1300 ($CHCl_3$–benzene, 20:80) were pooled and afforded 12.2 g of XIV after evaporation of the solvent. Crystallization from acetone–petroleum ether gave a sample: mp 145–146°, $[\alpha]_D^{26}$ +2.6° (*c* 0.9, in $CHCl_3$), λ_{max}^{Nujol} 5.81 and 8.11 μ.

Anal. Calcd for $C_{27}H_{41}ClO_5$: C, 67.50; H, 8.68. Found: C, 67.54; H, 8.69.

3β-Hydroxy-5α-chloro-6,19-oxidocholanic Acid (XV). To the acetoxy ester (12 g) (XIV), dissolved in 1200 ml of methanol, was added 12 g of K_2CO_3 in 60 ml of water. After refluxing the reaction mixture for 1 hr, the mixture was evaporated under reduced pressure, without heating. Water was added to the residue and a precipitate appeared after acidification with HCl. The precipitate was collected by filtration to yield 7.1 g (64%) of crude product. Two recrystallizations from acetone–petroleum ether gave a sample: mp 226–229°; $[\alpha]_D^{26}$ −13° (*c* 0.8, in dioxane); λ_{max}^{Nujol} 3.03, 3.18, and 5.81 μ.

Anal. Calcd for $C_{24}H_{37}O_4Cl$: C, 67.82; H, 8.88. Found: C, 67.95; H, 9.05.

3-Oxo-6,19-oxidochol-4-en-24-oic Acid (XVI). 3β-Hydroxy-5α-chloro-6,19-oxidocholanic acid (7 g) was dissolved in 400 ml of pyridine and cooled in an ice bath. Chromic trioxide (40 g) was added in portions with stirring and the reaction mixture was left standing at room temperature overnight. The incubation mixture was then poured into an excess of water, acidified with glacial acetic acid, and extracted with three, 700-ml portions of ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and concentrated to dryness to give 6.3 g of residue. The residue was dissolved in chloroform and chromatographed over a silicic acid–Celite (9:1) column (3.6 × 38 cm). The column was eluted with chloroform and the gradual addition of methanol; 23-ml fractions were collected. Fractions 340–950 were combined and evaporated to dryness to give 4.1 g (62.6%) of XVI. Recrystallization from acetone–petroleum ether afforded a sample: mp 157–158.5°; $[\alpha]_D^{26}$ −83.2° (*c* 0.9, in dioxane); λ_{max}^{Nujol} 5.81, 6.01, and 6.14 μ; $\lambda_{max}^{alcohol}$ 241 mμ (ϵ 11,000).

Anal. Calcd for $C_{24}H_{34}O_4$: C, 74.59; H, 8.87. Found: C, 74.89; H, 9.26.

Transformation of 3-Oxo-6,19-oxidochol-4-en-24-oic Acid (XVI). N. restrictus was grown for 4 days in the Bushnell Haas salt medium containing 300 μg/ml of cholesterol. This served as the inoculum (12%) for 6 l. of Difco nutrient broth (15, 2-l. erlenmeyer flasks).

At 0 hr, XVI (1.5 g) was dissolved in 60 ml of dimethylformamide and distributed equally among the flasks. The fermentation was continued for 100 hr on a rotary shaker. The culture broth was then acidified and extracted with three, 2.5-l. portions of ethyl acetate–chloroform (2:1) mixture. The organic extract was washed with water, dried over sodium sulfate, and evaporated to dryness to yield 1.13 g of residue. The residue was chromatographed over a cellulose powder column using the aqueous layer of ethanol–benzene–water (1:4:4) as the stationary phase. The mobile phase was the benzene layer and 20-ml fractions were collected. Fractions 85–146 were pooled and subjected to thick-layer chromatography (silica gel HF). The residue (353 mg) was streaked on four thick-layer plates and developed with acetone–chloroform (2:8). The bottom ultraviolet-absorbing band was scratched out and eluted with acetone. Several recrystallizations from acetone–petroleum ether gave a sample of XVIII (218 mg): mp 231–234°; $[\alpha]_D^{26}$ −66° (*c* 0.8, in dioxane); $\lambda_{max}^{alcohol}$ 241 mμ (ε 11,000); λ_{max}^{Nujol} 3.00, 5.82, 5.90, and 6.06 μ. Mass spectrum gave 374.

Anal. Calcd for $C_{22}H_{30}O_5$: C, 70.56; H, 8.08. Found: C, 70.62; H, 8.16.

6,19-Oxidoandrost-4-ene-3,17-dione from XVI. The fermentation procedure was the same as the *N. restrictus* fermentation of XVI, except *Nocardia sp.* (ATCC 19170) was used. A total 200 mg of XVI was incubated with the microorganisms in two, 2-l. erlenmeyer flasks. After the usual work-up 215 mg of residue was obtained. Thick-layer plate chromatography of the residue (two plates) using acetone–chloroform (2:8) as the mobile phase afforded 29.4 mg of 6,19-oxidoandrost-4-ene-3,17-dione (XVII) after scratching out the upper ultraviolet band and elution. Recrystallization from acetone–petroleum ether gave a sample (mp 182–185°) identical with respect to mixture melting point and infrared spectrum with an authentic sample.

Acknowledgments

The authors wish to express their gratitude to Dr. H. Herzog and K. Heuber for the gift of some steroids used in this work.

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