MICROBIAL DEGRADATION OF STEROLS AND STEROL SIDE CHAINS

BY

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TABLE OF CONENTS

Page

LIST OF FIGURES

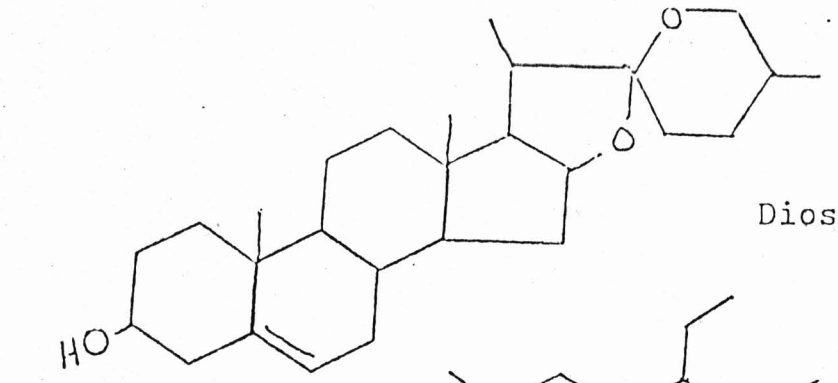
LIST OF TABLES

INTRODUCTION

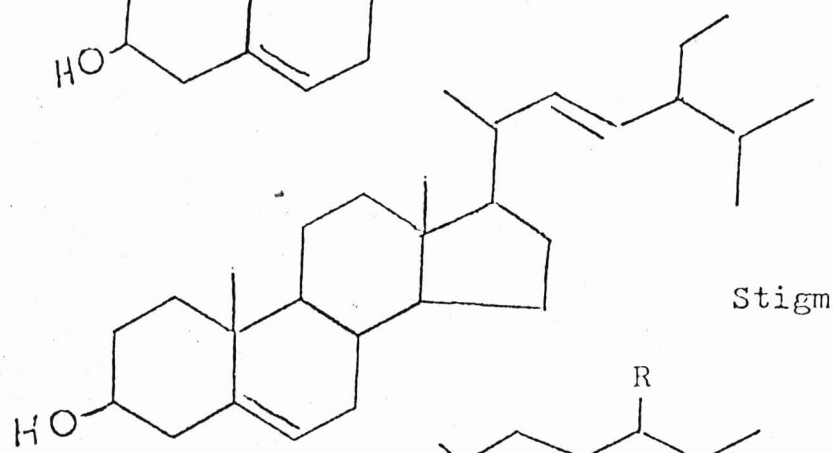
The commercial preparation of steroid hormones for the pharmaceutical industry is limited to a few sources as starting materials which can be economically converted into steroids. At present the two major sources are the diosgenins (a type of sapogenin) (Figure 1) which are mainly isolated from the Dioscorea species and stigmasterol (Figure I) which is mainly obtained from soybeans (46). In recent years the demand for steroid drugs has increased tremendously and the need for cheap sources of steroid precursors has led to investigations of the ability of microorganisms to convert sterols to compounds useful as intermediates in the preparation of anti-inflamatory steroids and those steroids useful as oral contraceptives.

The complexity of the steroid nucleus has until now, limited the possibility of an economical method for total synthesis. There have, however, been techniques developed which chemically cleave the side chains of certain sterols to produce precursors for conversion to the desired steroids. The Upjohn Company has used a process for chemically making progesterone from stigmasterol by oxidation of the unsaturated side chain of stigmasterol

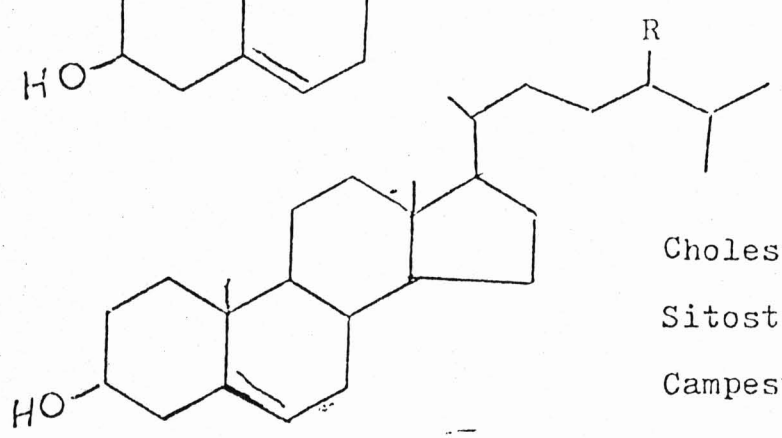
Figure I. Structures of Sterol Molecules



Diosgenin



Stigmasterol



Cholesterol, R= H

Sitosterol, R=C_2 H_5

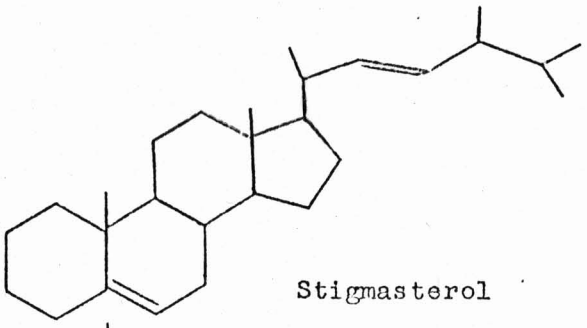
Campesterol, R= CH_3

(Figure II). The recent development of a system of remote oxidation for the removal of the cholesterol side chain to produce andrastane (67) may prove useful in the future.

There are many sources of the naturally occurring steroids such as the soybean sterols and other plant sterols (21) and a by-product of the paper pulping industry called tall oil (14). Generol 122^{R}, a mixture of sterols (see Materials and Methods) which is produced as a by-product material during the extraction of stigmasterol from soybeans consists mainly of a mixture of sitosterol (Figure I) and campesterol (Figure I). The tall oil sterols are approximately 85% sitosterol and campesterol (14). These compounds which cannot readily be converted to steroids when oxidized chemically, have attracted much attention as abundant sources of starting material for many different microbiological processes which have been developed in recent years to utilize these materials for the production of steroid compounds.

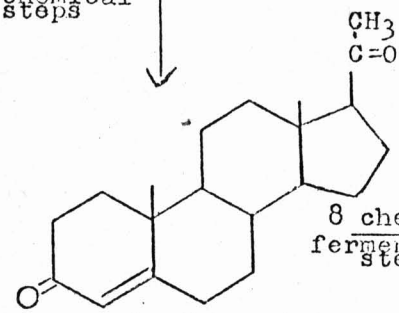
The first report of microbial utilization of steroids was when Soehngen (66) showed that a soil Mycobacterium could grow using cholesterol as the sole source of organic carbon. It wasn't until much later that Tak (70) also working with a Mycobacterium sp. showed that cholesterol actually disappeared from the medium. Cholesterol degradation in the soil was shown by Turfitt

Figure II. The Production of Steroid Hormones by the Upjohn Process.



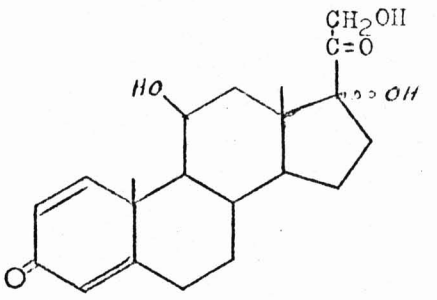
Stigmasterol

4 chemical steps



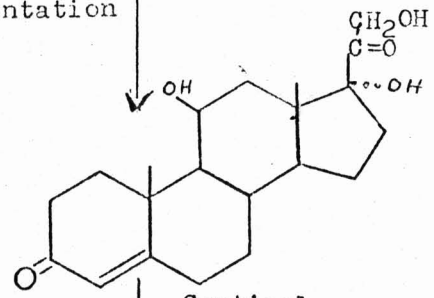
Progesterone

8 chemical and fermentation steps



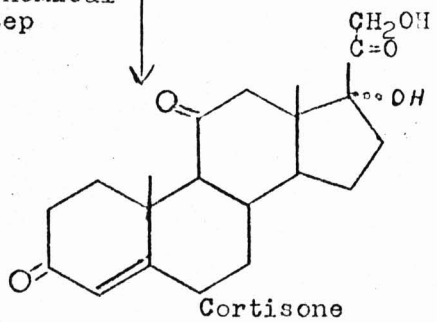
Prednisolone

6 chemical and fermentation steps



Cortisol

1 chemical step



Cortisone

(72), when he isolated a strain of Proactinomycetes erythropolis from soil enrichment cultures which were degrading cholesterol. This organism also degraded cholesterol in pure culture, as did several strains of Mycobacterium phlei (73).

In contrast to Turfitt's results which indicated that cholesterol degradation in soils was primarily due to Nocardia (72, 73) and only in well aerated non-acidic soils (72), Schatz et al (49) isolated a variety of rod-shaped gram negative bacteria from many types of soil which could degrade cholesterol. With these isolations they demonstrated that a variety of microorganisms were capable of utilizing cholesterol.

One of the first characterizations of cholesterol degradation products was made by Horvath and Kramli in 1947 (23). Working with an Azotobacter they isolated Δ^4-cholesten-3-one, $\Delta^{5,7}$-cholestadiene-3-one, and methyl-heptanone. The latter compound was supposed to have been formed by the cleavage of the side chain between C-17 and C-20, however, its appearance may have been due to chemical oxidation.

Turfitt (74) was the first to isolate a ring degradation product, Windaus keto acid in the fermentation of Δ^4-cholesten-3-one by Proactinomycetes erythropolis. This compound was also isolated by Stadtman et al (68) from a

fermentation of cholesterol with a Mycobacterium. Additionally Stadtman et al (68) showed that 4-^{14}C-cholesterol releasted $^{14}CO_2$ indicating an A ring cleavage between C-3 and C-4 or C-4 and C-5.

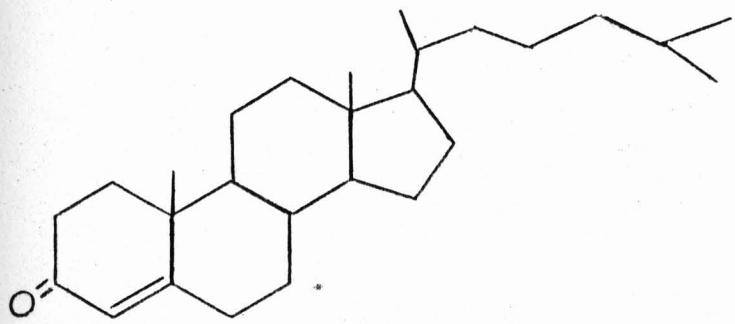
Santer and Ajl (48) collected $^{14}CO_2$ produced from ^{14}C-4-testosterone by a species of Pseudomonas. These observations led to speculations that the pathway for microbial degradation of the sterol nucleus started with an A ring cleavage. However, with the exception of the catabolism of eburicoic acid by Glomerella fusarioides (29) and 4-hydroxy-4-cholesten-3-one by Nocardia restrictus and Nocardia corallina (30), there have been few other observations of an A ring cleavage occurring as the first stage of the degradation sequence.

The possibility that the attack of the sterol molecule takes place simultaneously at several points on the molecule was postulated by Loomeijer (32). This hypothesis is supported by the results of Stadtman et al (68) and those of Peterson and Davis (45). Stadtman et al working with a Mycobacterium found that $^{14}CO_2$ was released from ^{14}C-26-cholesterol. Peterson and Davis working with a Streptomycete found the opposite result; fermentations with ^{14}C-26-cholesterol released $^{14}CO_2$ faster than ^{14}C-4-cholesterol. These results indicate multiple points of attack.

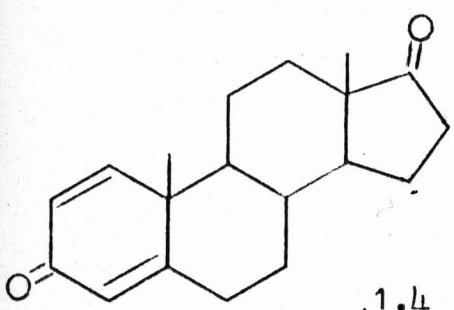
The isolations of many other different types of organisms capable of degrading sterols have been reported. These include Flavobacterium (6), Pseudomonas (48,77), Streptomyces (44), Agrobacterium (77) and Aeromonas (77), among others. Arima et al in their screening of 1589 strains of microorganisms (132 actinomycetes, 276 bacteria, 276 yeast, 905 molds), noted complete degradation of cholesterol in the following genera: Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protaminobacter, Serratia, and Streptomyces. No complete degradation was seen in the yeasts or molds, however, these organisms did carry out conversions of the cholesterol molecule mainly to Δ^4-cholesten-3-one (Figure III). This compound was, in fact, the most often observed product of cholesterol metabolism (5).

The main pathway of the degradation of the sterol nucleus by bacteria is shown in Figure IV. The first step in the breakdown of Δ^4-androsten-3,17-dione (I) is either 1,2 dehydrogenation or 9α hydroxylation depending upon the genus (17). In Pseudomonas species the 1,2 dehydrogenation is followed by 9α hydroxylation whereas in Nocardia the order is reversed (17). The $\Delta^{1,4}$-3-keto, 9α hydroxylated molecule (IV) then undergoes a spontaneous rearrangement to 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione

Figure III. The structures of Δ^4-cholesten-3-one and $\Delta^{1,4}$-androstadiene-3.17-dione

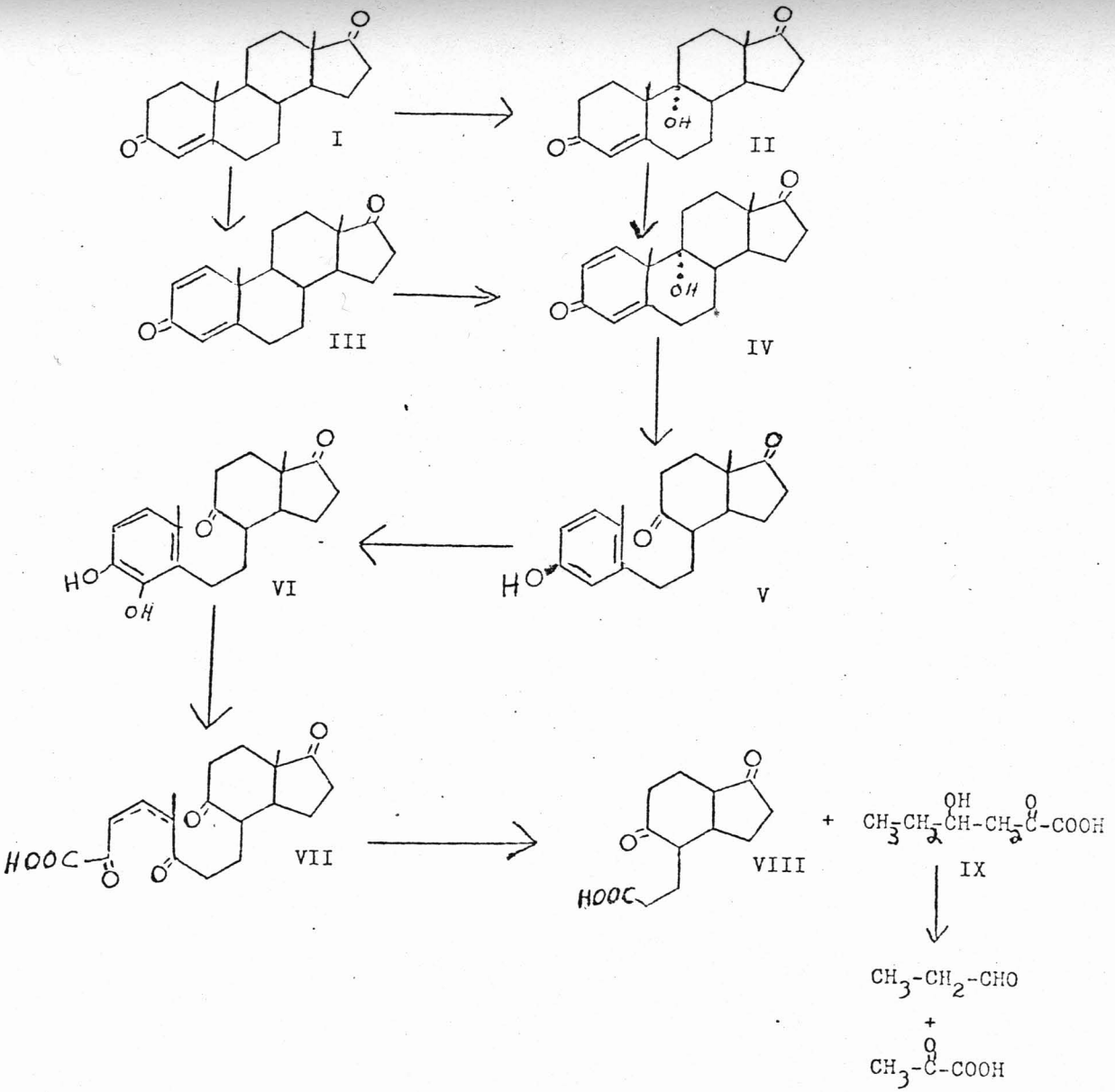


Δ^4-cholesten-3-one



$\Delta^{1,4}$-androstadiene-3,17-dione

Figure IV. Pathway for the Degradation of the Sterol Nucleus.


I
II
III
IV
V
VI
VII
VIII
IX
OH
HO
HOOC
CH_3-CH_2-CHO
$CH_3-\overset{O}{\overset{\|}{C}}-COOH$

(V) which is the first product of ring degradation (17). Sih and Rahim (54) observed this ring opening reaction in cell free extracts of Nocardia restrictus while studying the 9α hydroxylase enzyme. They showed that the 9α hydroxylase needs an electron acceptor such as flavo-protein to be active. Further studies of this enzyme showed that it was inhibited by Zn^{++}, Ca^{++}, Hg^{++}, and phenazine methyl sulfate (12). Recently Strijewski and Wagner (69) purified the 9α hydroxylase enzyme system and showed it to be a complex of proteins which act as an electron transport chain. This complex does not contain cytochrome P_{450} and is activated by NADH, Mg^{++}, and Ca^{++}. Other enzymes involved in the early stages of sterol metabolism have been studied by several groups (1,8,9,25,34,47,53). These include: The enzyme converting the 3-hydroxyl of cholesterol to the 3-keto function (34); The enzymatic isomerization of the $\Delta^{5(6)}$ or $\Delta^{5(10)}$ functions to the Δ^{4} function (25); and the 1,2 dehydrogenating enzyme (53,47,1). The latter enzyme was shown to act by trans-diaxial loss of the 1α and 2β hydrogens (47). The 1,2-dehydrogenase enzyme has been shown to be a flavo-protein (53,47).

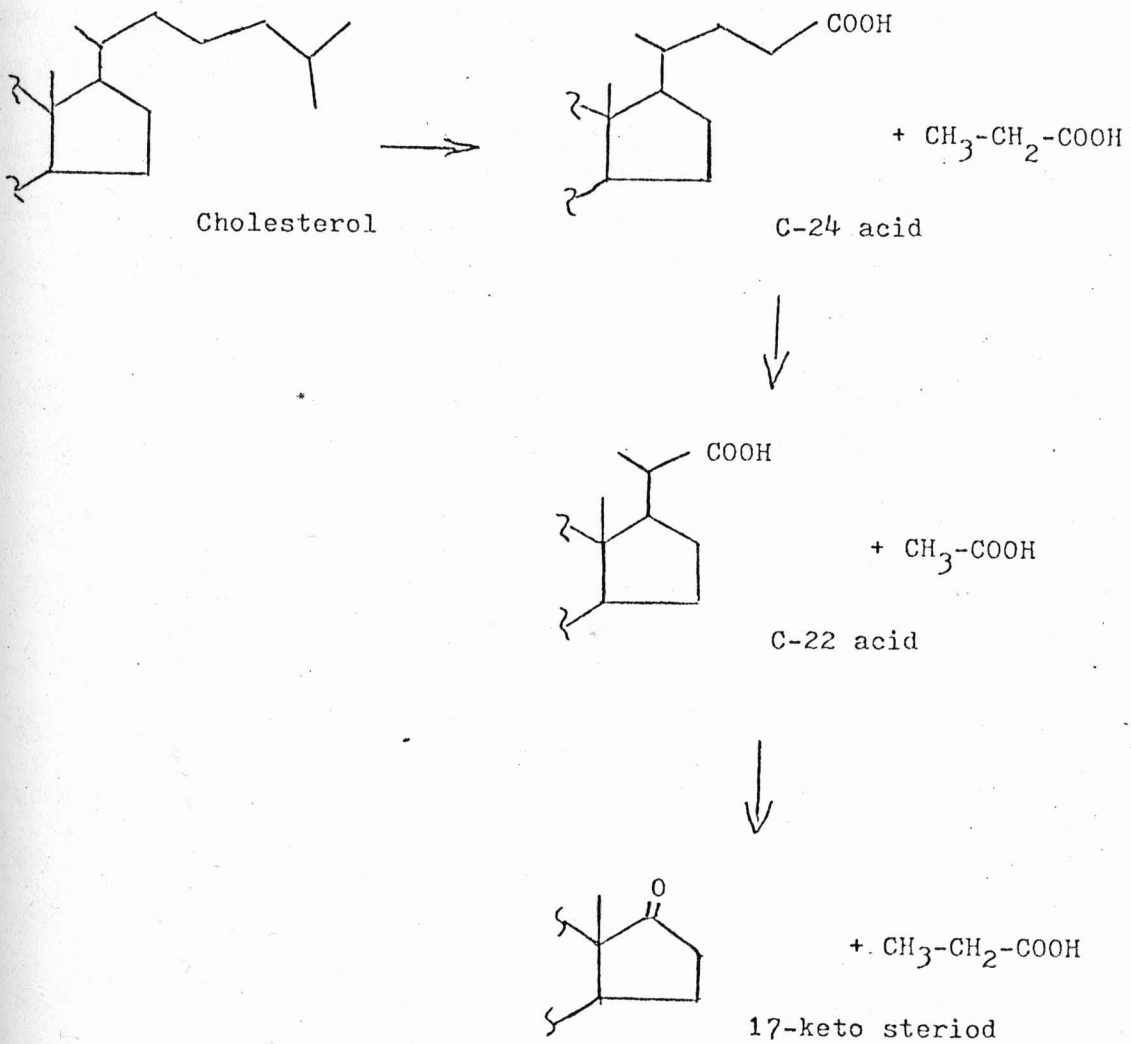
After the cleavage of ring B of the sterol molecule the next steps in the ring degradation are the hydroxylation at C-4 of the 9,10-seco phenol followed by the A ring cleavage to 4(5), 9(10)-diseco-3-hydroxyandrosta-1(10),

2-diene-5,9,17-trione-4-oic acid (VII) (57). This cleavage step is unusual in that it is catalyzed by a dioxygenase whereas most of the other enzymes in the sterol degradation scheme are monooxygenases (19). Although the 4-hydroxylated product was not isolated by Sih and his co-workers (19) confirmation of this pathway came when Holden et al (22) isolated 9,10-secophenols which were hydroxylated at C-4 when 17 βmethyl-B-Nor-testosterone was incubated with Protaminobacter ruber.

The final step in the degradation of the sterol ring system that has been identified is the cleavage of the diseco molecule to 3 aα H-4α-[3'-propionic acid] -7aβ methyl-hexahydro-1,5-indane dione (VIII) (57) and 2-keto-3-hexenoic acid (IX) (19). The latter compound is further degraded to pyruvic acid and propionaldehyde (19).

The pathway for the degradation of the sterol side chain which was elucidated in a brilliant series of experiments by Sih and his co-workers (58-61) is shown in Figure V. 19-oxygenated cholesterol was used as a substrate because ring degradation of this molecule is inhibited while allowing side chain degradation (54). Intermediates were isolated from fermentations of 19-oxygenated cholesterol with Norcardia restrictus which showed that the side chain is degraded via a mechanism analogous to the β-oxidation of fatty acids. The first product isolated was the C-24

Figure V. Pathway for the Degradation of the Cholesterol C 17 Side Chain.


COOH
+ CH_3-CH_2-COOH
Cholesterol
C-24 acid
COOH
+ CH_3-COOH
C-22 acid
O
+ CH_3-CH_2-COOH
17-keto steriod

acid which is formed after the oxidative cleavage of cholesterol to the C-24 acid and propionic acid. This was shown by using ^{14}C-26,27-cholesterol and by isolating ^{14}C propionic acid which was labeled equally in C-1 and C-3. The next step is the cleavage of the C-24 acid to the C-22 acid with the release of acetic acid. Finally the C-22 acid is cleaved to the 17-keto steroid with the release of another molecule of propionic acid. Supportive evidence of this pathway was given by Zaretskaya et al (81) when they isolated 27-hydroxy-4-cholesten-3-one and 27-hydroxy-4-sitosten-3-one from fermentations of cholesterol and sitosterol by a Mycobacterium. This was the first isolation of a sterol molecule which was hydroxylated in the side chain. This reaction is necessary for the β-oxidation pathway. The hydroxylation at C-26 of cholesterol was shown in Mycobacteria by Galli-Kienle et al (18) and in Nocardia by Lefebvre et al (30). Lefebvre was also able to isolate the C-24 acid and showed the release of propionic acid into the media, thus further confirming the pathway of sterol side chain degradation proposed by Sih for the genera Nocardia, Arthrobacter, Mycobacterium, and Corynebacterium.

Since many of the industrially important transformations of steroid molecules are performed microbiologically (35), it follows that microbial cleavage of the

side chain of cholesterol and phytosterols can also be an industrially important process. The possibility that such a process could be developed was evident when Turfitt isolated 3-keto-4-androsten-17β-carboxylic acid (lacking the side chain) from the fermentation of Δ^4-cholesten-3-one by Nocardia erythropolis (18). It was with Whitmarsh's (79) observation that $\Delta^{1,4}$-androstadien-3,17-dione was produced from cholesterol in the presence of 8-hydroxyquinoline during a fermentation with Mycobacterium that the utility of a microbial process for the production of intermediates of steroid drug preparations was actually shown.

Sih and Rahim (45) showed that steroid molecules modified at C-19 would not undergo ring degradation but would be transformed into an aromatic A ring compound. The chemical modifications of the sterol molecule included 19-Nor-19-hydroxy, and 19-oxo androstadienediones. The next step was to modify the cholesterol molecule itself to prevent ring degradation while allowing side chain cleavage. Sih et al (56) showed the production of 6,10-oxido estrone by Nocardia restrictus from cholesterol which had been chemically modified to 3 β-acetoxy-5-acetoxy-19-hydroxy cholesterol and from 19-hydroxysitost-4-4n-4-one was also shown (55). Other products have been made from modified sterol molecules. These products include:

estrone from 19-Nor-$\Delta^{1,3,5(10)}$-cholestatriene-3-ol by a Nocardia, equilin from 19-hydroxy-cholestra-4,7-diene-3-one by a Mycobacterium (16), a variety of 17-keto steroids from altered sterols by Brevibacterium SPP. (51), $\Delta^{9(11)}$ estrone from hydroxy cholesten-3-acetate by Corynebacterium sp. (33), 3α 5-cyclo- 6β -19-oxido-5α-androstan-17-one or the corresponding sitosterol and stigmasterol derivatives by Arthrobacter and Corynebacterium (52), 17-keto-3,5 cyclo steroids from the 3,5 cyclosterols (i-steroids) (75), and estrone from a variety of steroids modified at both C-19 and C-10 (such as 10 carbonyl, 19-nor or 10,19 hydroxy) (63).

As was mentioned previously, Whitmarsh (79) showed that steroid side chain degradation could occur without concurrent degradation of the ring structure of unmodified steroids when fermentations were carried out in the presence of 8-hydroxyquinoline. The accumulation of $\Delta^{1,4}$-androstadiene-3,17-dione in these fermentation suggested that the 8-hydroxyquinoline was preventing the 9α hydroxylation reaction. Wix (80) showed that 8-hydroxyquinoline was acting by chelating free Fe^{++} which is required by the 9α hydroxylase. Compounds such as αα 'dipyridyl and 8-hydroxyquinoline, which are effective chelators of Fe^{++} and Cu^{++}, were shown effective in blocking the sterol ring degradation in a number of bacterial genera as shown by

Arima (4). These include: Arthrobacter, Corynebacterium, Brevibacterium, Bacillus, Microbacterium, Protaminobacter, Serratia, Pseudomonas, Nocardia, Mycobacterium and Streptomyces.

In a systematic investigation of a large number of compounds as metabolic inhibitors Nagasawa and his co-workers (40-43) showed that the compounds effective in causing the accumulation of 17-keto steroids from cholesterol included lipophilic chelating agents, redox dyes, and certain metallic ions. Table I lists the compounds effective in the inhibition of sterol ring degradation. Nagasawa found that αα 'dipyridyl was the most effective of these inhibitors in fermentations with Arthrobacter simplex. He also discovered that the efficiency of the side chain cleaving activity was dependent upon the structure of the side chain itself. The efficiency of side chain cleavage followed the following rules: 1) increasing the length or a branch at C-24 decreased efficiency; 2) a double bond at C-22 decreased efficiency; 3) a Δ^7 double bond gives marked resistance to attack. Thus the efficiency of side chain cleavage by Arthrobacter simplex is cholesterol, β-sitosterol (type 1 above) stigmasterol (type 1 and 2) ergosterol (type 1,2,3).

Another approach to the conversion of sterols into useful products involves the use of mixed culture

Table I

Agents Effective in Selective Inhibition of Sterol Ring Degradation

Mechanism of Action	Compound
Chelating agents for Fe^{++}	$\alpha\alpha'$-dipyridyl
	1,10-phenanthroline
	8-hydroxyquinoline
	5-nitro-1,10-quinoline
	cupferron
	diphenylthiocarbzone
	diethyldithiocarbamate
	isonicotinic acid hydrazid
	xanthogenic acid
	o-phenylenediamine
	4-isopropyltropolone
	tetraethylthiuramdisulfide
Metal ions replacing Fe^{++} or blocking -SH functions	Ni^{++}
	Co^{++}
	Pb^{++}
	$SeO_3^{=}$
	$AsO_2^{=}$
Redox dyes	methylene blue
	resazurin

fermentations. Awata (7) has devised a system where members of the genera Arthrobacter, Brevibacterium, Corynebacterium or Nocardia are mixed 1:1 with Pseudomonas aeruginosa. It is necessary to add rhamnolipid (a compound consisting of two rhamnose residues and two 1-β-hydroxydecanoic acid residues produced by strains of Pseudomonas aeruginosa) along with the cholestrol substrate. This system converts cholesterol to $\Delta^{1,4}$-androstadiene-3,17-dione in yields of up to 60%.

The use of mutants of sterol degrading organisms which are blocked for ring degradation has proven useful in the production of 17-keto steroids from sterols. The isolation and utility of such mutants was shown by Marsheck (36) and Kraychy (28) when they isolated two mutants of a soil Mycobacterium. These organisms Mycobacterium NRRL B3683 and NRRL B3805 produce $\Delta^{1,4}$-androstadiene-3,17-dione and Δ^{4}androsten-3,17-dione respectively from cholesterol, sitosterol, tall oil sterols (a mixture of campesterol and sitosterol) (15) and Δ^{4}-3-keto sterols. The mutants made conversions of cholesterol to 17-keto steroids of up to 90% at sterol concentrations of 1 g/L.

Another investigation into the isolation of mutant organisms was not so successful. Cargile and McChesney (11) attempted to specifically enrich for mutants capable only of side chain degradations by using penicillin

enrichment techniques and replicate plating. Using these techniques they isolated cultures of mutants which would grow on cholesterol but not on testosterone. These mutants were able to maintain the ability to grow on cholesterol but not on testosterone. However, no steroid metabolites could be recovered from fermentations in minimal media with cholesterol as the sole fermentable carbon source or complex media containing cholesterol.

The extremely low solubility of cholesterol in water (1.8 µg/ml) (20) has created a great many problems in the investigation of sterol metabolism by microorganisms. To overcome some of these problems Buckland et al attempted a fermentation of cholesterol using washed cells and high concentrations of non-aqueous solvents (8,9). In these experiments they showed that the cholesterol oxidase in washed cells a Nocardia species would function in organic solvents. They achieved rates of conversion of cholesterol to Δ^4-cholesten-3-one of 7 g/hr when 100 g of frozen packed cells were thawed and suspended in 200 ml of CCl_4 containing 16% w/v of cholesterol. When other solvents such as toluene, hexadecane, and ethylether were substituted for CCl_4 the conversion of cholesterol to Δ^4-cholesten-3-one was less efficient. In analogous experiments Lilly et al (31) showed that the conversions of pregnenolone to progesterone, 17-hydroxypregnenolone to 17-hydroxyprogester-

one, and dehydroepiandrosterone to Δ^4-androsten-3,17-dione by Nocardia rhodochrous (NCIB 10554) could also take place in CCl_4.

A method for improving the conversion of β-sitosterol to $\Delta^{1,4}$-androstadiene-3,17-dione was developed by Martin and Wagner (38,39). The technique involves the addition of lipophilic organic adsorbants (Amberlite XAD-2 and XAD-4) to fermentations of cholesterol and β-sitosterol by a Nocardia species in a medium containing αα'dipyridyl (to prevent ring degradation). In this system the XAD-2 increases the total production of $\Delta^{1,4}$-androstadiene-3,17-dione by the absorption of this metabolite to the resin. This absorption prevents further degradation of the 17-keto steroids and increases the relative concentration of cholesterol or sitosterol thus favoring the reaction leading to product formation.

The ability of microorganisms to take up sterols from media is an important aspect of sterol metabolism. The uptake of cholesterol by Mycobacterium was shown to be an energy dependent process (13) which results in the formation of cholesteryl-fatty acids (50). The uptake of testosterone into membrane vesicles of Pseudomonas testosteron was shown to be NAD^+ dependent and resulted with a concentration of cholesterol on the inside of the membrane vesicles of about 800 times the media concentration (78).

The search for sources of steroid precursors which can be converted by fermentation into steroid compounds has by no means been limited to those compounds which have been discussed above. Other fermentations utilizing less common starting materials are summarized in Table II.

The volume of research which has been directed toward sterol compounds and sterol metabolism is so large that many reviews have been published covering various aspects of sterol metabolism and conversion. Those by Heftman (21), Marsheck (35), Martin (37), Sih and Whitlock (62), and Vezina et al (76) are especially noteworthy.

In the present study attempts have been made to isolate microorganisms capable of complete degradation of sterols. These organisms have been investigated for their utility to selectively degrade sterol side chains using some of the techniques described above.

Table II

Microbial Production of Steroid Compounds from Various Sterol Starting Material

Starting Material	Product	Microorganism	Reference
sapogenins	16-keto steroids	*Fusarium sp.*	26
		Vetricilium sp.	27
		Stachylidium sp.	27
	17-keto steroids	*Mycobacterium pheli*	3
phytoecdysones	progesterone	*Rhizopus sp*	10
		Curvularia sp.	10
ecdysones	rubrosterone	*Fusarium*	71

MATERIALS AND METHODS

Materials

Steroid compounds and other chemicals used in this study were acquired from the following sources: Generol 122^{R} (see Table III) from General Mills Chemical, Inc.; cholesterol (USP) from Vitamins, Inc.; 5-cholesten-3-ol ethylether, 5-cholesten-3-ol benzoate, 5-cholesten-3-ol acetate, 5-cholesten-3-ol n-propionate, and 5-cholesten-24b-ethyl-3-ol acetate from Steraloids, Inc. (these compounds showed a single spot in thin layer chromatography); $\Delta^{1,4}$-androstadiene-3,17-dione (~97%), Δ^{4}-androsten-3,17-dione, and testosterone from Sigma Chemical Co.; β-sitosterol (a mixture of sitosterol and campesterol) from Applied Science Laboratories; N-methyl-N'-nitrosoguonidine from Aldrich Chemical Company, Inc.; Potassium penicillin G from Pfizer Inc.; αα'dipyridyl from Sigma Chemical Co.; Polyoxyethylen 120 sorbitan monooleate (tween 80^{R}) from J. T. Baker Chemical Company. Samples of highly purified (>99%) cholesterol and sitosterol were a generous gift from Dr. J. W. Rowe and Mr. A. H. Conner.

Table III

Composition of Generol 122[R]

Compound	% of Steroids
Sitosterol	59.2
Campesterol	30.2
Stigmasterol	5.4
Cycloartenol	2.2
24-methylene cycloartenol	1.9
Cholesterol	1.0
Total steroids	92-95 %
Non-steroid compounds	5-8 %

Isolation of Sterol Degrading Cultures

One gram samples of soil gathered from the greenhouses of the University of Wisconsin Botany Department were suspended in 10 ml of 0.1 M phosphate buffer (ph 7.0), and 2 grams ground Generol 122^{R}. These samples were placed in small bottles and incubated at 30^{o}C. The samples were shaken twice daily. At weekly intervals 0.1 ml aliquots were taken. The aliquots were used to inoculate 10 ml of medium A in a 1 x 6 inch test tube capped with plastic closures (Bellco Glass Company). The test tubes were incubated at 30^{o}C on a rotary shaker (New Brunswick Scientific Company) at 300 rpm. After four days incubation the tubes were removed and an aliquot was tested for disappearance of the sterols. Choloform (½ volume) was used to extract the aliquots. The extracts were dried under a stream dry nitrogen, dissolved in 0.2 ml of $CHCl_3$ and spotted on silica gel HF-254 thin layer chromatography plates (Brinkmann). The plates were developed in benzene: ethylether (1:1). Samples which showed degradation of the sterols were diluted and plated on nutrient agar (Difco) containing 0.5 g/L of Generol 122^{R}. Well separated colonies were chosen and used to inoculate tubes of medium A (Table IV) and incubated at 30^{o}C on a rotary shaker at 300 rpm. Isolates which degraded the sterols were streaked on nutrient agar plates to obtain individual colonies.

Table IV

Medium A for Isolation of Sterol Degrading Cultures

Generol 122^{R}	0.5 grams
Glucose	5.0
Monosodium glutamate	4.0
KH_2PO_4	1.0
NaCl	1.0
$FeSO_4$-$7H_2O$	0.003
$MgSO_4 \cdot 7H_2O$	0.7
Yeast Extract (Difco)	1.0
Distilled water	1.0 liter

Isolated colonies from sterol degrading cultures were used to inoculate slants of nutrient agar: These served as the stock cultures. Stock cultures were maintained on medium B (Table V) under refrigeration.

Isolation of a Mixed Culture from Sewage

A medium consisting of 100 ml of pH 7 0.1 M phosphate buffer containing 0.1% $(NH_4)_2SO_4$, 0.5 grams Generol 122^R, and two drops of tween 80^R in a 300 ml Erlenmeyer flask was inoculated with 5 ml of raw untreated sewage collected at the Madisom, Wisconsin sewage treatment plant. The culture was incubated at 30^{o}C and 300 rpm in a New Brunswick Scientific Company environmental shaker. Aliquots of 0.1 ml were taken at weekly intervals diluted and plated on medium B agar (Table V). Individual colonies arising on these plates were used to inoculate slants of medium B. The slants were incubated at 30^{o}C for 24 hrs and used to inoculate 1 x 6 inch test tubes filled with 10 ml of medium C (Table VI) and capped with plastic closures (Bellco Glass Company). The tubes were incubated at 30^{o}C and 300 rpm for 24 hours at which time 0.05 ml of 100 mg/ml solution of Generol 122^R in N,N-dimethylformamide. The incubation was continued for four days at which time the tubes were extracted with $CHCl_3$ and analyzed by thin layer chromatography.

Table V

Medium B for Growth and Maintenance of the Sterol Degrading Cultures

Nutrient Broth (Difco)	8.0 grams
Glucose	10.0
Distilled water	1.0 liter
Agar	15 g/1

Stirred jar fermentations with the isolated mixed culture were run as described below (Fermentations section) except that the media used were those listed in Tables VI and VII.

Fermentations

Fermentations were carried out in 250 ml Erlenmeyer flasks plugged with cotton, or 1 x 6 inch test tubes with plastic caps (Bellco Glass Company). Incubations at 30° and 37°C were carried out in a New Brunswick Scientific Company controlled environment incubator shaker. Incubations at 25°C were run on a New Brunswick Scientific Company platform shaker. All shaken fermentations were aerated by shaking the flasks at 300 rpm.

Stirred jar fermentations were carried out in a 12 liter glass stirred jar apparatus (New Brunswick Scientific Company). The jars were filled with 8 liters of medium E (Table VIII) and the apparatus was assembled and sterilized at 121°C for one hour. The fermentor was then placed in a New Brunswick Scientific Company model FS-314 fermentation drive unit. Inoculum was 500 ml of nutrient broth cultures which were grown at 30°C and 300 rpm in a New Brunswick Scientific Company incubator shaker for 24 hours. The temperature of the fermentor was maintained at 30°C, aeration was at 4 liters/minute and the impellor speed was 300 rpm.

Table VI

Medium C for Cultivation of the Mixed Culture Isolated from Sewage

Nutrient Broth (Difco	8.0	grams
Yeast Extract	1.0	
Mannitol	10.0	
Distilled water	1.0	liter

Table VII

Medium D for Cultivation of the Mixed Culture Isolated from Sewage

NaCl	1.0 grams
$MgSO_4$ (anhydrous)	0.1
KH_2PO_4	0.5
Na_2HPO_4 (anhydrous)	1.2
$(NH_4)_2HPO_4$	1.0
Mannitol	10.0
Distilled water	1.0 liter

Table VIII

Medium E Minimal Salts Medium for Cultivation of *Nocardia* #22

Sterols (Generol 122^{R})	1.0 gram
$(NH_4)_2SO_4$	3.6
$MgSO_4 \cdot 7H_2O$	0.01
KH_2PO_4	13.6
$Na_2HPO_4 \cdot H_2O$	14.2
Na_3citrate	0.5
Trace metals solution	0.1 ml
Distilled water	1 liter

Trace metals solution (add 0.1 ml for each gram of carbon source)

$ZnSO_4 \cdot FH_2O$	0.144 gram
CuCl	0.0495
$MnSO_4 \cdot H_2O$	0.169
$CoCl_2 \cdot 6H_2O$	0.0012
NaCl	0.584
$CaCl_2 \cdot 2H_2O$	2.94
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0007
Conc. HCl	2 ml
Distilled water	1.0 liter

Taxonomic Methods

1. Sugar tests

The ability of the cultures to ferment sugars was tested using Durham tubes (150 mm x 16 mm outer tubes with 75 mm x 10 mm inverted inner tube capped with plastic caps--Bellco Glass Company) containing 7 ml of medium F (Table IX) which was supplemented with 0.5% of one of the following sugars: Glucose, galactose, fructose, mannose, ribose, maltose, sucrose, lactose, mannitol, or glycerol. The Durham tubes were inoculated with 0.5 ml of a 24 hour culture which was grown in nutrient broth.

2. Morphological observations

24 hour cultures which were grown in nutrient broth were used to inoculate plates of nutrient agar. Growth on the plates was removed with a sterile loop and observed in wet mounts. Observations were made at 18, 24, and 48 hours after inoculation to observe motility, and branching of the mycelial organisms. Gram stained preparations were prepared as described in Skerman (64) at 12 and 24 hours after inoculation. Acid fast stains were prepared as described in Skerman (64) using 24 hour cultures.

Table IX

Medium F for Sugar Tests

Peptone (Difco)	10.0 grams
Yeast Extract (Difco)	7.0
Bromcreasol purple (Allied Chemical Company)	0.004
Distilled water	1.0 liter

Analytical Methods

1. Extraction of fermentation samples

Samples of fermented media were extracted with 1/2 volume of chloroform. The samples were shaken using a vortex^{R} mixer and the chloroform layer was removed and dried under nitrogen.

2. Thin layer chromatographic method

Dried samples were dissolved in 0.2 ml $CHCL_3$ and 5 µl was spotted on silica gel HF-254 plates (Brinkmann). The plates were developed in benzene:ethylether (1:1). The plates were sprayed with sulphuric acid and charred at 100°C in a drying oven (Grieve Corporation).

3. Gas-liquid chromatographic method

The gas-liquid chromatography methods were developed and carried out by Mr. A. H. Conner, Forest Products Laboratories, U. S. Department of Agriculture, Madison, Wisconsin. Samples were dried in a vacuum oven at 60°C for 2-24 hours. One ml of dry pyridine and 0.25 ml N, O-bis-(trimethyl silyl)-acetamide (Pierce Chemical Company) were added to the samples. The samples were mixed on a Vortex^{R} mixer and heated at 100°C for no more than ten minutes. The samples were dried under a stream of nitrogen, 0.25 ml of ethylether:methanol (10:1) was added, and

the samples were again dried under a stream of nitrogen. The internal standard n-hexacosane was added on a basis of 1 mg for each theoretical 10 mg of sample. One ml of ethylether:methanol (10:1) was added. A small amount of methanol was added to any samples which were cloudy or showed a precipitate. Two µl of each sample was chromatographed in Research Specialties model 600 gas chromatograph using a 1.63 m x 4 mm i. d. glass column packed with 2.5% SE-30 (Supelco, Inc.) and 1.5% QF-1 (Allied Chemical Co.) on 100-120 mesh Gas Chrom Q (Allied Chemical Co.). Compounds were detected using a model 660-1 (Research Specialties) flame ionization detector.

Mutations

1. N-methyl-N'-Nitrosoguanidine

0.1 ml of a 4 mg/ml aqueous solution of N-methyl-N'-nitrosoguanidine (Aldrich Chemical Company, Inc.) was added to 10 ml of a 22 hour culture growing on medium B (Table V). This mixture was incubated without shaking at 30°C for 15 and 30 minutes. The cultures were centrifuged twice and resuspended in medium E (Table VIII). The cultures were centrifuged again and resuspended in medium E (Table VIII) with $\Delta^{1,4}$-androstadiene-3,17-dione instead of Generol 122^{R} as the sole carbon source, and incubated for 24 hours. Potassium penicillin G (Pfizer) was added

to give 250 U/ml and the cultures were incubated for five hours. The cultures were plated on nutrient agar and incubated for 24 hours. Nutrient agar plates were replicated onto medium E agar (Table VIII) with 0.5 g/L of Generol 122^{R}, $\Delta^{1,4}$-androstadiene-3,17-dione, or testosterone as the sole carbon source.

2. Mutations with ultra violet light

Ten ml of a 24 hour culture which was growing on medium A (Table IV) was diluted to 20 ml with medium A and exposed to an ultra violet source (model MLA-85 George W. Gates and Company). The lamp is a high pressure mercury vapor arc which gives u.v. light of wave lengths of 225.1 to 313.1 nm. Aliquots of 0.1 ml were taken at 15, 20, and 30 minutes of exposure and added to 9.9 ml of medium A (Table IV). Dilutions of these samples were plated on nutrient agar plates. After 24 hours incubation individual colonies were taken and used to inoculate 10 ml of medium A (Table IV) in 1 x 6 inch test tubes. Tubes were incubated at 30^{o}C and 300 rpm in a New Brunswick Scientific Company environmental shaker for 4 days and extracted with 1/2 volume of $CHCL_3$.

αα'dipyridyl Inhibition of Ring Cleavage

This method for the inhibition of ring degradation is based on Nagasawa et al (40). Ten ml of medium B (Table V) in a 1 x 6 inch test tubes were inoculated from a 48 hour old nutrient agar slant of the sterol degrading cultures. The inoculum was incubated at 25°, 30°, and 37°C at 300 rpm on rotary shakers. After 48 hours growth these cultures were used to inoculate 50 ml of the same medium. The cultures were incubated at the same temperature as the inoculum for 20 hours when 0.5 g/L or 1.0 g/L of Generol 122^{R} or cholesterol was added as a solution in dimethylformamide (Aldrich Chemical Company). Six hours after the sterol addition 124 mg/L or 248 mg/l of αα'dipyridyl was added in ethanol solution. The incubation was continued for two or four days at 25°, 30°, and 37°C. The cultures were then extracted with $CHCL_3$ and analyzed.

Mixed Culture Fermentations

1. Isolation of rhamnolipid

Rhamnolipid was isolated from a fermentation of Pseudomonas aeruginosa ATCC 7700 according to the method of Jarvis and Johnson (24). A 24 hour medium G (Table X) agar slant of Ps. aeruginosa ATCC 7700 was used to inoculate 250 ml of medium G (Table X) in 2 L Erlenmeyer flasks. After 4 days incubation at 30°C and 300 rpm in a New

Table X

Medium G for Cultivation of *Pseudomonas aeruginosa* ATCC 7700

Peptone (Difco)	40 grams
Glycerol	30
Distilled water	1.0 liter
Agar	15 grams

Brunswick Scientific Company environmental shaker, the culture was centrifuged and the broth was acidified to pH 2 with H_2SO_4 and refrigerated for 2 days. The crystals thus formed were collected by filtration and taken up in a small volume of ethylether. The rhamnolipid was precipitated from the ethylether with petroleum ether. The solvent was filtered off and the rhamnolipid was dried under aspiration and washed with water. From 1 liter of broth 0.5 grams of crystalline material was obtained. The melting point of the isolated material was 85°C which was the same as the material isolated by Jarvis and Johnson (24).

2. Mixed culture fermentation method

The method used for mixed culture fermentations was based on that of Awata (7). Pseudomonas aeruginosa ATCC 7700 grown on an agar slant of medium G (Table X) was used to inoculate 50 ml of medium H (Table XI) in a 250 ml flask. Nutrient agar slants of the sterol degrading cultures were grown for 48 hours at 30°C and then used to inoculate 1 x 6 inch test tubes containing 10 ml of medium I (Table XII). The flasks and tubes were shaken at 30°C for 24 hours and used to inoculate 50 ml of medium I (Table XII) at a rate of 3% by volume of a sterol degrading culture and 3% by volume of the Pseudomonas culture. At the time of inoculation 1.0 g/L of Generol 122^R in

Table XI

Medium H for Growth of *Pseudomonas aeruginosa* ATCC 7700

KH_2PO_4	3.0 grams
NH_4NO_3	2.0
NaCl	0.25
$Na_2HPO_4 \cdot H_2O$	1.0
$MgSO_4 \cdot 7H_2O$	0.5
Yeast Extract (Difco)	0.1
Distilled water	1.0 liter

Table XII

Medium I for Mixed Culture of Sterol Degrading Cultures and *Pseudomonas aeruginosa* ATCC 7700

Yeast Extract (Difco)	5.0 grams
Nutrient Broth (Difco)	2.5
$MgSO_4$ (anhydrous)	0.5
Glucose	5.0
Distilled water	1.0 liter

dimethylformamide solution and 0.02% rhamnolipid (0.1 ml of a 40 mg/ml ethanol solution) were added. The flasks were incubated for 48 hours at 30°C and 300 rpm in a New Brunswick Scientific Company environmental shaker, extracted and analyzed.

RESULTS

Isolation of Sterol Degrading Cultures

Soil samples gathered from the University of Wisconsin Botany Department greenhouses were used as sources for sterol degrading organisms. Fifteen soil samples of 1 gram each were diluted with 10 ml of 0.1 M phosphate buffer, supplemented with 2 grams of Generol 122^{R} (ground to a fine powder) and incubated at 30^{o}C. Aliquots of 0.1 ml from these enrichments were used to inoculate 1 x 6 inch test tubes containing 10 ml of medium A and capped with plastic closures (Bellco Glass Company). After incubation for four days on a rotary shaker the tubes were extracted with chloroform and tested by silica gel thin layer chromatography for reduction of the amount of starting material compared to uninoculated controls. Aliquots from the fermentations which showed complete or nearly complete loss of the starting material were streaked onto plates of medium B and incubated at 30^{o}C for two days. Individual colonies from these plates were used to inoculate tubes of medium A which were incubated on rotary shakers for four days at 30^{o}C. Those cultures which showed

degradation of the starting material when compared to uninoculated controls were retained as degrading cultures. Out of the fifteen soil samples, 48 cultures were selected which degraded sterols completely and 40 cultures which made conversions of the sterols to the Δ^4-3-keto analogs.

Of the 48 cultures initially designated as sterol degrading cultures 18 were subsequently discovered as mixed cultures. These cultures were contaminated by a large spore forming bacillus which when isolated by boiling broth cultures would not degrade sterols. The active organism in these mixed cultures could not be isolated from the spore former by subsequent streaking and dilution on medium B and were discarded. Of the remaining cultures 4 failed to survive transfer on agar slants of medium B and 2 were mixed cultures. The components of these 2 mixed cultures were isolated by streaking on medium B and isolating the various colony types involved. Each of these two cultures produced two organisms (25-1 and 25-2 from #25 and 34-1 and 34-2 from #34) which showed sterol degrading activity.

Table XIII shows the results of gas-liquid chromatagraphic analysis of the various sterol degrading cultures after incubation in test tubes for four days. The initial amount of sterol (Generol 122^R or cholesterol) was

Table XIII

Degradation of Sterols by Purified Cultures

Organism	Generol 122 Remaining (%)	Cholesterol Remaining (%)	Comments
1	10	t*	Δ^4-3-keto sterols produced from Generol 122^R
2	t	none	
3	2	none	Δ^4-3-keto sterols produced from Generol 122^R
4	t	t	
5	t	t	Δ^4-3-keto sterols produced from Generol 122^R
8	6	t	Δ^4-3-keto sterols produced from Generol 122^R
9	t	2	
10	8	2	Δ^4-2-keto sterols produced from Generol 122^R
11	4	t	Δ^4-3-keto sterols produced from Generol 122^R
12	2	t	Δ^4-3-keto sterols produced from Generol 122^R
13	t	t	
14	10	t	Δ^4-3-keto sterols produced from Generol 122^R
15	10	t	Δ^4-3-keto sterols produced from Generol 122^R
16	12	t	Δ^4-3-keto sterols produced from Generol 122^R and cholesterol
21	10	2	
22	2	2	
23	8	t	Δ^4-3-keto sterols produced from Generol 122^R and cholesterol

Table XIII (Continued)

Organism	Generol 122 Remaining (%)	Cholesterol Remaining (%)	Comments
24	16	t	
25-1	12	t	
25-2	14	t	Δ^4-3-keto sterols produced from Generol 122^R
26	8	2	Δ^4-3-keto sterols produced from Generol 122^R and cholesterol
27	8	2	Δ^4-3-keto sterols produced from Generol 122^R
29	4	t	Δ^4-3-keto sterols produced from Generol 122^R and cholesterol
30	4	t	Δ^4-3-keto sterols produced from Generol 122^R
31	14	none	Δ^4-3-keto sterols produced from Generol 122^R
32	26	none	Most of the remaining Generol 122^R is Δ^4-3-keto sterols
33	8	t	Δ^4-3-keto sterols produced from Generol 122^R
34-1	none	none	
34-2	2	t	trace of cholesterol remaining is Δ^4-cholesten-3-one

t* = trace amount

5 mg/tube. Trace amounts were less than 0.1 mg and could not be accurately measured by gas-liquid chromatagraphic analysis.

Identification of the Sterol Degrading Cultures

Taxonomic studies were performed using the programmed identification scheme of Skerman (64) as a guide. The majority of the sterol degrading isolates were observed to have life cycle type of growth. The morphological changes were typical of organisms from the genera Nocardia and Arthrobacter. Life cycles were observed in both Gram stained preparations and wet mount preparations. Preparations made from the growth of the organisms on plates of medium C were observed by Gram stain at 12 and 24 hours after inoculation. Wet mount preparations were observed after 18, 24, and 48 hours growth on medium B.

The assignment of organisms to Arthrobacter or Nocardia is primarily dependent upon the morphology of young cultures and the final state of the older cultures. Young cultures of the genus Nocardia are characterized by well defined mycelia which show distinct branching. Older cultures fragment into rods which may be difficult to distinguish from large cocci. Young cultures of Arthrobacter are either rods on mycelia which show no branching or very rudimentary branching. Older cultures of Arthrobacter

fragment completely to cocci. The two major considerations for assignment of organisms to Arthrobacter or Nocardia are: 1) branching in young cultures and 2) complete fragmentation to cocci or rods in older cultures.

In addition to the morphological observations, all cultures were examined for their ability to ferment carbohydrates to acid and gas. The cultures were also examined for their Gram and acid fast staining characteristics.

Several of the isolates did not exhibit any morphological variation. These cultures were tested (in addition to the tests mentioned above) for their ability to reduce nitrate to nitrite, their ability to undergo mixed acid fermentations (methyl red test), their ability to produce acetoin from glucose (Voges-Proskauer test), the ability to oxidativly utilize glucose (Hugh-Leifson test for oxidative or fermentative metabolism of glucose), motility, the production of acetic acid from ethanol, and their ability to degrade cellulose.

None of the sterol degrading cultures produced acid or gas from glucose, galactose, fructose, mannose, ribose, maltose, sucrose, lactose, mannitol, or glycerol. None of the organisms were acid fast. The results of the taxonomic studies are summarized in the following tables.

Table XIV

Taxonomic Characteristics of Sterol Degrading Organisms Which Display a Life Cycle Type of Growth

Culture	Gram Stained Preparations		Wet Mount Preparations			Tentative Classification
	12 hr	24 hr	18 hr	24 hr	48 hr	
2	Gram positive rods singly and in pairs	Gram positive cocci	non-motile rods	non-motile rods & cocci	clumps of cocci	Arthrobacter
3	Gram positive rods singly and in pairs	Gram positive cocci	non-motile rods	non-motile rods & cocci	clumps of cocci	Arthrobacter
4	Gram positive rods	Gram Positive cocci	non-motile rods & cocci	non-motile rods & cocci	clumps of cocci	Arthrobacter
5	Gram positive rods	Gram positive cocci	non-motile rods	non-motile rods	non-motile cocci	Arthrobacter
8	Gram positive rods	Gram positive cocci	non-motile rods & cocci	non-motile rods & cocci	non-motile cocci	Arthrobacter
9	Gram positive rods singly, pairs and short chains	Gram positive cocci	small non-motile rods	non-motile rods	non-motile cocci	Arthrobacter
10	Gram positive rods	Gram positive cocci	non-motile rods, singly pairs and short chains	non-motile rods	non-motile cocci	Arthrobacter
11	Gram positive rods	Gram positive cocci	non-motile rods	non-motile rods & cocci	non-motile cocci	Arthrobacter
12	Gram negative rods singly & in pairs	Gram positive cocci	non-motile rods in short chains	non-motile rods	non-motile cocci	Arthrobacter
13	Gram positive rods	Gram positive cocci	non-motile rods, singly pairs and short chains	non-motile rods	non-motile cocci	Arthrobacter
14	Gram positive rods and mycelial fragments which show branching	Gram positive small rods	non-motile rods and mycelial fragments showing branching	non-motile rods and mycelia	non-motile small fat rods	Nocardia

Table XIV (Continued)

Culture	Gram Stained Preparations		Wet Mount Preparations			Classification
	12 hr	24 hr	18 hr	24 hr	48 hr	
18	Gram positive mycelia with distinct branching	Gram positive rods	non-motile mycelial fragments	non-motile mycelial fragments and small rods	non-motile small rods	Nocardia
22	Gram positive rods and mycelial fragments showing branching	Gram positive rods and mycelial fragments	non-motile rods and branched mycelia	non-motile rods and branched mycelia	non-motile small fat rods	Nocardia
25-1	Gram positive rods	Gram positive cocci	small motile rods	small rods and cocci	small rods and cocci	Arthrobacter
25-2	Gram positive rods and branching mycelia	Gram positive rods	non-motile rods and mycelial fragments	non-motile rods and mycelial fragments	non-motile short fat rods	Nocardia
27	Gram positive branching mycelia	Gram positive small rods	non-motile rods and branching mycelia	non-motile rods and mycelia	small fat rods in clumps	Nocardia
29	Gram positive branching mycelia	Gram positive small rods	branching mycelia	branching mycelia	small rods in clumps	Nocardia
30	Gram positive branching mycelia	Gram positive rods	non-motile rods and branching mycelia	non-motile rods and mycelial fragments	small fat rods in clumps	Nocardia
31	Gram positive rods singly and short chains	Gram positive cocci	non-motile rods singly and short chains	non-motile rods	non-motile cocci	Arthrobacter

Table XIV (Continued)

Culture	Gram Stained Preparations		Wet Mount Preparations			Classification
	12 hr	24 hr	18 hr	24 hr	48 hr	
32	Gram positive rods singly and short chains	Gram positive cocci	non-motile rods singly and short chains	non-motile rods singly and short chains	non-motile cocci	Arthrobacter
33	Gram positive rods singly and in pairs	Gram positive cocci	non-motile short fat rods singly and in pairs	non-motile rods	non-motile cocci	Arthrobacter
34-1	Gram positive rods	Gram positive cocci	non-motile rods singly and short chains	non-motile rods	non-motile rods	Arthrobacter
34-2	Gram positive rods and branching mycelial fragments	Gram positive small rods	branching mycelia fragments	branching mycelia fragments	non-motile small rods	Nocardia

Table XV

Taxonomic Characteristics of the Sterol Degrading Cultures
Which Do Not Display Life Cycles

Organism	Gram Stain	Nitrate Reduction Test	Vogue-Proskauer Reaction	Methyl Red Test	Hugh-Leifson Reaction	Degradation of Cellulose	Acetic Acid from Ethanol	Tentative Classification
16	negative	-	negative	-	oxidative	-	-	Pseudomonas
21	negative	+	negative	-	oxidative	-	-	Pseudomonas
23	negative	+	negative	-	oxidative	-	-	Pseudomonas
24	negative	-	negative	-	oxidative	-	-	Pseudomonas
26	positive	+	negative	-	oxidative	-	-	Brevibacterium

Mutations

Attempts were made to select sterol degrading mutant cultures that would no longer degrade the sterol nucleus while retaining the ability to degrade the sterol side chains. Two systems of mutation were used, N-methyl-N'nitrosoguanidine as the mutagenic agent, and ultra violet light.

Mutations with nitrosoguanidine were carried out using isolates which were shown to be sensitive to potassium penicillin G in an agar diffusion assay. Cultures were designated as penicillin sensitive if they had a zone of clearing of at least 11 mm around a 6.35 mm paper disc containing 5 units of potassium penicillin G (Pfizer, Inc.). The discs were placed on plates of medium B agar which was seeded with the culture to be tested. The zones were observed after 48 hrs incubation at 30°C. Cultures #5, 9, 13, 14 and 22 were sensitive to penicillin. These cultures were grown in 1 x 6 inch test tubes containing 10 ml of medium B. After 24 hrs growth 4.0 mg of nitrosoguanidine in 0.1 M Tris at pH 7.0 was added. After 15 minutes the cultures were centrifuged twice, resuspended in medium D with $\Delta^{1,4}$-androstadiene-3,17-dione in place of sterols as the sole carbon source, and incubated for 24 hrs. After incubation 250 U/ml of potassium penicillin G was added. Table XVI shows the $O.D._{660}$ of the cultures

after each stage of treatment. Growth of the cultures on minimal media containing $\Delta^{1,4}$-androstadiene-3,17-dione was good. The penicillin treatment was effective in lysing culture 22. Cultures 5 and 14 were moderately affected by the penicillin treatment and culture 13 was only slightly affected by the penicillin. After exposure to penicillin the cultures were diluted and plated on medium B (agar). After 24 hours incubation at 30°C the plates which showed well separated colonies were replicated onto medium E with sterols, $\Delta^{1,4}$-androstadiene-3,17-dione, or testosterone as the sole fermentable carbon source. Growth on the replicate plates was sparce, especially on the plates containing sterols as the carbon source. Mutant colonies could not be detected by the replicate method.

Isolated colonies from the medium B plates of the cultures treated with nitrosoguanidine were used to inoculate test tubes containing 10 ml of medium A. Thirty isolated colonies of each of the six cultures were tested. Thin layer chromatographic analysis of these mutant cultures showed none that were able to convert sterols to $\Delta^{1,4}$-androstadiene-3,17-dione. Fifteen of the mutant cultures no longer degraded sterols.

Ultra violet light was used as a mutagenic agent for cultures #6, 9, 13, 22, 27, and 29. The organisms were grown for 48 hours on medium B (broth) and exposed to ultra

Table XVI

Growth of Sterol Degrading Cultures After Various Steps in Nitrosoguanidine Mutations

Culture	Nitrosoguanidine Treatment	Centrifugation After Nitrosoguanidine Treatment	24 hrs Growth in $\Delta^{1,4}$-androstadiene-3,17-dione Media	4 hrs exposure Potassium Penicillin G Treatment
5	0.46	0.38	0.61	0.56
9	0.8	0.54	1.10	0.95
13	2.0	0.85	1.25	1.20
14	1.1	0.57	0.90	0.80
22	1.5	0.135	0.50	0.36

*Growth was measured by the change in the $O.D._{660}$. O.D.'s were measured in a Bausch and Lomb Spectronic 20^{R} using whole cultures.

violet light from a high pressure mercury vapor arc lamp 30 cm from the surface of a petri plate containing 20 ml of culture broth being stirred continuously by a magnetic stirrer. Aliquots were taken at intervals during exposure for a kill analysis. The aliquots were diluted and plated on medium B (agar) and incubated for 24-48 hours until colonies were visible. Analysis of the survival kinetics of the mutation treatment showed that exposure of 15-30 minutes gave 99% kill (Figure VI). Colonies from these plates were used to inoculate 1 x 6 inch test tubes of medium A and incubated for four days at 30°C on rotary shakers. As many colonies were selected from a single plate as could be easily isolated. This method of choosing colonies somewhat increases the chance of selecting a mutant clone.

None of the survivors of ultra violet light exposure showed conversion of sterols to $\Delta^{1,4}$-androstadiene-3,17-dione. However, 10% of the survivors tested lost the ability to degrade sterols. The results of the ultra violet mutations are summarized in Table XVII.

One of the mutants of organism #9 which showed loss of sterol degrading activity was used for a mutation by ultra violet light. Of the 100 colonies tested only two cultures regained sterol degrading activity and none converted sterols to 17-keto steroids.

Figure VI. Survival of Sterol Degrading Cultures Following Exposure to Ultra Violet Light.

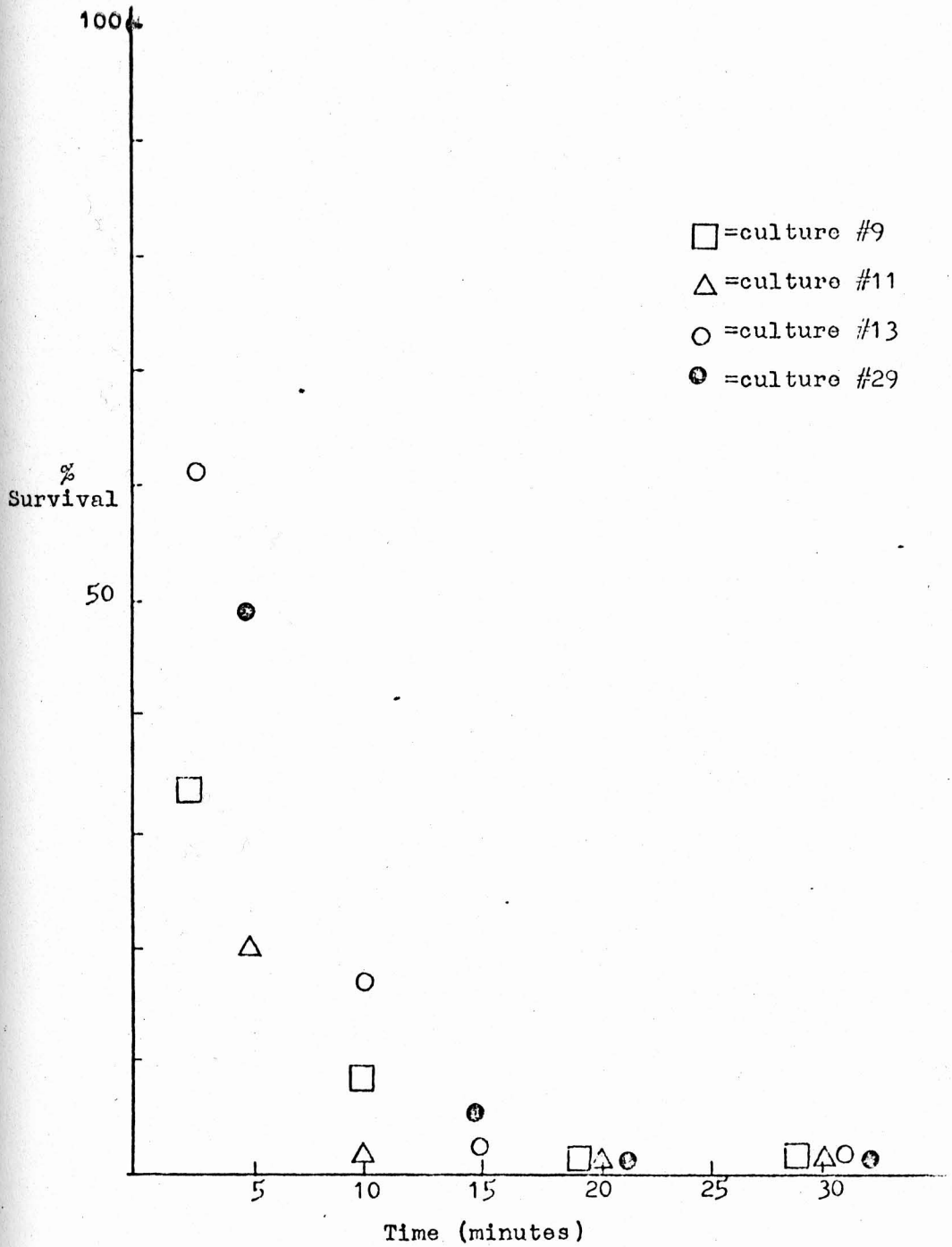

100
% Survival
50
□=culture #9
△=culture #11
○=culture #13
❶ =culture #29
5
10
15
20
25
30
Time (minutes)

Table XVII

Results of Ultra Violet Mutations of Sterol Degrading Organisms

Organism	Number of Survivors Tested	Number of Survivors that Lost Sterol Degrading Activity
6	50	4
9	680	24
11	100	7
13	100	15
27	40	4
29	200	31

Inhibition of Sterol Ring Degradation by αα'dipyridyl

A number of investigators have proven the ability of αα'dipyridyl to inhibit microbial sterol ring degradation without affecting the cleavage of the sterol side chains (80, 4, 40-43, 38, and 39). The ability of this inhibitor to promote selective degradation of sterol side chains was tested with the various isolates. All of the isolates were tested in 1 x 6 inch test tubes containing 10 ml of medium and incubated at 30°C and 300 rpm.

Each culture was tested for its ability to convert cholesterol and Generol 122^{R} to $\Delta^{1,4}$-androstadiene-3,17-dione in the presence of αα'dipyridyl (124 mg/L). Table XVIII shows the results that were obtained from gas-liquid chromatographic analysis of the chloroform extracts of these fermentations. The results showed only trace amounts of $\Delta^{1,4}$-androstadiene-3,17-dione in the chloroform extracts.

Flask fermentations were run to provide enough material for gas-liquid chromatographic analysis. In these studies ten organisms which had demonstrated the ability to accumulate $\Delta^{1,4}$-androstadiene-3,17-dione in the presence of αα'dipyridyl were chosen for further study. These organisms were #1, 3, 9, 12, 14, 22, 26, 27, 32, and 34-2.

Fermentations were carried out at 25°, 30°, and 37°C. Generol 122^{R} and cholesterol were added 20 hours

Table XVIII

$\Delta^{1,4}$-androstadiene-3,17-dione Production by Sterol Degrading Cultures from Generol 122^{R} and Cholesterol in the Presence of αα'dipyridyl

Organism	Generol 122^{R} Remaining* (%)	$\Delta^{1,4}$-androstadiene-3,17-dione from Generol 122^{R}	Cholesterol Remaining** (%)	$\Delta^{1,4}$-androstadiene-3,17-dione from Cholesterol
1	10	+	t***	+
2	+	+	none	+
3	2	+	none	+
4	t	+	t	+
5	t	+	t	+
8	6	+	t	+
9	t	+	2	+
10	8	+	2	+
11	4	+	t	+
12	2	+	t	+
13	t	+	t	+
14	10	+	t	+
15	10	-	t	-
16	12	-	t	-
21	10	+	2	-
22	2	+	2	+
23	8	-	t	-
24	16	-	t	-
25-1	12	-	t	-
25-2	14	-	t	-
26	8	+	2	+
27	8	+	2	+
29	4	+	t	-
30	4	-	t	+

Table XVIII (Continued)

Organism	Generol 122^{R} Remaining* (%)	$1,4$-androstadiene-3,17-dione from Generol 122^{R}	Cholesterol Remaining** (%)	$1,4$-androstadiene-3,17-dione from Cholesterol
31	14	-	none	-
32	26	-	none	+
33	8	+	t	+
34-1	none	+	none	+
34-2	2	-	t	+

*Control tubes containing 5 mg of Generol 122^{R} but no αα'dipyridyl.

**Control tubes containing 5 mg of cholesterol with no αα'dipyridyl.

***t = trace amounts.

after inoculation to a final concentration of 0.5 g/L and αα'dipyridyl was added at 26 hours after inoculation to final concentrations of 124 or 248 mg/L. The fermentations were continued for four days after the addition of the inhibitor.

Tables XIX and XX summarize the results of these fermentations. Many of the fermentations showed only trace amounts of $\Delta^{1,4}$-androstadiene-3,17-dione and most samples showed extensive microbial degradation of the starting material. In some samples the only material recovered was $\Delta^{1,4}$-androstadiene-3,17-dione.

To increase the recovery of steroid material the amount of starting material was increased to 1.0 g/L. αα'dipyridyl was added to 124 mg/L. The fermentations were run at 25°, 30°, and 37°C and 300 rpm for four days. The results of these fermentations are shown in Table XXI.

An additional experiment was carried out using αα'dipyridyl to inhibit sterol ring structure degradation. The conditions for this experiment were sterols at 0.5 g/L; αα'dipyridyl at 124 mg/ml and incubation was at 30°C and 300 rpm. The results of this experiment are summarized in Table XXII.

Table XIX

Inhibition of Sterol Ring Degradation of Generol 122^{R} by αα'dipyridyl

Organism	αα'dypridyl mg/L	Incubation Temp. °C	Recovered Material Converted to $\Delta^{1,4}$-androstadiene-3,17-dione (%)	Sterol Material Recovered (%)
1	124	30	9	23
1	248	30	9	24
1	124	25	9	23
1	248	25	16	13
1	124	37	16	27
3	124	25	9	24
3	248	25	10	19
9	124	30	30	6
9	248	30	39	5
9	124	25	26	8
9	248	25	21	10
12	124	30	3	85
12	124	25	22	19
12	248	25	9	23
12	124	37	21	10
14	124	30	3	80
14	124	25	4	54
32	124	30	15	28
32	124	25	16	13
32	248	25	8	27
34-2	124	37	8	26

Table XX

Inhibition of Cholesterol Ring Degradation by αα'dipyridyl

Organism	αα'dipyridyl mg/L	Incubation Temp. °C	Recovered Material Converted to $\Delta^{1,4}$-androstadiene-3,17-dione (%)	Sterol Material Recovered (%)
1	124	37	50	4
1	248	37	100	2
3	248	25	100	2
3	124	37	25	8
9	124	37	21	20
9	248	37	46	8
32	124	25	100	2
32	124	37	50	3
32	248	37	33	5

Table XXI

Inhibition of Sterol Ring Degradation of 1.0 g/L Generol 122^{R} or Cholesterol by αα'dipyridyl

Organism	Sterol Added	Temperature of Incubation (%)	Recovered Material Converted to $\Delta^{1,4}$-androstadiene-3,17-dione (%)	Sterol Material Recovered (%)
1	Generol 122^{R}	25	2	26
1	Generol 122^{R}	37	1	32
1	Cholesterol	25	t*	6
1	Cholesterol	30	25	2
1	Cholesterol	37	t	0.4
3	Generol 122^{R}	25	t	17
3	Generol 122^{R}	37	t	20
3	Cholesterol	25	t	2
3	Cholesterol	37	40	2
9	Generol 122^{R}	25	4	20
9	Generol 122^{R}	30	3	16
9	Generol 122^{R}	37	t	22
9	Cholesterol	25	10	4
9	Cholesterol	30	43	3
9	Cholesterol	37	t	2
12	Generol 122^{R}	25	2	26
12	Generol 122^{R}	30	1	32
12	Generol 122^{R}	37	t	29

Table XXI (Continued)

Organism	Sterol Added	Temperature of Incubation (%)	Recovered Material Converted to $\Delta^{1,4}$-androstadiene-3,17-dione (%)	Sterol Material Recovered (%)
12	Cholesterol	25	t	14
12	Cholesterol	30	20	2
12	Cholesterol	37	t	.4
14	Generol 122^{R}	25	2	26
14	Generol 122^{R}	37	t	35
14	Cholesterol	25	t	9
14	Cholesterol	37	50	1
32	Generol 122^{R}	25	2	17
32	Generol 122^{R}	30	3	14
32	Generol 122^{R}	37	1	28
32	Cholesterol	25	11	4
32	Cholesterol	30	17	2
32	Cholesterol	37	33	1
34-2	Generol 122^{R}	25	3	23
34-2	Cholesterol	25	t	16

*t = trace amounts

Table XXII

Production of $\Delta^{1,4}$-androstadiene-3,17-dione from Generol 122^{R} and Cholesterol by Sterol Degrading Cultures After 48 Hours in the Presence of αα'dipyridyl

Organism	Sterol Added	Temperature of Incubation (%)	Recovered Material Converted to $\Delta^{1,4}$-androstadiene-3,17-dione (%)	Sterol Material Recovered (%)
1	Generol 122^{R}	25	4	21
1	Generol 122^{R}	30	6	14
1	Generol 122^{R}	37	5	17
1	Cholesterol	25	9	9
1	Cholesterol	37	100	1
3	Generol 122^{R}	25	t	10
3	Generol 122^{R}	37	8	21
3	Cholesterol	25	t	32
3	Cholesterol	25	t	2
9	Generol 122^{R}	25	6	14
9	Generol 122^{R}	30	6	13
9	Generol 122^{R}	37	t	19
9	Cholesterol	25	3	23
9	Cholesterol	30	75	3
9	Cholesterol	37	100	2
12	Generol 122^{R}	25	4	18
12	Generol 122^{R}	30	4	18
12	Generol 122^{R}	37	t	22

Table XXII (Continued)

Organism	Sterol Added	Temperature of Incubation (%)	Recovered Material Converted to $\Delta^{1,4}$-androstadiene-3,17-dione (%)	Sterol Material Recovered (%)
12	Cholesterol	25	33	2
12	Cholesterol	30	100	1
12	Cholesterol	37	50	2
14	Generol 122^{R}	25	3	28
14	Generol 122^{R}	37	t	24
14	Cholesterol	25	t	16
14	Cholesterol	37	t	6
32	Generol 122^{R}	25	4	21
32	Generol 122^{R}	30	4	20
32	Generol 122^{R}	37	t	34
32	Cholesterol	25	t	27
32	Cholesterol	30	75	3
32	Cholesterol	37	50	2
34-2	Generol 122^{R}	25	2	44
34-2	Cholesterol	25	5	2

Characterization of the Unusual Metabolic Product of Nocardia #22

The growth of Nocardia #22 in medium E with Generol 122^R as the sole carbon source was accompanied by the accumulation of compound which showed up on thin layer chromatography plates as a spot with an R_f of about 0.24 in benzene:ethylether (1:1). It was initially supposed that this compound was a steroid due to similarity of its R_f in the thin layer chromatography system with those of other steroid products. The compound produced a brownish orange spot on charring. Table XXIII gives the thin layer chromatography data for various steroid molecules.

The compound was not produced in large enough amounts in small scale fermentations to allow characterization, so a stirred jar fermentation was undertaken. A 12 L glass stirred jar fermentation apparatus (New Brunswick Scientific Company) was filled with 8 L of medium E using 1 g/l of Generol 122^R as the carbon source. The apparatus was assembled and sterilized for one hour at 121^{o}C. After cooling, the fermentor was inoculated with 500 ml of a 24 hour nutrient broth culture of Nocardia #22. The culture was aerated a 4 L air/min and stirred at 300 rpm.

After five days incubation at 30^{o}C the broth was extracted with 4 L chloroform. The chloroform extract was taken to dryness on a rotary evaporator, dissolved in a

small volume of chloroform, and applied to a thin layer chromatography plate. After development the spot corresponding to the Nocardia #22 metabolite was scraped from the plate and compound was eluted from the gel using chloroform. Thirteen mg of material was recovered from about 0.5 g of Generol 122^{R} using this process. The compound was further purified by filtration and crystallization from hexane.

The metabolite was characterized by the following data: mp 199^{o}C (dec.); Corr. NMR (60 MHz, $CDCl_3$) 1.00 (3H, S, $C\underline{H}_3$), 3.51 (1 H, M, -C$\underline{H}$-O), and 7.70 (1 H, S, HOOC=C$\underline{H}$-O); λ_{max}^{EtOH} nM:237 with a shoulder at 230; V_{max}^{KBr} cm^{-1}: 3400-2400(COOH), 1740 (pentanone), 1680 (COOH), 1630 (C=C), and 1225, 1195 (C-O). The compound was methylated with a slight excess of CH_2N_2 to give the methyl ester which was >95% pure by thin layer chromatography and gas-liquid chromatography. This compound gave the following data: NMR (60 MHz, -CCl_4): 0.96 (3H, S, $C\underline{H}_3$), 3.47 (1H, M, C$\underline{H}$-O), 3.67 (3H, S, $COOC\underline{H}_3$) and 7.47 (1 H, S, CH_3OOC-C=C$\underline{H}$-O); λ_{max}^{EtOH}nM (ε):240 (12,300); V_{max}^{KBr} $cm^{-}1$: 1740 (pentanone), 1710 ($COOCH_3$), and 1620 (C=C). Mass spectroscopy of the methylated compound gave a molecular ion at 264 which is consistent with the formula $C_{15}H_{20}O_4$. Figure VIII shows the structural formula which is consistent with the data, and the first two mass fragments obtained by mass

Table XXIII

Mobilities of Steroid Compounds in Thin Layer Chromatography

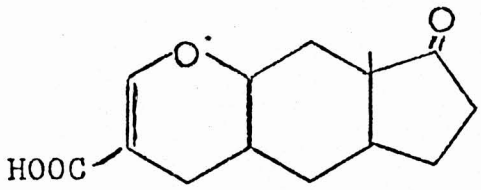
Compound	R_f in Solvent System A[1]	R_f in Solvent System B[2]	Color[3]
$\Delta^{1,4}$-androstadiene-3,17-dione	0.66	0.27	Orange
Δ^{4}-androsten-3,17-dione	0.75	0.39	Green
Sitosterol (cholesterol)	0.71	0.50	Pink
20α-hydroxymethyl-4-pregnen-3-one	0.57	0.30	Yellow
20α-hydroxymethyl-1,4-pregnadien-3-one	0.48	0.22	Brown
#22 Product	0.9	0.24	Brown

[1]Solvent system A = $CHCl_3$:Me_2CO (5:1)

[2]Solvent system B = Benzene-Et_2O (1:1)

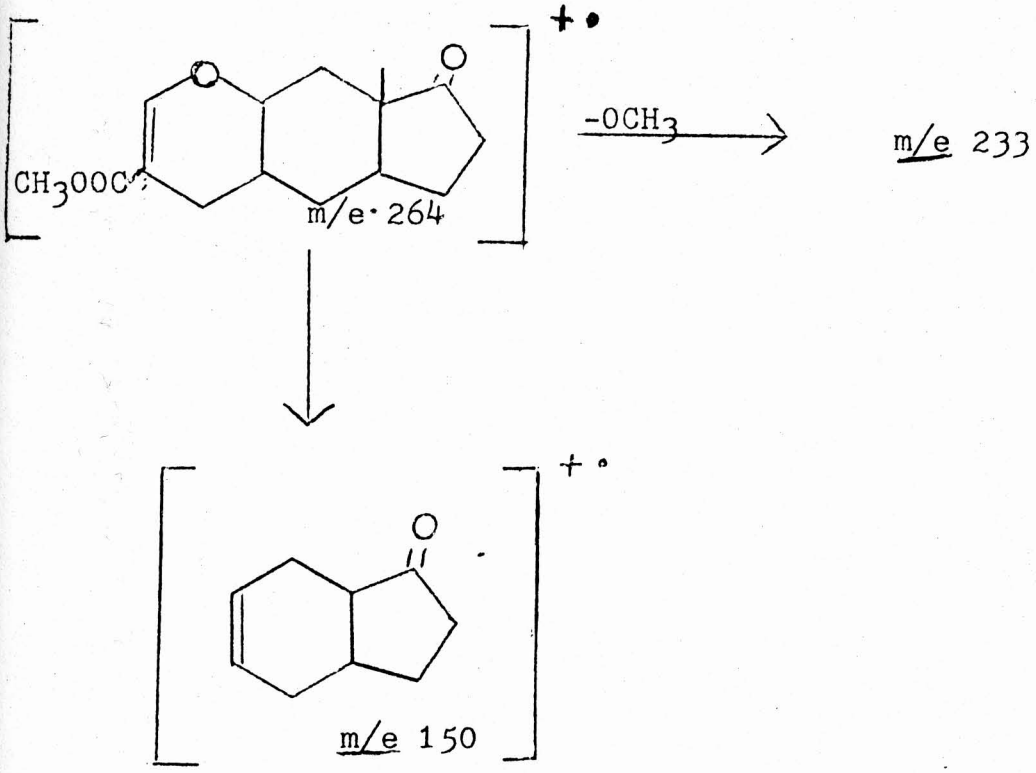
[3]On charring at 100°C with H_2SO_4 spray

Figure VII. The Metabolite Produced by Norcadia #22 from Sterols.



Compound X

Figure VIII. Major Mass Fragments of Methylated Compound X.


+•
CH3OOC
m/e 264
-OCH3
m/e 233
+•
m/e 150

spectroscopy. Further confirmation of this structure was provided by ^{13}C-NMR.

Fermentations of Nocardia #22 using pure sitosterol and pure cholesterol as starting material showed production of compound X--(Figure VII) by thin layer chromatography analysis. The metabolic route by which this compound is produced is unknown but is obviously different from the major pathway for sterol degradation proposed by Sih (54, 57, 19) (Figure II in Introduction) since the first ring cleavage is at C-9 of the sterol molecule where the corresponding bond is unbroken in compound X.

Fermentations of C-3 Modified Sterols

Each of the sterol degrading organisms was grown in 1 x 6 inch test tubes, capped with plastic caps (Bellco Glass Company), containing 10 ml of medium B containing 0.5 g/L of the following C-3 modified sterols: 5-cholesten-3-ol ethylether, 5-cholesten-3-ol benzoate, 5-cholesten-3-ol acetate, 5-cholesten-3-ol-n-propionate, and 5-cholesten-24b-ethyl-3-ol acetate. Fermentations were carried out at 30°C on a rotary shaker at 300 rpm for four days.

Analysis of the chloroform extracts of these fermentations showed no degradation products of either 17-keto steroids or cholesterol. Since cholesterol was not produced, it is apparent that the groups at C-3 block

recognition by sterol degrading enzymes or by transport systems in the cells.

Mixed Culture Fermentations

The sterol degrading cultures and Pseudomonas aeruginosa ATCC 7700 were co-fermented with 0.5 g/L of Generol 122^{R}. The fermentations were carried out at 30^{o}C and 300 rpm in an incubator shaker. Rhamnolipid (a compound isolated from Pseudomonas aeruginosa ATCC 7700 -- see Methods section) was added to the fermentation at a rate of 0.2%. After two days of fermentation the flasks were analyzed. No conversion of the starting material to 17-keto steroids was found. The cultures also failed to degrade the sterol material with virtually all of the starting material being recovered.

Isolation of a Mixed Culture from Sewage

An enrichment culture containing 5.0 ml raw untreated sewage and 100 ml pH 7.0 phosphate buffer supplemented with 0.1% $(NH_4)_2SO_4$ and 0.5 grams Generol 122^{R} was incubated at 30^{o}C and 300 rpm in a New Brunswick Scientific Company incubator shaker. After 22 days of incubation an aliquot was diluted and plated on Medium B agar. Individual colonies were selected and used to inoculate slants on medium C agar. After 24 hours incubation at 30^{o}C the slants were used to inoculate 1 x 6 inch test tubes of media C.

The tubes were incubated for 24 hours at 30°C and 300 rpm in a New Brunswick Scientific Company environmental shaker at which time 0.5 g/L of Generol 122^{R} was added in N,N-dimethylformamide solution. The tubes were returned to the shaker and the incubation was continued for four days.

Chloroform extracts of the test tube fermentations were tested by thin layer chromatography and three isolates showed conversion of Generol 122^{R} to $\Delta^{1,4}$-androstadiene-3,17-dione. These isolates were characterized as Gram positive rods which did not produce spores. The cultures were not acid fast in the initial characterizations and were characterized as Corynebacterium by the methods described in the Materials and Methods section.

Subsequent work with one of these cultures iVIII24 revealed that it was contaminated by an acid fast rod which had not appeared in the original characterization. This organism was shown to be identical (by comparison of mycolic acid content) with Mycobacterium NRRL 3683 isolated by Marsheck et al (36). This organism was being studied in the laboratory at the time. The other two cultures which had the ability to convert sterols to $\Delta^{1,4}$-androstadiene-3,17-dione were also found to contain Mycobacterium NRRL 3683. The second organism in the three cultures was the Corynebacterium that was originally classified. The growth of the Corynebacterium species was much greater than the

Mycobacterium on nutrient agar slants and this masked the presence of the Mycobacterium. As the yield of conversion products increased in subsequent sub-cultures the appearance of the culture on the slants began to change. The proportion of Mycobacterium NRRL 3683 increased to the point where they could be seen in acid fast stained preparations and could be isolated by streaking.

The mixed culture had the ability to convert Generol 122^R to $\Delta^{1,4}$-androstadiene-3,17-dione in a defined media (medium D) at a much greater efficiency than Mycobacterium NRRL 3683 alone. This property of the mixed culture can be seen in Table XXIV which compares the results of stirred jar fermentations using the mixed culture iVIII24 and Mycobacterium NRRL 3683. The conversion of Generol 122^R to $\Delta^{1,4}$-androstadiene-3,17-dione in stirred jars by iVIII24 in defined media nearly equal to that of Mycobacterium NRRL 3683 in nutrient medium.

Table XXIV

Stirred Jar Fermentations with *Mycobacterium* NRRL 3683 and Culture iVIII24

Culture	Media[1]	Initial Generol 122[R] Concentration (g/L)	Efficiency of Conversion to $\Delta^{1,4}$-androsta-dine-3,17-dione (%)	Sterol Material Recovered (%)
3683	C	1.0	91	97
3683	C	1.0	90	71
3683	C	0.5	95	62
3683	D + 0.05% casamino acids[2] (Difco)	1.0	10	31
3683	D + 0.25% casamino acids[2] (Difco)	1.0	7	28
3683	D + 0.5% yeast extract	1.0	23	44
iVIII24	D	0.5	99	27
iVIII24	D	1.0	82	49
iVIII24	D + 1.0% yeast extract	0.5	4	8
iVIII24	D + 1.0% casamino acids	0.5	50	60

[1]Media is described in the Materials and Methods section; C is a nutrient media, D is a defined media.

[2]Casamino acids were vitamin free.

DISCUSSION

In preliminary experiments soil samples from various locations on the University of Wisconsin campus were diluted and used to inoculate nutrient agar plates. Individual colonies which developed on the agar plates were used to inoculate test tubes containing nutrient medium and 0.5 g/L of Generol 122^{R}. Approximately 300 colonies were tested in this manner. Of these isolates, eight were found to decrease the level of Generol 122^{R} when compared to uninoculated controls and 30 produced Δ^{4}-3-keto sterols from Generol 122^{R}. Sterol degrading cultures were even more readily isolated from enrichment cultures consisting of 10 ml of phosphate buffer, 1 gram of soil and 2 grams of Generol 122^{R}. When these enrichment cultures were incubated at 30^{o}C for 10-14 days about 25% of the colonies arising on nutrient agar plates had the ability to degrade sterols.

The cultures isolated in this study were characterized as belonging to the genera Arthrobacter, Nocardia, Pseudomonas, and Brevibacterium. Members of other genera of bacteria have also been shown to degrade sterols (5). The majority of the isolates proved to be either

Arthrobacter or Nocardia. This observation is consistent with the results of Turfitt's original report of cholesterol degrading cultures in soil (72). In contrast to the organisms isolated by Turfitt (72, 73) the cultures isolated in this study were able to completely degrade 0.5 g/L of sterols in four days in medium containing organic nutrients.

Organisms which degrade only the side chain of sterols have been developed by Marsheck et al by mutation (28,36). Examples of these organisms are Mycobacterium NRRL 3683 and Mycobacterium NRRL 3805 which were developed from a soil organism which converted cholesterol to small amounts of $\Delta^{1,4}$-androstadiene-3,17-dione. The amount of conversion was increased by successive mutations. The organisms which were isolated in the present study did not accumulate $\Delta^{1,4}$-androstadiene-3,17-dione as a by-product of sterol degradation. Attempts to mutate several of the isolates such that they acquire the ability to convert sterols to 17-keto steroids have been unsuccessful. It is possible that such a mutant could be discovered owing to the fact that a single enzymatic reaction (the 9α - hydroxylation or 1,2 dehydrogenation is essential for initiation of degradation of the sterol ring structure, but not for side chain degradation.

The 9α-hydroxylase reaction can be inhibited to prevent the sterol ring from being degraded while allowing the side chain to be removed (80). One of the most potent inhibitors of the 9α-hydroxylase enzyme is αα'dipyridyl (37,40). Several of the organisms isolated in this laboratory were found to produce $\Delta^{1,4}$-androstadiene-3,17-dione from Generol 122^{R} and cholesterol upon exposure to αα'dipyridyl. The inhibition of sterol degradation by αα'dipyridyl was not complete as is shown by the low recoveries of the sterols added to the fermentations. In several cases the major component of the $CHCl_3$ extractions of fermentations of media containing cholesterol and αα'dipyridyl was $\Delta^{1,4}$-androstadiene-3,17-dione (Tables XVII-XX). The amount of added cholesterol which was recovered in these fermentations was very low but the fact that the conversion product was the major component extracted would be useful in a large scale process where the separation of the starting material from the product is a major consideration.

Certain chemical modifications of the sterol ring structure such as the hydroxylation at C-19 inhibit the degradation of the sterol nucleus without affecting the side chain degradation (56,63). Several sterol derivatives modified by esterification at C-3 (see Materials and Methods section) were used as substrates in fermentation with the

sterol degrading isolates. The organisms failed to convert these cholesterol derivatives to 17-keto steroids. Further, the sterol derivatives were not degraded by the isolates. The fact that the C-3 modified sterols were not degraded may have been caused by the inability of the enzymes involved in sterol metabolism to recognize these derivatives. The inability of the isolates used in this study to degrade sterols derivatized at C-3 is not always noted, as Sih and Bennett (53) found that enzymes from Nocardia restrictus could cleave the acetate group from cholesterol-3-acetate.

Another approach to the conversion of 17-keto steroids from sterols involves the mixed culture method of Awata (7). In this method the fermentation of cholesterol is accomplished by a mixed culture system including a strain of Pseudomonas aeruginosa and a sterol degrading organism. The addition of the Pseudomonas metabolite, a rhamnolipid (first described by Jarvis and Johnson-24) greatly enhances the conversion of cholesterol to $\Delta^{1,4}$-androstadiene-3,17-dione. In the present studies, rhamnolipid producer, Pseudomonas aeruginosa ATCC 7700 was used as a source of rhamnolipid and also as the second component in the mixed culture experiments. None of the sterol degrading cultures converted Generol 122^{R} or cholesterol to $\Delta^{1,4}$-androstadiene-3,17-dione when tested by the method of Awata (7). The degradation of the sterols

was also inhibited in these experiments. These results are surprising in light of the fact that Awata reports that representatives from several genera, including Arthrobacter, Nocardia, and Brevibacterium are able to convert cholesterol to $\Delta^{1,4}$-androstadiene-3,17-dione in mixed cultures with Pseudomonas aeruginosa IFO 3447 or IFO 3081 (7). The fact that the Pseudomonas aeruginosa strain used in the present study was different from those used by Awata may be an important consideration even though the strains used by Awata were considered as sources of rhamnolipid.

Early investigations into the mechanism of cholesterol ring cleavage by Turfitt (74) and Stadtman (68) indicated that cleavage of the A ring occurred first in the reaction (the isolation of Windaus keto acid from fermentations of cholesterol by Mycobacteria). Although Windaus keto acid was not isolated in this study, one of the sterol degrading isolates produced a product when using cholesterol and sitosterol as the sole carbon sources which could not have been produced by the sterol degradation pathway proposed by Sih (55,57,62). In this compound (Figure V) the C9-C10 bond of the original sterol molecule is still intact. This is the bond that is normally broken first in sterol degradation (17). The biogenesis of this compound is unclear and remains to be investigated.

CONCLUSIONS

The isolation of sterol degrading cultures was undertaken with the development of a system of selective sterol side chain cleavage as the ultimate goal. During the course of this investigation, several aspects of sterol degradation were examined.

1. The isolation of sterol degrading cultures from soil samples was readily accomplished. Approximately 2% of the colonies arising on nutrient agar from soil dilutions had the ability to degrade sterols. Additionally, 10% of the isolates had the ability to convert sterols to Δ^4-3-keto sterols. Isolation of sterol degrading cultures from soil samples enriched with sterols was found to be more efficient than isolation directly from soil. Approximately 25% of the colonies arising on nutrient agar from dilutions of enrichment cultures had the ability to degrade sterols. Of the 28 sterol degrading cultures characterized, 14 were Arthrobacter, 8 were Nocardia, 5 were Pseudomonas, and 1 was a Brevibacterium. The large number of Arthrobacter species isoalted is interesting. Since all the characterized isolates were isolated from

enrichment cultures it would be interesting to determine whether the distribution of genera of organisms capable of degrading sterols is the same in unenriched soil as it is in enrichment cultures.

2. The selection of a mutant strain of a sterol degrading organism that it will convert sterols to 17-keto steroids was also investigated. The potential for development of such a mutant was hampered by the inability of the organisms to grow on selective media after replica plating. This inability to selectively enrich for mutant clones left only random selection of isolated colonies arising after a mutational treatment. Limitations in space available reduced the number of mutants which could be tested at one time. This coupled with the fact that the slightest cross contamination of a mutant which could convert sterols to 17-keto sterols with the wild type would mask the activity of the mutant by degrading the 17-keto product reduced the efficiency of the selection process. A single mutation in the 9α-hydroxylase could potentially produce the desired phenotype since the 9α-hydroxylase function is essential for the ring opening reaction. This enzyme is a complex of proteins (69) and therefore has more target sites for mutational events. These observations would indicate that the isolation of a mutant clone blocked for ring degradation should be a more frequent

event. Testing more survivors of mutational treatments could possibly produce a culture with the ability to convert sterols to 17-keto steroids. The number of survivors of mutational treatments of sterol degrading organism which had lost the ability to degrade sterols was approximately 10%. This high frequency of mutation indicates that at least portions of the sterol degrading system are easily mutated. It would seem, therefore, that mutants which could produce 17-keto steroids from sterols should be more readily isolated than is indicated by the present studies. More extensive examinations of the survivors of mutational events might give the desired mutants. It is also possible that mutations which destroy the function of ring degrading enzymes affect the affinity of sterol molecules for the side chain degrading enzymes, because of an enzyme complex system of sterol degradation. The possibility of an enzyme complex involved in sterol degradation is indicated by the rate of sterol degradation (1 g/L in less than 4 days) and the inability to detect degradation intermediates by thin layer chromatography.

3. The inhibition of sterol ring degradation by αα'dipyridyl was effective in causing the conversion of Generol 122^R and cholesterol to $\Delta^{1,4}$-androstadiene-3,17-dione in 19 of the 28 isolates (Table XVIII). Of these only 7 produced more than trace amounts of $\Delta^{1,4}$-androsta-

diene-3,17-dione. Absolute amounts of $\Delta^{1,4}$-androstadine-3,17-dione were small, as were the amounts of material recovered. Investigations into the optimal conditions for the production of 17-keto steroids from Generol 122^{R} and cholesterol in the presence of αα'dipyridyl by the isolates used in this study could improve the conversion. Investigations into the oxygen dependence of the conversion, the use of washed or immobilized cells, and continuous culture conditions might prove profitable. Several of the fermentations yielded $\Delta^{1,4}$-androstadiene-3,17-dione as the major product when the chloroform extracts were analyzed. These fermentations could be useful in industry because the desired product was virtually the only compound extracted. Even though the absolute amount of product was low the high purity in the extraction is a desirable feature.

4. The observation that the sterol degrading cultures did not degrade the C-3 modified sterols is interesting. The possibility exists that other modifications of the sterol molecule would be effective in selectively preventing sterol ring degradation by one or more of the sterol degrading cultures. It is possible that the C-3 modified compounds are not transported into the cells of the degrading cultures rather than not being recognized by the sterol degrading enzymes.

5. The conversion of sterols to 17-keto sterols in mixed cultures of Pseudomonas aeruginosa and the sterol degrading isolates was not found. The use of a strain of Pseudomonas aeruginosa other than that reported by Awata (7) may be the reason for the failure of this experiment. Experiments varying the cultural conditions, the media, the incubation of the inoculum, the time of addition of sterols, the ratio of sterol degrading culture to Pseudomonas culture in the inoculum, and the amount of rhamnolipid added to the fermentation could produce conditions under which the conversion of sterols to 17-keto steroids could proceed.

6. The isolation of a mixed culture of Mycobacterium NRRL 3683 and a Corynebacterium species from a sewage sample points out some aspects of mixed cultures. This mixed culture was able to convert Generol 122^{R} to 17-keto sterols under conditions which were not conducive to this conversion by pure cultures of the Mycobacterium NRRL 3683. These conditions included fermentations in chemically defined media and were nearly as efficient as fermentations by pure cultures of Mycobacterium NRRL 3683 grown in complex media. The ability of the mixed culture to convert sterols to 17-keto sterols in defined media could be advantageous to industry. The use of defined media is better from a quality control point of view in that the components of the media are less variable than is the case with

complex media. The fact that the culture is iVIII24 was isolated as a mixed culture points out the limitations of isolating pure cultures from isolated colonies. The Mycobacterium component of the mixture grew more slowly than the Corynebacterium component and was not noticed on slants or streak plates until well after the initial isolation.

Mixed cultures can be useful in producing conditions favorable for specific reactions as in the conversion of sterols to 17-keto sterols in defined media by culture iVIII24 and the mixed culture system fermentations described by Awata (7).

Mixed cultures may also mask the organism responsible for a given reaction as was the case of iVIII24 and several of the cultures which were isolated form soil as sterol degrading organisms. In these latter cultures the functional organism could not be isolated from the contaminating spore forming bacteria. On initial isolation of the mixed cultures containing these spore forming bacteria it was assumed that the spore forming bacillus was the organism of value. Boiling these cultures purified the spore forming bacteria but destroyed the ability of the culture to degrade sterols. The activity of mixed cultures may be dependent upon the presence of both components. The isolation and characterization of functional mixed cultures

could lead to improvements in the production of many different products by fermentation.

7. The isolation and characterization of the metabolite produced by Nocardia #22 while growing in minimal media with Generol 122^{R} or cholesterol as the sole fermentable source of carbon was undertaken. This compound (Figure V) suggests an alternate pathway of sterol degradation. In the accepted pathway of sterol degradation by microorganisms the C-9 - C-10 bond is the first bond in the ring structure broken. In the compound isolated from Nocardia #22 the bond corresponding to the C-9 - C-10 bond is still intact. This observation leads to the conclusion that there is more than one pathway for the degradation of sterols by microorganisms.

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