MICROBIOLOGICAL AGENCIES IN THE DEGRADATION OF STEROIDS

II. Steroid Utilization by the Microflora of Soils

G. E. TURFITT

Home Office Forensic Science Laboratory, Nottingham, England

Received for publication July 14, 1947

An investigation of the degradation of cholesterol by soil microorganisms has shown that the initial oxidation of this compound is due, both *in situ* and in pure culture, to the activities of members of a single genus, *Proactinomyces* (Turfitt, 1944*a*). During the cultural work a considerable number of organisms, both molds and bacteria, persisted with the strains of *Proactinomyces* through many subcultures, but when isolated and inoculated into synthetic medium with cholesterol as sole C source, were unable to survive. Since *Proactinomyces* oxidation results in the formation of Δ^4-cholestenone, and subsequently in actual molecular fission (Turfitt, 1944*b*, 1947), it may well be that the further products resulting from *Proactinomyces* oxidation constituted a substrate for the growth of the attendant organisms.

In the case of steroid compounds lacking the intact hydrocarbon C_{17} side chain, oxidations at —OH groups giving the corresponding keto compounds have been reported with several groups of organisms, *Flavobacterium dehydrogenans* (Arnaudi, 1942), *Flavobacterium androstenedionicum* (Ercoli and Molina, 1944), *Flavobacterium carbonilicum* (Molina and Ercoli, 1944), *Alcaligenes faecalis* (Schmidt, Hughes, Green, and Cooper, 1942; Hughes and Schmidt, 1942), *Escherichia coli* (Schmidt and Hughes, 1944), and pseudodiphtheria bacilli (Zimmermann and May, 1944). These investigations, however, have been concerned essentially with ketone formation, and, in general, alternative carbon sources such as serum or yeast water have been included in the cultures with the object of obtaining a vigorous multiplication of the bacterial cells and a consequent high dehydrogenase concentration. Synthetic media with cholic acid as the sole carbon source have been used by Schmidt, Hughes, Green, and Cooper (1942) in oxidations with *Alcaligenes faecalis*, and triketocholanic acid has been isolated as the end product of the bacterial action. In this instance it is clear that the carbon available for growth has been derived from a breakdown of the bile acid molecule.

The only general restrictive influence on bacterial utilization thus far reported is due to the side chain, and the available evidence suggests that the modified natural sterol skeleton is probably susceptible to attack by a variety of organisms.

EXPERIMENTAL RESULTS

Although the basic ring structure of all steroid compounds is fundamentally the same, the variety of compounds resulting from the introduction of various

substituents is extremely wide and is in addition vastly increased by the complex stereoisomeric configurations in the steroid rings. It was clearly impracticable to investigate more than a limited selection of compounds, and a choice was made with a view to determining the influence on the microbiological utilization of (1) the length and nature of the side chain, (2) the presence of free and esterified —OH groups, (3) the presence of ketonic groups in the more usual C_3 and C_{17} positions, (4) the presence of free or "blocked" double bonds, (5) the *cis*- and *trans*-decalin configurations of rings A and B, (6) stereoisomerism at C_5, and (7) the presence of benzenoid rings in place of the complete saturation of the polyhydrocyclopentanophenanthrene skeleton.

Isolation technique. The process of selective isolation used in the investigation of the cholesterol-decomposing organisms of soils (Turfitt, 1944*a*) was again adopted, although certain modifications were considered desirable owing to the varied physical and chemical properties of the steroid substrates.

The soil samples were restricted to the British Isles, and the 20 specimens taken constituted a range covering both virgin and cultivated ground. In each instance the sample was collected from immediately below the soil surface in a sterile 4- by 1-inch tube. No attempt was made to investigate the microflora present at a greater depth.

Sterilization of the individual steroid materials to be used in the cultures presented a somewhat difficult problem. In the case of cholesterol, steam sterilization was entirely effective, but this method was obviously unsuitable with other steroids. Δ^4-Cholestenone, for example, has a melting point of 80 C and on cooling separates as a hard, solid mass. Again, there can be no guarantee that in the case of some of the less stable steroids heat treatment does not result in a slight decomposition yielding a material containing traces of impurity. The same considerations hold with regard to sterilization by ultraviolet light, since with ergosterol, and probably also in minor degree with certain other steroids, molecular transformation results. A successful solution of the problem was achieved by recrystallization of the compounds from suitable solvents, with filtration and drying conducted under aseptic conditions. The materials were stored in sterile tubes and were tested for sterility before use by streaking on both nutrient agar and wort agar plates.

Bacteria and actinomycetes. Conical culture flasks (100-ml), each containing 30 ml mineral salt solution (NH_4NO_3, 0.1 per cent; K_2HPO_4, 0.025 per cent; $MgSO_4 \cdot 7H_2O$, 0.025 per cent; NaCl, 0.0005 per cent; $FeSO_4 \cdot 7H_2O$, 0.00001 per cent) were autoclaved at 115 C for 10 minutes, and approximately 1 mg steroid was introduced aseptically.

Molds. In devising a method to ensure a normal surface development of mold mycelium, a distinction was drawn between the slightly soluble carboxylic acids, cholic acid, and 3-hydroxy-Δ^5-cholenic acid, and the insoluble steroid compounds. For the former, 100-ml Gates' culture flasks, each containing 50 ml of mineral salt solution ($NaNO_3$, 0.2 per cent; KH_2PO_4, 0.1 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; KCl, 0.05 per cent; $FeSO_4 \cdot 7H_2O$, 0.01 per cent), were sterilized by steaming for 1 hour on each of 3 successive days, and ap-

proximately 1 mg steroid was aseptically added. In the case of the insoluble compounds petri dishes were employed; each contained a thick, compact layer of glass wool previously purified by acid and alcohol treatment and just covered with the mineral salt solution. The dishes were sterilized by steaming, and approximately 1 mg steroid was sprinkled with aseptic precautions over the surface.

Approximately 0.5 ml of a heavy aqueous suspension of each of the 20 soil samples were transferred to each of the flasks and dishes containing the various steroid compounds, and the cultures were incubated aerobically at 25 C. After 7 days a loopful from each vessel was transferred to a duplicate containing fresh medium, this procedure being repeated three times. From the final cultures transfers were made on (a) nutrient agar, (b) casein agar, and (c) Czapek-Dox agar. The organisms which appeared on these plates were isolated and incubated in pure culture with the various steriod-containing media. Increased bacterial count or development of mold mycelium, together with alteration of the pH of the medium, was regarded as evidence of steroid utilization.

Description and distribution of isolated organisms. In this investigation of the aerobic organisms of soils, 20 soil samples and 20 steroid compounds, under two separate cultural conditions, involved 800 initial cultures. After the subsequent "purification" cultures, numbers of organisms were isolated which failed to survive on the appropriate pure steroid. No mention is made of these organisms in table 1, which summarizes the numbers and general types of steroid-decomposing organisms isolated from particular classes of soils. Strains of *Proactinomyces* are indicated by "P," and the numbers of strains isolated are given in parentheses. Gram-negative rods are indicated by "gm-."

Description of isolated strains. In this survey, 313 of the 355 cultures of bacteria isolated consisted of gram-positive rods, or of long or short filaments breaking up in older culture into short rods or coccoid forms. The organisms have been cultured on a wide variety of media, and in the majority of cultures, especially upon the less rich media, aerial mycelium was produced in greater or less degree; in no instance was there evidence of spore formation upon examination by the method of Orskov (1923). In cultural and morphological characteristics the organisms fall essentially within the genus *Proactinomyces* and for the most part have the softness and translucency of the α-type of colony (Umbreit, 1939). In several cases the strains did not show a strict agreement with the characteristics of known types, but the divergencies were insufficient to justify new species rank, and they have been regarded rather as variants of existing species. The divergencies were particularly marked in respect to acid-fastness, a character which was found to be influenced markedly by the composition of the culture medium. This feature of *Proactinomyces* has previously been reported by Jensen (1931, 1932) in a detailed taxonomic study of the genus.

The 298 cultures of this group have thus been classified as follows: *P. opacus* (135 cultures), *P. erythropolis* (126 cultures), *P. globerulus* (17 cultures), *P. coeliacus* (8 cultures), *P. aquosus* (5 cultures), *P. crystallophagus* (5 cultures), and *P. agrestis* (2 cultures).

TABLE 1

Steroid-decomposing organisms from varied soil types

STEROID	SOIL TYPES (4 SAMPLES EACH TYPE)				
	Acid Sand	Loam	Marl	Alkaline Peat	Arable
Stigmasterol	P (3)	P (4)	P (4)	P (2)	P (6)
β-Sitosterol	P (4)	P (4)	P (5)	P (3)	P (4)
Ergosterol	P (3)	P (5)	P (3)	P (3) gm (1)	P (6) gm (2)
Coprosterol	P (3)	P (3)	P (3)	P (2)	P (5)
Dihydrocholesterol	P (4)	P (4)	P (4)	P (3)	P (7)
epi-Dihydrocholesterol	P (3)	P (3)	P (4)	P (3)	P (6)
Cholesterol acetate	P (3)	P (4)	P (3)	P (2)	P (5)
Coprosterol acetate	P (3)	P (4)	P (4)	P (3)	P (6)
Cholesteryl chloride	None	None	None	None	None
Cholesterol acetate dibromide	None	None	None	None	None
Dicholesteryl ether	P (4)	P (4)	P (4)	P (2)	P (4)
Δ^4-Cholestenone	P (4)	P (5)	P (4)	P (3)	P (6)
Coprostanone	P (3)	P (3)	P (4)	P (3)	P (5)
Androsterone	P (4) gm (2)	P (5) gm (1)	P (5)	P (3)	P (6) gm (1)
trans-Dehydro-androsterone	P (4) gm (3)	P (4) gm (2)	P (4) gm (1)	P (2) gm (1)	P (6) gm (3)
Progesterone	P (3) gm (1)	P (3) gm (1)	P (3) gm (1)	P (3)	P (4) gm (2)
3-Hydroxy-Δ^5-cholenic acid	P (4) gm (3) molds (1)	P (5) gm (2)	P (4) gm (1)	P (3) gm (1)	P (5) gm (2) molds (1)
Cholic acid	P (4) gm (5) molds (2)	P (4) gm (3) molds (1)	P (3) gm (1)	P (3)	P (4) gm (2) molds (1)
α-Oestradiol	P (1)	None	None	None	P (2)
Oestrone	None	None	None	None	P (1)

Many of these individual cultures, of course, originating from the same soil samples, were quite obviously identical. In addition, two cultures have been provisionally identified as *Mycobacterium phlei*. An authentic strain of *M. phlei* has previously (Turfitt, 1944*a*) been found unable to utilize cholesterol, and in consequence tests for cholesterol-decomposing ability have been conducted with a number of standard strains of this organism. The property, which was fairly vigorous in some strains, was entirely lacking in the majority and could not, furthermore, be stimulated by enrichment cultures. Closely comparable findings have been experienced with cultures of *Mycobacterium smegmatis* and *Mycobacterium stercoris*.

The β-group of *Proactinomyces* was represented by 13 cultures which have been thus identified: *P. asteroides* (8 strains), *P. farcinicus* (4 strains), and *P. paraffinae* (1 strain).

In addition to the *Proactinomyces*, 42 cultures of gram-negative bacteria were obtained. Of these, 29 were short rods with 1 to 5 polar flagella and on asparagine agar (Georgia and Poe, 1931) developed the green fluorescence of *Pseudomonas*. The blue pigment pyocyanin, typical of *Pseudomonas aeruginosa*, was not detected even in glycerol peptone agar (Gessard, 1891; Turfitt, 1936). Neither these nor the 13 cultures of gram-negative nonfluorescent organisms have as yet been satisfactorily classified, but they are being incorporated in a further study of steroid utilization specifically by gram-negative organisms. Ercoli (1938), in the attempted bacterial reduction of male sex hormones to etiocholane derivations, has described the culture of *Pseudomonas fluorescens*, and also of *Escherichia coli*, in meat broth in the presence of 200 mg androstenedione. No hydrogenation products could be isolated, but 86 mg of unchanged dione were recovered. Similar results were obtained with *trans*-dehydroandrosterone. This apparent utilization of the steroid by these organisms is thus in accord with the present results indicating that in such modified steroids gram-negative bacteria play a not inappreciable part.

The only instances in which isolated molds were able to survive repeated transfer in pure culture were with the free acids, cholic acid, and 3-hydroxy-Δ^5-cholenic acid. In all, six molds (identified as species of *Penicillium*, *Aspergillus*, and *Altenaria*) were found to yield a few straggling hyphae on the surface of the medium with the petri dish, glass wool technique. A definite mycelial felt never developed. There was no change in the pH of the medium, and no detectable ketone formation. Steroid decomposition by these organisms was clearly of a negligible order, and they were accordingly not further investigated.

ACKNOWLEDGMENT

The author wishes to express his thanks to Professor A. Ercoli, University of Milan, for cultures of his new species of *Flavobacterium* and to Mrs. A. E. Oxford, Rothamsted Experimental Station, for her valuable comments concerning the descriptions of the *Proactinomyces* species.

SUMMARY AND CONCLUSIONS

Cultural conditions are described by which the microflora of soils have been tested for ability to utilize a variety of steroid materials.

Steroids generally, with a very few special exceptions such as halogen-substituted derivatives, are attacked by *Proactinomyces* of soils, and these are clearly the predominant organisms in steroid decomposition.

With steroid compounds in which the C_{17} side chain is modified or lacking, certain gram-negative bacteria, expecially of the fluorescent type, can utilize the molecule.

Although species of *Penicillium*, *Aspergillus*, and *Altenaria* have been found to survive repeated transfer in pure culture on soluble carboxylic acid derivatives, the paucity of the growth and the lack of evidence of steroid decomposition are taken to indicate that these fungi are of small significance in the utilization of steroids in nature.

REFERENCES

ARNAUDI, C. 1942 *Flavobacterium dehydrogenans* (*Micrococcus dehydrogenans*) und seine Fähigkeit zur Oxydation von Steroiden sowie Substanzen aus der Sexualhormonreihe. Zentr. Bakt. Parasitenk., II, **105**, 352–366.

ERCOLI, A. 1938 Zur Frage der biologischen Bildung der Ätiocholan-Derivate. Ber. deut. chem. Ges., **71B**, 2198.

ERCOLI, A., AND MOLINA, L. 1944 Sui batteri che determinano l'ossidazione di steroidi ad attività ormonale. I. Un nuovo stipite di flavobacterium: il *flavobacterium androstendionicum*. Boll. ist. sieroterap. milan., **23**, 158–163.

GEORGIA, F. R., AND POE, C. F. 1931 Study of bacterial fluorescence in various media. I. Inorganic substances necessary for bacterial fluorescence. J. Bact., **22**, 349–361.

GESSARD, C. 1891 Des races du bacille pyocyanique. Ann. inst. Pasteur., **5**, 65-78.

HUGHES, H. B., AND SCHMIDT, L. H. 1942 Oxidation of steroids by *Alcaligenes faecalis*. Proc. Soc. Exptl. Biol. Med., **51**, 162–163.

JENSEN, H. L. 1931 The definition and sub-division of the genus *Actinomyces*. Proc. Linnean Soc. N. S. Wales, **56**, 345–370.

JENSEN, H. L. 1932 The identity of certain species of *Mycobacterium* and *Proactinomyces*. Proc. Linnean Soc. N. S. Wales, **57**, 364–376.

MOLINA, L., AND ERCOLI, A. 1944 Sui batteri che determinano l'ossidazione di steroidi ad attività ormonale. II. Isolamento e caratteristiche di un nuovo stipite di flavobacterium: *il flavobacterium carbonilicum*. Boll. ist. sieroterap. milan., **23**, 164–167.

ORSKOV, J. 1923 Investigations into the morphology of the ray fungi. Copenhagen.

SCHMIDT, L. H., AND HUGHES, H. B. 1944 Conversion of hydroxy-steroids to keto-steroids. U. S. P. 2, 360, 447.

SCHMIDT, L. H., HUGHES, H. B., GREEN, M. H., AND COOPER, E. 1942 Studies on bile-acid metabolism. II. The action of *Alcaligenes faecalis* on cholic acid. J. Biol. Chem., **145**, 229–236.

TURFITT, G. E. 1936 Bacteriological and biochemical relationships in the pyocyaneus-fluorescens group. I. The chromogenic function in relation to classification. Biochem. J., **30**, 1323–1328.

TURFITT, G. E. 1944*a* Microbiological agencies in the degradation of steroids. I. The cholesterol-decomposing organisms of soils. J. Bact., **47**, 487–493.

TURFITT, G. E. 1944*b* The microbiological degradation of steroids. 2. Oxidation of cholesterol by *Proactinomyces* spp. Biochem. J., **38**, 492–496.

TURFITT, G. E. 1947 The microbiological degradation of steroids. 4. Fission of the steroid molecule. Biochem. J. *In press*.

UMBREIT, W. W. 1939 Studies on the *Proactinomyces*. J. Bact., **38**, 73–89.

ZIMMERMANN, W., AND MAY, G. 1944 Bacterial oxidation of steroids. Zentr. Bakt. Parasitenk., I, Orig., **151**, 462–466.