The Microbiological Degradation of Steroids

3. OXIDATION OF HYDROXY-STERIODS TO KETO-DERIVATIVES BY *PROACTINOMYCES* SPP.

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(Received 6 October 1945)

Recent work on bacterial oxidation of substances in the steroid group has demonstrated the possibility of converting certain hydroxy-steroids into the corresponding keto-derivatives by microbiological means. Thus, by the use of a 'bacterial mixture' cultured on Milan yeast, Mamoli (1938) obtained androstenedione from *trans*-dehydroandrosterone, and progesterone from pregnenolone; with cholesterol as substrate, however, no oxidation occurred. In later work, Mamoli (1944) used '*Corynebacterium mediolanum*' to effect oxidation of certain steroid alcoholic hydroxyl groups capable of dehydrogenation. Similar results were reported by Ercoli (1940) with '*Micrococcus dehydrogenans*' cultured on an aerated yeast extract buffered at pH 6.8. The inhibitory influence of the C₁₇ side-chain which both of these workers have experienced has not been encountered by the present author (Turfitt, 1944) working with species of the genus *Proactinomyces*. In view of the readiness with which these organisms oxidized cholesterol to Δ^4 -cholestenone, it appeared likely that the dehydrogenase mechanism might be generally effective with all hydroxy-compounds of the cyclopentanopolyhydrophenanthrene series. A selection has accordingly been made of various common hydroxy-steroids, and these compounds have been subjected to the oxidizing action of *Proactinomyces* species.

EXPERIMENTAL

The experimental technique is essentially that previously described (Turfitt, 1944) for cholesterol oxidation. *P. erythropolis* Gray & Thornton, has been used throughout as the test organism for the quantitative work, but other *Proactinomyces* have been shown to function in an identical manner.

In each individual experiment a 250 ml. conical culture flask containing 100 ml. of mineral salt solution (NH₄NO₃ 0.1%; K₂HPO₄ 0.025%; MgSO₄·7H₂O 0.025%; NaCl 0.0005%; FeSO₄·7H₂O 0.00001%) and 0.5 g. CaCO₃ was autoclaved for 15 min. at 115°. An accurately weighed quantity of the finely powdered, sterile, hydroxy-steroid was added under aseptic conditions, and the medium inoculated with a 24 hr. agar slope culture of *P. erythropolis*. The culture was incubated in darkness for a period of 7–28 days at 25°.

Characterization of products of bacterial activity

From cholesterol (250 mg.). After 28 days, continuous chloroform extraction of the culture yielded 225 mg. of pale yellow solid. This was separated by the Girard technique into a ketonic (85 mg.), and a non-ketonic fraction (132 mg.) consisting of unchanged cholesterol. The ketonic fraction crystallized from methanol-acetone (3:2) in fine needles m.p. 80°. This product, and the dark red 2:4-dinitrophenylhydrazone m.p. 233° prepared from it, gave no depression of m.p. when mixed respectively with Δ^4 -cholestenone and its 2:4-dinitrophenylhydrazone. Yield of cholestenone: 34 %.

From stigmasterol (250 mg.). Chloroform extraction of the culture after 28 days gave 236 mg. of white residue. By means of the Girard 'T' reagent there was obtained from this 172 mg. of unchanged stigmasterol. The ketonic fraction (48 mg.) after recrystallization had m.p. 125°, not depressed in admixture with authentic stigmastadienone. The 2:4-dinitrophenylhydrazones prepared from the two compounds had an undepressed mixed m.p. 244° (decomp.). Yield of stigmastadienone: 19 %.

From β -sitosterol (250 mg.). A 28-day culture yielded 230 mg. chloroform-soluble material, from which were isolated 133 mg. unchanged β -sitosterol. From the ketonic fraction (91 mg.) there was obtained a crystalline solid of m.p. 83°; 2:4-dinitrophenylhydrazone m.p. 247°, which were characterized by reference to β -sitostenone and its derivative. Yield of β -sitostenone: 36 %.

From coprosterol (250 mg.). After incubation of the culture for 28 days, continuous chloroform extraction gave 228 mg. of yellowish residue. The unchanged coprosterol isolated by the Girard process amounted to 167 mg., whilst 52 mg. ketonic material were obtained. After purification this melted at 61°, and was identified as coprostanone by reference to an authentic specimen, and also by preparation of its semicarbazone which melted, after recrystallization from benzene, at 192°. Yield of coprostanone: 21 %.

From 3-hydroxy- Δ^5 -cholenic acid (250 mg.). After 7 days the solid organic content of the culture was in the form of large 'quartz-grain' crystals. Incubation was continued for a total period of 28 days,

during which time there was some appreciable evolution of gas from the culture. The medium was then acidified with diluted HCl, and extracted continuously with ether. Evaporation of the extract, after drying with anhyd. Na_2SO_4 , gave 235 mg. of a pale brown solid. The ketonic material (92 mg.), recrystallized from aqueous acetone, had m.p. and mixed m.p. with 3-keto- Δ^4 -cholenic acid 185° . Yield of 3-keto- Δ^4 -cholenic acid: 37 %.

From *trans*-dehydroandrosterone (250 mg.). Incubation for 4 days resulted in the appearance in the medium of sheaves of needles. In a 7-day culture the whole of the solid organic material in the medium was apparently present in the form of needles 5–10 mm. in length. Extraction of the culture with chloroform at this stage yielded 246 mg. of residue in the form of rosettes of slightly yellow needles. This residue was dissolved in 10 ml. ethanol, and to the solution were added 100 mg. digitonin in 5 ml. ethanol. The mixture was evaporated to dryness on purified cotton-wool, and extracted continuously with ether. On evaporation of the ether 239 mg. of yellowish needles were obtained; recrystallized from petroleum b.p. $40\text{--}50^\circ$, the colourless product melted at 169° , and gave no depression with authentic Δ^4 -androstene-3:17-dione. Characterization was completed by preparation of the enol benzoate m.p. 176° (decomp.). Yield of Δ^4 -androstene-3:17-dione: 96 %.

From testosterone (250 mg.). In a 7-day culture sheaves of needles up to 5 mm. in length were present, although they did not appear in the quantity observed with *trans*-dehydroandrosterone. Extraction of the culture with chloroform afforded 238 mg. of gummy product. This was chromatographed on Al_2O_3 from benzene-petroleum b.p. $40\text{--}50^\circ$ (1:1), developed with the same mixed solvents, and eluted with benzene. In this manner there were obtained 121 mg. of rosettes of white needles m.p. 154° (unchanged testosterone), and 117 mg. of needles which, after recrystallization from acetone-petroleum b.p. $40\text{--}50^\circ$ and further purification by high vacuum sublimation, had m.p. 171° . Characterization of this product as Δ^4 -androstene-3:17-dione was achieved from the mixed m.p. and by preparation of the enol benzoate m.p. 177° (decomp.). Yield of Δ^4 -androstene-3:17-dione: 47 %.

From Δ^5 -androstene-3-*trans*:17-*trans*-diol (250 mg.). Sheaves of needles were present in the medium after 4 days. Incubation was continued for a further 3 days, and the chloroform-soluble material then separated (229 mg.). With the Girard 'T' reagent this yielded 92 mg. of unchanged androstenediol, and 130 mg. of a ketonic material. This latter fraction was chromatographed on Al_2O_3 from benzene-petroleum b.p. $40\text{--}50^\circ$ (1:1), and eluted with benzene. Two products were thus obtained: first, 105 mg. of rosettes of fine white needles, recrystal-

lized from acetone-petroleum b.p. $40\text{--}50^\circ$, m.p. 153° (identical with testosterone), and secondly, 15 mg. white needles m.p. 170° (identified by m.p. and preparation of the enol benzoate m.p. 174° (decomp.) as Δ^4 -androstene-3:17-dione). Yields: testosterone 42 %; Δ^4 -androstene-3:17-dione 6 %.

DISCUSSION

The experiments recorded above, although not in any way exhaustive, are sufficiently representative to indicate the utility of the *Proactinomyces* method in effecting oxidation of steroid hydroxyl groups. In no instance is the oxidation of compounds containing the intact C_{17} side-chain completely inhibited, as hitherto reported for such bacterial oxidations; the yields of keto-derivatives do, however, appear in general to be somewhat lower than in the case of compounds where the side-chain is shortened or completely absent.

No clear estimate of the influence of stereo-isomerism upon the oxidation process can be obtained from this investigation, but the *cis*- and *trans*-decalin configurations of rings A and B apparently show no material differences in their effect upon the oxidation of the C_3 hydroxyl group.

It is of interest to note that the oxidation is limited to $-\text{OH}$ groups, and leaves both nuclear and side-chain double bonds unattacked.* The specificity of the reaction thus constitutes an interesting parallel with that of the Oppenauer oxidation. Although more lengthy than the chemical methods, the essentially simple nature of the process and the high degree of purity of the products resulting from the absence of wasteful side-reactions, render it in many instances preferable to the established chemical techniques for production of keto-steroids. Under the conditions described the yields are by no means optimum, and very appreciable increases can be obtained by suitable modification of the process; in one experiment, by introducing Δ^5 -androstene-3:17-diol into the medium in a non-inhibiting concentration of ethanol, the yield of testosterone was increased from 42 to 68 %.

The susceptibility of the C_3 $-\text{OH}$ to *Proactinomyces* oxidation is particularly well illustrated in the case of Δ^5 -androstene-3:17-diol, where oxidation at C_{17} is of a relatively low order. With testosterone, where there is no group available for oxidation at C_3 , the C_{17} hydroxyl undergoes quite extensive oxidation. This behaviour of the C_3 $-\text{OH}$ is in striking contrast to that of the corresponding group in cholic acid when this latter compound is subjected to CrO_3

* Since the steroid compound is being utilized as the sole C source a secondary oxidation undoubtedly occurs, but under the experimental conditions here employed it is inappreciable.

oxidation (Gallagher & Long, 1943), or to the oxidizing action of *A. faecalis* (Hoehn, Schmidt & Hughes, 1944); in both of these cases the C₃—OH is the last of three hydroxyl groups to undergo oxidation.

The bacterial cells of the *Proactinomyces* spp. evidently contain no enzymic mechanism capable of hydrolyzing ester-linkages, since cholesterol acetate under the above conditions was recovered unchanged, and androstenediol-17-benzoate yielded testosterone benzoate.

SUMMARY

Various of the more common hydroxy-steroids have been found to be oxidized to keto-derivatives by *Proactinomyces* spp., and the available evidence suggests the possible general nature of the method in the steroid series.

The author wishes to express his thanks to Dr O. Rosenheim, F.R.S., Prof. D. H. Hey, Dr E. R. H. Jones, and Dr G. A. D. Haslewood for gifts of specimens of several of the compounds mentioned in this publication.

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Studies of the Van den Bergh Reaction

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(Received 6 October 1945)

Van den Bergh & Muller (1916) showed that the red pigment azobilirubin* was formed when a solution of diazotized sulphanilic acid was added to sera from patients with obstructive jaundice. On the other hand, with normal sera and sera from patients with other forms of jaundice the addition of ethanol was essential for the reaction to take place. The reaction occurring in the absence of ethanol was termed the direct reaction, while that occurring only in the presence of ethanol was termed the indirect reaction. The cause of the difference in behaviour of the two types of sera remains obscure, but possibilities which have been considered are (a) the existence of two forms of bilirubin in the two types of case (Van den Bergh, 1918; Grunenberg, 1923 *a, b*; Soejima, 1927; Newman, 1928; Fowweather, 1932), (b) combination of bilirubin with protein or other interfering substances in sera giving only the indirect reaction (Bollman, Sheard & Mann, 1927; Barron, 1931) and (c) the presence of catalytic substances in sera giving

the direct reaction (Acil & Goldgruber, 1932). Van den Bergh & Muller showed that sera giving the indirect reaction either failed completely to react with the diazo reagent in the absence of ethanol or reacted very slowly indeed (a delayed direct reaction), but they made no attempt to assign differences in clinical significance to these last two types. Feigl & Querner (1919), Lepehne (1920) and McNee (1922) subdivided the direct reaction into prompt and biphasic types. In contrast to the rapid completion of formation of azobilirubin in the prompt reaction, in the biphasic reaction formation of azobilirubin proceeded rapidly during the first minute after addition of the reagent but then continued at an obviously slower rate during the following 15 min. Such biphasic reactions were believed to be due to the presence of prompt reacting and delayed reacting bilirubins in varying proportions. After much controversy the general consensus of opinion was well expressed by Harrison (1937) who observed that 'the direct test (qualitative or quantitative) is of doubtful value and in routine work might be omitted'.

Malloy & Evelyn (1937) who determined total bilirubin by the formation of azobilirubin in the presence of methanol, replaced the latter by water, and claimed that the proportion of direct bilirubin could be determined by measuring azobilirubin 30 min. after addition of the diazo reagent. More-

* Established custom and the need for a short name are our reasons for using the term 'azobilirubin' for the red pigment produced by the reaction between bilirubin and an acid solution of diazotized sulphanilic acid. It is an unsatisfactory name, however, since Fischer and his colleagues have shown that the reaction consists of a splitting process as well as a coupling and that the red pigment is probably a mixture of isomeric diazotized hydroxy dipyrromethenes (Fischer & Orth, 1937).