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The Microbiological Degradation of Steroids

4. FISSION OF THE STEROID MOLECULE

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During recent years the presence of an enzyme system capable of effecting the oxidation of hydroxy-steroids to the corresponding keto derivatives has been clearly demonstrated in a number of bacterial species. The investigations of the Italian schools of Ercoli and Arnaudi have resulted in the isolation from yeast of several new organisms, *Flavobacterium dehydrogenans* (Arnaudi, 1939), *F. androstenedionicum* (Ercoli & Molina, 1944), and *F. carbonilicum* (Molina & Ercoli, 1944), which, in general, oxidize the nuclear hydroxyl groups of steroid compounds devoid of the 17 side chain. Zimmermann & May (1944) obtained similar oxidations by the action of pseudodiphtheria bacilli. In the bile acid series stepwise oxidation of the hydroxyl groups has been achieved using *Alcaligenes faecalis* (Schmidt, Hughes, Green & Cooper, 1942), and more recently, *Escherichia coli* (Schmidt & Hughes, 1944). The independent investigations of the flora of soils by Tak (1942), and by the present author (Turfitt, 1943, 1944a), have shown that the cholesterol-decomposing organisms fall essentially within the genus *Proactinomyces*. These organisms are of particular interest in the breakdown of steroids since their oxidizing action is not, as reported for other organisms, inhibited completely by the presence of the intact side chain. Their place in the economy of nature is discussed later.

Whilst this primary oxidation of steroid hydroxyl groups is firmly substantiated, actual breakdown of the steroid molecules, yielding distinct chemical

fission products, has not hitherto been recorded. Evidence of a secondary action of this nature has, however, been obtained during large-scale work on the oxidation of cholesterol to cholest-4-en-3-one (Turfitt, 1944b). With cholesterol as the sole carbon source, energy for growth must inevitably, in view of the non-autotrophic character of *Proactinomyces*, have resulted from utilization of the sterol carbon. During the course of the work certain small fractions were obtained which undoubtedly represented products of degradation, but attempts to isolate definite compounds were unsuccessful. A detailed account of the various methods by which information has been gained as to the course of the degradation process is given in the present publication.

EXPERIMENTAL

Foci of bacterial attack

Fission of the steroid skeleton by bacterial oxidation might be expected, by analogy with chemical methods, to occur primarily at one of several susceptible points, viz. OH groups, double bonds, or the side chain. With a view to determining whether the presence or absence of each of these points exerts any material effect upon the bacterial oxidation, a series of compounds covering different combinations of OH, double bond and side chain has been subjected to the oxidizing action of *P. erythropolis*.

In each experiment, performed in triplicate, 50 mg. finely powdered, sterile compound were introduced with aseptic

precautions into a 250 ml. conical culture flask containing 100 ml. mineral salt solution, previously sterilized at 115° for 15 min. (The mineral salt solution referred to throughout this publication had the following composition: NH_4NO_3 , 0.1; K_2HPO_4 , 0.025; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025; NaCl , 0.0005; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00001%.) Inoculation was performed from 24 hr. agar-slope cultures of *P. erythropolis* (strain 15/G), and the cultures incubated at 25°. At intervals of 4, 7 and 11 days test samples of the cultures were treated with Universal Indicator (British Drug Houses Ltd.), production of acid being taken as indicating steroid decomposition.

A summary of the results of these experiments is given in Table 1. With those compounds in which the side chain is shortened either partially or completely to $>\text{CO}$, degradation is apparently more rapidly initiated. When, on the other hand, the hydrocarbon side chain is intact, blocking of the hydroxyl group or of the double bond, or of both, does not prevent attack, although it seems to occasion

solution of *o*-nitrobenzaldehyde in 2*N*-NaOH contained in a 100 ml. tube. At the end of 2 months the contents of the tube were warmed for 5 min. on the water bath, cooled and extracted with 5 ml. chloroform; the red or blue colour characteristic of the CH_3CO -grouping under these conditions was not obtained. The experiment was repeated with like result using cholestane as substrate. Superficially this would appear to indicate that during this steroid decomposition volatile ketones and aldehydes are not produced, and in particular that the side chain is neither partially nor completely removed in the manner encountered in chemical oxidations. The results, however, are equally consistent with the theory that volatile compounds are produced, and are at once utilized by the organisms. In support of this theory it has been found by cultural experiments that *P. erythropolis* readily utilizes acetone, methyl isohexyl ketone and laevulaldehyde when each of these substances constitutes the sole available carbon source in the culture medium.

Table 1. Steroid utilization by *P. erythropolis*

Compound	Points available			Universal indicator		
	—OH	Double bond	Side chain	4 days	7 days	11 days
Cholesterol	+	+	+	YR*	R	R
Stigmasterol	+	+	+	YR	R	R
Sitosterol	+	+	+	YR	R	R
Coprosterol	+	—	+	Y	R	R
Dihydrocholesterol	+	—	+	Y	R	R
Cholesterol acetate	—	+	+	YR	R	R
Coprosterol acetate	—	—	+	Y	R	R
Cholestane	—	—	+	Y	R	R
3-Hydroxychol-5-enic acid	+	+	+	R	R	R
			(modified)			
<i>trans</i> Dehydroandrosterone	+	+	—	R	R	R
Androsterone	+	—	—	R	R	R
Androsterone acetate	—	—	—	R	R	R

* R = Red (pH < 4); Y = Yellow (pH 6.5); reaction of original medium = Green (pH ~ 8).

some slight delay. Unless, therefore, there exists some other unsuspected point of oxidative attack, this would appear to be presumptive evidence of side-chain disruption.

Attempted isolation of volatile products

During the classical work on sterol structure two volatile materials were isolated which had resulted from oxidative disruption of the side chain, viz. acetone, originating from the terminal isopropyl group (Windaus & Neukirchen, 1919), and methyl isohexyl ketone representing removal of the complete side chain (Windaus & Resau, 1913). Attention has thus first been directed towards determining whether the *Proactinomyces* oxidation proceeds along a similar course with the production of volatile ketones or possibly laevulaldehyde.

A 1 l. conical flask containing 250 ml. mineral salt solution and 2.5 g. CaCO_3 was autoclaved at 115° for 15 min., and after cooling 2 g. finely powdered sterile coprosterol acetate were added. The flask and contents were inoculated with *P. erythropolis* (strain 15/G) and incubated at 25° for 2 months, during which time sterile O_2 was slowly bubbled through the culture and thence through 25 ml. saturated

Degradation of cholest-4-en-3-one

A quantitative study has been made of the production of cholest-4-en-3-one from cholesterol by *Proactinomyces* oxidation, and of its subsequent decomposition.

Six 250 ml. conical culture flasks, each containing 100 ml. mineral salt solution and 0.5 g. CaCO_3 , were autoclaved at 115° for 15 min., and into each flask were introduced aseptically 50 mg. finely powdered cholesterol previously sterilized by steaming in a sealed tube. After inoculation from 24 hr. agar-slope cultures of *P. erythropolis* (strain 42/G), the flasks were incubated at 25° for periods of $\frac{1}{2}$, $\frac{1}{4}$, 1, 2, 3 and 4 months respectively. After the appropriate incubation period, each culture was removed and thoroughly extracted with chloroform; the extract was washed, dried with anhydrous Na_2SO_4 and evaporated. Curve A (Fig. 1) shows the decrease in total chloroform-soluble material throughout the culture period. The residues were redissolved in chloroform. Residual cholesterol (curve B) was determined by the method previously described (Turfit, 1943), and 3-ketosteroids (curve C) by the Callow modification (Callow, Callow & Emmens, 1938) of the original Zimmermann procedure (Zimmermann, 1936). In order to

determine decomposition products present as either soluble or insoluble Ca salts, the aqueous suspension from the initial chloroform extraction was acidified with diluted HCl, and re-extracted with chloroform; at no time, however, was more than 1.5 mg. residue obtained from the extracts. The total chloroform-soluble material was accounted for almost entirely by cholesterol + cholest-4-en-3-one, and it was evident that degradation products of cholest-4-en-3-one only existed in the cultures in minute amounts.

Fig. 1. Variation of total chloroform-soluble material (curve A), cholesterol (curve B), and 3-ketosteroids (curve C) in culture of *P. erythropolis* on cholesterol.

In an endeavour to isolate individual breakdown products in sufficient quantity for characterization, a considerable number of large-scale experiments was tried, each using 200 g. cholest-4-en-3-one prepared chemically from cholesterol by the Oppenauer oxidation (Oppenauer, 1941). In spite of the wide variety of experimental conditions no success was achieved, and it became increasingly apparent that the primary products of fission of the molecule were themselves attacked by the organisms in preference to the parent steroid, and that trapping of any acidic products as Ca salts did not prevent their further breakdown.

Continual removal of the breakdown products from the sphere of action of the organisms appeared to be the only logical method of isolating any intermediates. No suitable process for the purpose could be found recorded in the literature, and a technique was finally perfected employing the continuous culture apparatus shown in Fig. 2, which was kindly constructed to the author's specification by Messrs Quickfit and Quartz, King's Norton, Birmingham. Finely powdered sterile cholest-4-en-3-one (20 g.) was introduced into the previously autoclaved 2 l. reaction vessel A, which was then connected to the sterile unit B containing 3 l. mineral salt solution, and to the receiver units U and C. With the tap T open, culture medium was allowed to siphon into A, and a heavy inoculum of *P. erythropolis* (strain 42/G) was introduced. A fresh supply of sterile medium was connected to vessel A, and the tap adjusted so that the rate of flow from the nozzle N was approximately 1 drop/sec. Three litres of fresh culture medium were thus admitted during approx. 24 hr. Throughout the period of incubation (2 months) the reaction vessel was immersed in a water bath maintained at 25°. Outflow of medium occurred via the side arm S where loss of solid particles of steroid was prevented by the loose-fitting glass-wool plug. Sterile O₂ was passed continuously through the medium at the rate of approx. five bubbles/min., whilst the particles of the

sediment at the bottom of the reaction vessel were gently agitated at intervals of a few hours. Further bacterial activity in the effluent was prevented by allowing the issuing drops to pass through a 1 in. layer of ether in the unit U, the ether being replenished from time to time to maintain this layer. The ether-saturated effluent was collected in the 3 l. flask C.

Fig. 2. Apparatus for continuous culture and collection of products.

After 2 months' incubation the assembly was dismantled, the contents of the reaction vessel filtered, and the filtrate added to the pooled effluents; the residue (A) was dried in an air oven at 25°. By evaporation under reduced pressure of the ether-saturated spent medium, there were obtained 500 ml. of a pale straw-coloured liquid with a heavy granular deposit (B).

The detailed examination of the fractions A and B is given schematically in Figs. 3 and 4.

Characterization of products III and IV

Product III. On standing, the gum deposited rosettes of white needles. By repeated fractional crystallization from light petroleum (b.p. 100–120°) two products were obtained: (a) 4 mg. white plates, m.p. 150°. A specimen of the keto-acid of the formula

Fig. 3. Fractionation of solid residue from degradation of cholest-4-en-3-one.

Fig. 4. Fractionation of fluid product of degradation of cholest-4-en-3-one.

prepared by the original method of Windaus (1906) gave no depression of the melting point in admixture with this product. (b) 9 mg. slightly yellow crystals, m.p. 245°. Methyl derivative, prepared by means of diazomethane, and crystallized from light petroleum (b.p. 40–60°), had m.p. 130°, not depressed when mixed with methyl 3-keto-aetiochol-4-enate.

Product IV. The prisms, m.p. 96–98°, were readily soluble in water, the solution being neutral to litmus, slightly soluble in 90% ethanol, and insoluble in ether, chloroform and light petroleum. N, S, P and halogens were absent. On heating, the product melted and then charred with a 'burnt-sugar' odour. The solution did not reduce Fehling's solution, but gave a positive Molisch test. A red colour was obtained by heating with resorcinol in HCl, but no reaction was obtained with phloroglucinol in HCl. 2:4-Dinitro-phenylhydrazine also gave a negative response.

Owing to the small yield of product no further attempts to characterize the material were possible. At present, speculation as to the origin of this product is somewhat premature, but if it should be found to be a genuine product of cholestenone degradation it would produce a most valuable clue to the ultimate fate of the ring structure. The likelihood is, however, that it is a product of synthesis, a view which would seem to be supported by the fact that carbohydrates are not in general susceptible to utilization by *Proactinomyces* spp.

The products isolated and characterized as a result of the above process demonstrate the course of the initial fission of the cholestenone molecule under *Proactinomyces* attack. *iso*Caproic acid and 3-keto-aetiochol-4-enic acid are obviously complementary products of the fission of the side chain at C₂₀–C₂₂, the 21–CH₃ being lost completely. This evidence of the removal of the entire side chain affords an explanation of the failure to isolate the intermediates, 3-ketochol-4-enic acid, 3-ketonorchol-4-enic acid and 3-ketobisnorchol-4-enic acid which would be expected to result from a stepwise breakdown of the side chain. A simultaneous rupture of ring A is indicated by the production of the keto acid already known to result from hypobromite oxidation of cholestenone. The extreme smallness of the yields fully justifies the theory that the initial breakdown products are themselves rapidly utilized, so that the unidentified materials (I) and (II) must be taken either to represent secondary degradation products, or to contain products derived from the simultaneous oxidations at the two susceptible points.

Degradation of 3-ketochol-4-enic acid

The use of 3-hydroxychol-4-enic acid as substrate in *Proactinomyces* cultures leads to a large and rapid increase in bacterial population, and the fall in pH of the medium occurs somewhat earlier than in the case of cholesterol. Since the structure of the primary oxidation product, 3-ketochol-4-enic acid, is essentially that of cholest-4-en-3-one, apart from

the modified side chain, further oxidation should follow a similar course in both compounds.

A quantity of 10 g. of 3-ketochol-4-enic acid was treated in the continuous culture apparatus as described under cholestenone, and the 'spent' medium and residual solid substrate carried through an identical extraction process. A weight of 8.7 g. of unchanged 3-ketochol-4-enic acid was recovered, and 3-ketoaetiochol-4-enic acid isolated in 7 mg. yield.

No conclusive evidence of a fission of ring A could be obtained, since the only other materials isolated consisted of uncrystallizable yellowish brown gums.

Degradation of cholesterol acetate

Previous experience with cholesterol acetate as substrate has been that, whilst the bacterial count increases and acid is produced, the steroid is largely recovered unchanged. From this it has been assumed that *Proactinomyces* possess no enzymic mechanism capable of hydrolyzing the esterified linkage, thus permitting oxidation of the liberated OH group.

A quantity of 20 g. cholesterol acetate has been treated according to the process described above, incubation in this instance being continued for a total period of 3 months. Even after this time 18.9 g. of the initial material were recovered unchanged. *iso*Caproic acid was obtained in 10 mg. yield, indicating a disruptive oxidation of the side chain, but repeated attempts to isolate a crystalline product from the 26 mg. of brownish gum expected to contain the acetate of 3-hydroxyaetiochol-5-enic acid were unsuccessful.

Since no evidence of any kind was obtained in this experiment of an actual ring fission, it still appears that in the case of cholesterol acetate there is but a single focus of initial bacterial attack.

Degradation of 3:7:12-triketocholanic acid

In the oxidation of cholic acid by *Alcaligenes faecalis* (Hoehn, Schmidt & Hughes, 1944) stepwise oxidation of the three hydroxyl groups occurred, the 3(α)-OH being oxidized last, and 3:7:12-triketocholanic acid was obtained as end product in 85% yield. The general method and detailed isolation technique introduced by these workers has been followed in an investigation of *Proactinomyces* attack on cholic acid, and essentially similar results have been obtained. The further, disruptive oxidation of 3:7:12-triketocholanic acid is of considerable interest, since each of the three keto groups should theoretically provide a susceptible point for bacterial attack, in addition to the probable attack on the shortened side chain. A complicating factor, however, from the cultural standpoint is the greater solubility of cholic acid and the fact that growth of *Proactinomyces* is inhibited in concentrations above approximately 1500 mg./100 ml.

The continuous culture process described in detail above was employed in slightly modified form. Initially, the empty reaction vessel A was autoclaved and connected with the

units *B* and *UC*, 10 ml. water containing the combined surface growth of three 24 hr. agar-slope cultures of *P. erythropolis* (strain 42/G) being subsequently introduced into the reaction vessel. The flask *B* and contents, 3 l. mineral salt solution + 1.0 g. cholic acid, sterilized by autoclaving 5 min. at 115°, were replaced as required in order to maintain a continuous flow of solution at approx. 1 drop/sec. After 2 months' incubation, culture medium containing 52 g. cholic acid had been utilized. The spent medium, including the final contents of the reaction vessel, was evaporated under reduced pressure to 1 l. The resulting orange-coloured concentrate was rendered alkaline with ammonia and filtered. Solvent extraction of the residue (A) did not yield any identifiable product, neither was any material other than traces of brown gum obtained by continuous extractions of the residue, after suspension in dilute HCl, with light petroleum (b.p. 40–60°) and ether. The filtrate (B) was acidified with dilute HCl, and cooled with ice. On standing, the heavy, granular precipitate became crystalline, and was filtered off and dried (27.3 g.). After recrystallization from ethanol this product had m.p. 234°; in admixture with authentic 3:7:12-triketocholic acid (m.p. 236°), a m.p. of 228° was obtained. The crystalline product was accordingly examined for the presence of intermediate oxidation products by the method described by Hoehn *et al.* (1944), but neither cholic acid nor any hydroxy-ketocholic acid was isolated. The product was dissolved in 60 ml. methanol, 1 ml. conc. H₂SO₄ added, and the solution gently refluxed for 15 min. No precipitate was obtained by cooling in ice, but on addition of 10 ml. water followed by further ice cooling for 24 hr., a deposit of rosettes of colourless needles was obtained, m.p. 222°. Repeated recrystallization from aqueous ethanol only raised the m.p. to 228°, which was depressed to 218° in admixture with a specimen of methyl 3:7:12-triketocholanoate. The product was hydrolyzed and the free acidic materials regenerated. Repeated fractional crystallization from ethanol-acetone-ether yielded 15.7 g. 3:7:12-triketocholic acid, m.p. 234°, and 19 mg. of a crystalline acid, m.p. 240°. Further recrystallizations of this latter product from acetone-ether did not raise the melting point. In admixture with authentic 3:7:12-triketocholic acid, m.p. 245°, no depression was observed.

The isolation of this product of degradation points to the side chain as the primary focus of attack on the molecule, and a careful search has failed to isolate any product arising from rupture of the ring system.

Oxidation of α -oestradiol

Proactinomyces oxidations in the steroid group have hitherto been concerned solely with compounds of saturated ring structure. In view of the readiness with which these organisms utilize simple aromatic compounds such as phenol and naphthalene (Gray & Thornton, 1928), it appeared probable that the benzenoid ring structure in steroid compounds would present no obstacle to *Proactinomyces* attack. In several small-scale experiments with *P. erythropolis*, however, whilst there was some slight increase in bacterial numbers, the pH of the medium remained unchanged, and the whole of the starting material was recovered unaltered. These unexpected initial failures were at first attributed to a diminution in

the oxidative capacity of the particular strain used, but similar lack of success was encountered with typical strains of all the known *Proactinomyces* species. A more detailed study of the process was accordingly made as follows.

Finely ground sterile α -oestradiol (100 mg.) was introduced aseptically into 250 ml. sterile mineral salt solution contained in a 500 ml. conical culture flask. An extremely heavy inoculum, the growth from twelve 24 hr. agar-slope cultures of *P. erythropolis* (strain 143/N), was added. The culture was incubated at 25° for 2 months, during which time the flask was gently rocked, and a steady stream of sterile oxygen was bubbled through the culture. After the incubation period the medium was filtered, and the residue (A) dried in an air oven at 25°. The filtrate (B) was extracted successively with light petroleum (b.p. 40–60°), ether and chloroform, and this extraction was repeated after acidification of the aqueous liquid with dilute H₂SO₄; evaporation of the extracts yielded only minute amounts of brown resinous gums. The dried residue (A) was extracted continuously with chloroform in a Soxhlet for 3 hr., and the extract evaporated to dryness under reduced pressure. The residue was dissolved in 15 ml. 75% ethanol, and shaken with three successive 5 ml. quantities of light petroleum (b.p. 40–60°). The ethanol phase was separated, and examined for unchanged α -oestradiol and for oestrone by a modification of the technique described by Szego & Samuels (1943). On evaporation under reduced pressure 97.3 mg. almost colourless residue was obtained. This was dissolved in 20 ml. 15% acetone-light petroleum (b.p. 40–60°) and run on to a column (10 × 1 cm.) of 'adsorption alumina' (British Drug Houses Ltd.) previously treated with the same solvent. The column was washed with 80 ml. of acetone-light petroleum (b.p. 40–60°) mixture. The eluate was evaporated to dryness on a water bath, and the crystalline residue dissolved in 10 ml. ethanol, a separation of ketonic and non-ketonic materials being effected by the Girard (T) technique. The non-ketonic fraction consisted of 85.6 mg. almost pure α -oestradiol. Since the 5.5 mg. of brownish ketonic fraction could not be induced to crystallize an attempt was made to prepare a semicarbazone. 3.1 mg. long, colourless needles were thus obtained, and recrystallized from aqueous ethanol. No depression of the m.p. (256°, micro.) was observed in admixture with a prepared specimen of the semicarbazone of oestrone.

This experiment has been repeated with other *Proactinomyces* spp., but in no case has any actual degradation product been isolated, and oxidation at C₁₇ with formation of oestrone is detectable only when the bacterial population is abnormally high. Ercoli (1941) has already reported a similar experience with *Micrococcus dehydrogenans*.

During this work on α -oestradiol the interesting observation was made that many *Proactinomyces* strains, after prolonged culture upon the diol as sole carbon source, show a diminution in their capacity for oxidizing cholesterol and other steroids with the intact 17 side chain; typical examples are given in Table 2.

There is no immediately apparent explanation of this phenomenon, and the fact that it is not

Table 2. *Diminution of steroid-decomposing capacity*

Strain	Cholestenone from 100 mg. cholesterol; 2 months incubation	
	Before oestradiol culture (mg.)	After oestradiol culture (mg.)
<i>P. erythropolis</i> M 14	20	17
<i>P. erythropolis</i> M 18/G	25	9
<i>P. erythropolis</i> 42/G	31	23
<i>P. globerulum</i> H/53	17	12
<i>P. aquosus</i> T 5/G	29	20
<i>P. aquosus</i> T 29/G	12	8

encountered, or only in small degree, with various other *Proactinomyces* strains renders it almost impossible, without further cultural study, to formulate even a tentative hypothesis in explanation.

DISCUSSION

With the accumulation of data on the process of steroid degradation by the microflora of soils, the fundamental importance of these organisms in the economy of nature becomes apparent. The mechanism of the synthesis of the polyhydrocyclopentenophenanthrene compounds, both in the animal and vegetable kingdoms, is still obscure, and the complex processes of metabolism, resulting in the biogenesis of the sex hormones and other similar products, has still to be finally elucidated. These processes of synthesis, however, and the subsequent bacterial disposal of the steroid materials when divorced from the living organism may be regarded as constituting a natural 'steroid cycle'. Decomposition of steroids deposited in soils is, in general, a relatively rapid process, although in adverse environmental conditions bacterial activity is restricted and some steroid accumulation results.

In the oxidation of sterols with the 17 side chain intact *Proactinomyces* still appears to occupy a unique position, but a detailed investigation (Turfitt, 1947) of the organisms utilizing other steroids where the side chain is not present indicates that various Gram- organisms may also play a not inconsiderable role in the disposal of this type of compound, whilst moulds and actinomycetes are more or less inactive in this respect.

The products of steroid breakdown do not, so far as they have been individually isolated and investigated, appear to have any significance as plant growth stimulants. The possibility of phytohormone activity in the further disruptive products has been examined experimentally in the case of the germination of cress seeds, and also in the root formation and growth promotion of lettuce and tomato seedlings. Two methods were investigated in this connexion; in the first method, cholesterol, carefully purified via the dibromide, was intimately mixed with the experi-

mental soil, and the seeds or seedlings watered daily with a suspension of *P. erythropolis*. In the second technique, no steroid was initially added to the soil, but the latter was treated with a heavy aqueous suspension of *P. erythropolis*; subsequently, watering was carried out with aqueous solutions or suspensions of the crude isolates from the continuous culture method. Comparison with the corresponding controls, however, revealed no indication of stimulant action. In the absence of any contrary evidence, therefore, it must provisionally be considered that bacterial decomposition of steroids is essentially a process for the disposal of waste products.

SUMMARY

1. Further *Proactinomyces* oxidation of steroid ketones, obtained as primary oxidation products of hydroxy steroids, results in disruption of the molecules.

2. Yields of degradation products are small, due largely to their further bacterial decomposition. By a continuous culture process sufficient individual products may generally be isolated to permit of identification, but the bacterial oxidation is in no way comparable with chemical technique as a preparative method.

3. Two types of oxidation are encountered, and are frequently simultaneous processes, viz. ring fission at $>CO$ group, and complete removal of side chain giving the *aetiocarboxylic acid*.

4. Thus, cholest-4-en-3-one yields 3-keto*aetio*-chol-4-enic acid and *isocaproic acid* and Windaus's keto acid by disruption of ring A at C_3-C_4 .

5. Blocking of free hydroxyl at C_3 , as in cholesterol acetate, apparently prevents bacterial fission of the ring at this point, but *isocaproic acid* is again obtained from degradation of the side chain. The expected complementary product, the acetate of 3-hydroxy*aetio*-chol-5-enic acid, has not been isolated.

6. Side-chain fission of 3-ketochol-4-enic acid yields 3-keto*aetio*-chol-4-enic acid, but no definite product has been isolated as evidence of fission at the 3-keto group.

7. The side chain is also completely removed from 3:7:12-triketocholanic acid giving 3:7:12-triketo-*aetio*-cholanic acid, but again there is no conclusive evidence of ring fission at any of the susceptible points.

8. In α -oestradiol the benzenoid ring appears to have an inhibiting influence on the bacterial dehydrogenase system, and it is only when the bacterial population is abnormally high that some slight oxidation to oestrone occurs. No evidence of actual molecular degradation has been obtained.

9. Review of available information suggests the presence of a natural 'steroid cycle', degradation of steroids by soil microflora apparently providing a disposal mechanism for waste materials.

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The Mechanism of the Effect of X-rays on Bacterial Toxins

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PART 1. THE SENSITIVITY OF TOXINS TO X-RAYS

Relatively little work has so far been done on the effect of X-rays on bacterial toxins. Gerhartz (1909) studied the effect of X-rays on diphtheria toxin, and Fabre & Ostrovsky (1912) found a partial inactivation of this toxin following the addition of radium to the toxin solution. Ferroux & Muttermulch (1925) detoxified tetanus toxin with radium and observed that the loss of toxicity was accompanied by a loss of antigenicity. Baker (1935) showed that the sensitivity of tetanus toxin to radium was similar to that of Rous sarcoma virus and bacteriophage, and was less marked than the sensitivity of bacteria. Carpenter & Stokinger (1942), working with culture filtrates of *Clostridium botulinum*, *Cl. perfringens*, *Cl. tetani* and *Staphylococcus aureus*, showed that X-rays caused a partial inactivation of each toxin, tetanus toxin being the most resistant. Loiseleur & Crovisier (1943) were able to increase the effect of X-rays on tetanus toxin by bubbling oxygen through the solution during irradiation.

Most of these investigators, however, were able to obtain only a partial destruction of toxins, and the very wide range and unknown degree of purification of the preparations used in all these works make it quite impossible to draw any conclusions as to the actual sensitivity to X-rays and radium of the toxins

used. Moreover, the quantitative results of different experiments cannot be compared and cannot be used as a reliable basis for further investigations. We therefore decided to investigate the conditions favourable for the complete destruction of bacterial toxins by X-rays, the properties of the toxins after irradiation, and the influence of the medium on the sensitivity of toxins to X-rays.

For this purpose two toxins were used, tetanus toxin and *Staph. aureus* haemolysin.

EXPERIMENTAL

Methods

Media used for the production of toxins, and purification of the toxins. Two preparations of tetanus toxin were used.

(1) Crude tetanus toxin. *Cl. tetani* was grown according to Mueller & Miller (1942), and 3% tomato juice was added to the medium. The toxin solution obtained contained 4.5 mg. N/ml., and upon assay on mice was found to contain 1000 minimum lethal doses (Min.L.D.)/ml.

(2) Purified tetanus toxin. This toxin was obtained and purified according to Eaton & Cronau (1938). The purified solution contained 0.14 mg. N and 1000 Min.L.D./ml.

(3) *Staph. aureus* haemolysin. *Staph. aureus* was grown on the medium of Favorite & Haman (1941). The filtrate contained 0.95 mg. N/ml., and up to a dilution of 1:200 was capable of haemolyzing rabbit corpuscles. No purification of the haemolysin was attempted. The haemolysin was assayed as follows: 0.5 ml. of serial dilutions of the