MICROBIOLOGICAL AGENCIES IN THE DEGRADATION OF STEROIDS

I. The Cholesterol-Decomposing Organisms of Soils

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The disappearance of cholesterol added experimentally to soils has been shown (Turfitt, 1943) to result from the activities of microbiological agents. It is reasonable to presume that the degradation of the complex cholesterol molecule is accomplished by the coordinated activities of a variety of organisms. The soil protozoa, and even some of the more highly organised animal members of the soil population, may well play some role in the process, but during the further work on cholesterol disappearance recorded in this publication attention has been directed towards the bacteria and the mould fungi.

EXPERIMENTAL

Collection of soil samples

Samples representative of the chief types of British soils were collected in sterile tubes 4″ x 1″. Where the surface of the ground was covered with vegetation, or partially decomposed plant debris, this was removed, and the sample taken at the exposed soil surface. In the majority of cases, a further sample was taken from 6 to 12 inches below the surface, or at an even greater depth where thick peat strata were encountered.

Isolation of cholesterol-decomposing organisms

The organisms under consideration fall into three groups, viz. bacteria (aerobic and anaerobic), moulds, and actinomycetes, but the same general technique for their isolation was employed in each case, the only difference being in the culture medium. Table 1 shows the composition of the mineral salt solutions which, together with cholesterol as sole carbon source, constituted the media used throughout the present work.

The actual media were prepared from these basal solutions as follows:

Bacteria and actinomycetes. 50-ml conical culture flasks, each containing 10 ml mineral salt solution I, and approximately 0.001 g finely powdered cholesterol, were sterilised by steaming for 30 minutes on each of 3 consecutive days.

Moulds. 100 ml conical culture flasks, each containing 20 ml mineral salt solution II were autoclaved, and to each flask was then added, with aseptic precautions, approximately 0.001 g cholesterol from a tube of the finely-powdered material which had previously been sterilised by steaming. This modified procedure was adopted in order to ensure that the cholesterol should remain floating on the surface of the medium, and so facilitate normal development of mould mycelium.

Each soil sample was shaken with sterile distilled water, and the grosser particles allowed to settle. Approximately 0.5 ml of a heavy suspension was then transferred to each of three prepared flasks—(1) and (2) containing the bacterial culture medium described above, and (3) containing the mould culture medium. Flasks (1) and (3) were then incubated aerobically at 25°, and (2) under anaerobic conditions at the same temperature. After 7 days, transfers were made from these flasks to a duplicate set, and these also incubated. This process was repeated twice at 7-day intervals. A loopful of the contents of the final flask in each case was then streaked on a solid medium: sub-cultures (1) and (2) on nutrient agar, incubated aerobically and anaerobically respectively; (1) again on casein agar, for actinomycetes; and (3) on Czapek-Dox agar. Organisms which developed were isolated and tested for their ability to grow on the appropriate mineral-salt-cholesterol medium, the criteria for such growth being production of turbidity, change of pH of the medium, and actual cholesterol decomposition determined as described below.

TABLE 1

Mineral salt solutions

I. For bacteria and actinomycetes		*II. For moulds*	
NH_4NO_3	1.0 g	$NaNO_3$	2.0 g
K_2HPO_4	0.25 g	KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.25 g	$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.005 g	KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.0001 g	$FeSO_4 \cdot 7H_2O$	0.01 g
Distilled water to 1000 ml		Distilled water to 1000 ml	

Description of organisms isolated

A great number of microorganisms, both bacteria and fungi, were isolated which were able to grow in mixed culture, but a large proportion of these failed to survive repeated transfer in pure culture. No anaerobic bacteria and no actinomycetes were found capable of utilising cholesterol. Amongst the mould fungi many distinct species, chiefly of *Penicillium* and *Aspergillus*, formed an occasional thin mycelium with partially submerged hyphae, but in no case could the decomposition of cholesterol be detected.

The predominant bacteria appearing on the plates were white, opaque, raised colonies of gram-positive rods, breaking up in older cultures into purely coccoid forms. These bacteria grew readily in pure culture on mineral-salt cholesterol, and produced an acid reaction in the medium after 4 to 5 days. Associated with them, and only occasionally appearing in their absence, were thin, translucent colonies of gram-negative bacteria, often showing a green fluorescence. Many of these organisms survived repeated sub-culture, but showed no indication of ability to utilize cholesterol. It was originally considered that they might be deriving their energy from some impurity in the sterol, but by the use of cholesterol carefully purified via the dibromide this view was rendered untenable. In some instances the organisms have been shown to be facultative autotrophs, but

in general it is likely that they are secondary invaders, utilizing as carbon source some breakdown product of the parent sterol.

In order to determine whether this selective method of isolation was giving an inaccurate picture of the conditions prevailing in nature, certain soils were treated experimentally with cholesterol, and, after an interval of several weeks, platings were made from aqueous suspensions of the soils. The typical colonies of these organisms again appeared in high percentage on the plates.

It thus appears that cholesterol degradation in soils is initiated by a single group of gram-positive bacteria, the individual members of which are closely similar in morphological and cultural characters. The classification of these organisms is discussed below.

TABLE 2

Distribution of cholesterol-decomposing organisms in soils

SOIL TYPE	SOURCE AND VEGETATIVE TYPE	NUMBER OF SAMPLES EXAMINED	NUMBER OF ORGANISMS ISOLATED
Acid, well-drained sand	Oak and oak-birch associations (Sherwood Forest)	22	17
	Beech (Sherwood Forest, Epping Forest, Chiltern Plateau)	30	23
	Heath-land (Sherwood Forest, Pennine Moors, Yorkshire Moors, Central Scottish Highlands)	32	10
Acid to alkaline loam	Acid grassland (Malvern Hills)	2	2
	Neutral grassland (Trend and Thames Valleys)	31	34
	Beech associations (South Downs)	2	2
Alkaline marl	Beech and basic grassland (Chiltern Escarpment)	9	11
Alkaline peat	Fen-land (Wicken Fen, Cambridge; Norfolk Broads)	22	10
Deep, acid peat	Bog-land (Athlone, Dartmoor, Pennine plateaux)	16	2
Arable land	various	63	77

Distribution of cholesterol-decomposing organisms in soils

It has previously been found, in a survey of soil sterols (Turfitt, 1943), that sterol accumulation occurs under conditions of high acidity, waterlogging and lack of aeration, and where these conditions have been counteracted, as in arable soils, sterol turn-over is relatively rapid. By collecting the soil samples from the same general localities as those used in that survey, it was hoped to establish a relation between sterol turn-over and the relative abundance of cholesterol-decomposing organisms for the various types of soil. The distribution of these organisms throughout the soils examined is summarised in table 2. The accumulation of sterols in certain soil types is thus due to the inability of the cholesterol-decomposing bacteria to flourish under the adverse environmental conditions prevailing in these soils.

Classification of the cholesterol-decomposing bacteria

The general features of the cholesterol-decomposing organisms are essentially those of *Mycobacterium* as described by Gray and Thornton (1928). In recent taxonomic studies of the Order Actinomycetales (Jensen, 1931; Bergey, 1939) they are classified as the genus *Proactinomyces*, and this nomenclature is adopted throughout the present work. The typical morphological character upon which the genus is based is the formation of a 'mycelium' of rod-shaped forms or filaments showing some slight degree of branching; in older culture all evidence of branching is lost, and the cells divide into short rods and coccoid forms. The organisms thus constitute a group intermediate between the unbranched *Corynebacterium* and *Mycobacterium* and the richly-branching *Actinomyces*. Amongst the cholesterol-decomposing bacteria isolated in this work two new species have been identified in which branching is rare, and can be demonstrated only in very young cultures upon special media.[1] Their general characteristics, however, leave no doubt that they should be classed with *Proactinomyces*, and they may perhaps be regarded, along with *P. rubropertinctus* (Heffernan), as a first transitional stage towards *Mycobacterium*.

The 188 strains isolated which were capable of utilising cholesterol as sole carbon source have been identified as follows:

Proactinomyces erythropolis Gray and Thornton	144 strains
Proactinomyces aquosus n. sp.	21 strains
Proactinomyces globerulus Gray	14 strains
Proactinomyces coeliacus Gray and Thornton	8 strains
Proactinomyces restrictus n. sp.	1 strain

No other species of *Proactinomyces* was encountered, although, as described below, stock strains of the known species were all found to utilise cholesterol when grown in pure culture.

Proactinomyces aquosus n. sp.

Curved, uneven-sided rods: 0.4 to 0.6 by 0.4 to 3.0μ, occasionally longer to 4.5μ, with well-marked beading. Younger cultures, 10 to 11 hours, show longer rods up to 6.5μ with occasional slight branching; after 36 hours only in coccoid forms 0.3 to 0.5μ. Gram-positive. non-motile. Not acid-fast.

Nutrient agar colonies (2 days): 0.5 to 2 mm., irregularly round, flat, water-white, smooth, shining; edge entire.

Nutrient agar slope (24 hours): good growth, thin watery appearance, slightly raised, smooth, shining; edge entire.

Nutrient gelatin stab (1 month): compact, pinkish surface growth; no liquefaction; line of stab filiform, erose.

Nutrient broth: turbid, with stringy or flocculent deposit; little surface growth.

Litmus milk (14 days): unchanged or slightly acid.

Potato slope (2 days): poor to fair growth, thin, flat, cream-coloured becoming pale orange to orange-brown, smooth, shining, soft consistency; potato not darkened.

Carbohydrates (14 days): no acid or gas from glucose, sucrose, maltose, lactose.

Indole not produced.

[1] The simplification of the Dreschler method described by Jensen (1931) was used for this purpose.

Hydrogen sulphide not produced.
Nitrate reduced to nitrite by some strains.
Paraffin wax utilisable as C source.
Optimum temperature: 25°; little growth at 37°.
Obligate aerobe.
Distinctive characters: Most closely resembles *P. erythropolis* Gray and Thornton, but differs from this organism in shorter length of rods, comparative rarity of branching, and in nature of growth on solid media.

Proactinomyces restrictus n. sp.

Short, uneven-sided, beaded rods and coccoid forms: 0.3 to 0.5μ by 0.4 to 1.5μ. Rods up to 4.5μ in 10-hour cultures, with occasional very slight branching; after 24 hours only in coccoid forms 0.3 to 0.5μ in diameter.
Gram-positive. Non-motile. Not acid-fast.
Nutrient agar colonies (2 days): 0.5–1.5 mm., irregularly round, convex, white, smooth, shining, opaque; edge entire.
Nutrient agar slope (24 hours): restricted, filiform streak, raised, pinkish-white, smooth, shining; edge entire.
Nutrient gelatin stab (1 month): restricted surface growth; no liquefaction; line of stab filiform, erose.
Nutrient broth: turbid with fine powdery deposit; little surface growth.
Litmus milk (14 days): slightly acid.
Potato slope (2 days): good growth, flat, shining, pale orange colour, soft consistency; potato not darkened.
Carbohydrates (14 days): no acid or gas from glucose, sucrose, maltose, lactose.
Indole not produced.
Hydrogen sulphide not produced.
Nitrate rapidly reduced to nitrite.
Paraffin wax utilisable as C source.
Optimum temperature: 25°; little or no growth at 37°.
Obligate aerobe.
Distinctive characters: Cells are very short rods, never filaments. Very slight branching is detectable in 10-hour glucose-asparagine-agar cultures. Distinguished from *P. rubropertinctus* Heffernan by nature of growth on solid media, and by various biochemical features.

Attempted growth of known bacterial species on cholesterol

A wide range of known bacterial species (from the National Collection of Type Cultures, Lister Institute, London) was tested for cholesterol-decomposing ability by inoculating each culture into mineral salt solution with cholesterol as sole carbon source:

Chromobacterium violaceum Schroeter; *Alkaligenes faecalis* Castellani and Chalmers; *Vibrio cyclosites* Gray and Thornton; *Pseudomonas aeruginosa* Schroeter; *Pseudomonas fluorescens* Migula; *Phytomonas malvacearum* Erw. Smith; *Protaminobacter alboflavum* den Dooren de Jong; *Mycoplana bullata* Gray and Thornton; *Micrococcus piltonensis* Gray and Thornton; *Staphylococcus aureus* Rosenbach; *Sarcina lutea* Schroeter; *Streptococcus pyogenes* Rosenbach; *Escherichia coli* Migula; *Aerobacter aerogenes* Beijerinck; *Serratia marcescens* Bizio; *Proteus vulgaris* Hauzer; *Bacterium mycoides* Migula; *Bacillus megatherium* De Bary; *Pseudomonas pictorum* Gray and Thornton; *Bacillus subtilis* Cohn; *Bacillus tumescens* Zopf; *Bacillus closteroides* Gray and Thornton; *Corynebacterium xerose* Neisser and Kuschbert; *Corynebacterium pseudodiphthericum* Lehmann and Neumann; *Mycobacterium lacticola* Lehmann and Neumann; *Mycobacterium phlei* Lehmann and Neumann; *Proactinomyces agrestis*

Gray and Thornton; *Proactinomyces minimus* Jensen; *Proactinomyces polychromogenes* Valée; *Proactinomyces crystallophagus* Gray and Thornton; *Proactinomyces rubropertinctus* Heffernan; *Proactinomyces coeliacus* Gray and Thornton; *Proactinomyces actinomorphus* Gray and Thornton; *Proactinomyces erythropolis* Gray and Thornton; *Proactinomyces globerulus* Gray; *Proactinomyces paraffinae* Jensen.

None of these strains, with the exception of the *Proactinomyces* species and *Mycobacterium lacticola* proved capable of utilising cholesterol as a source of energy, although growth of several organisms, notably *P. aeruginosa*, *P. vulgaris*, *A. aerogenes*, *A. faecalis*, persisted through several subcultures. The property of decomposing cholesterol with the formation of an acid reaction in the basal salt medium already described thus seems to be a characteristic of the genus *Proactinomyces*, and it may well prove a useful additional diagnostic feature. *M. lacticola* presents an apparent anomaly, but the author is inclined to the view that the organism represents, in effect, a transitional stage even nearer to *Mycobacterium* than that of *P. aquosus* and *P. restrictus* described above.

TABLE 3

Extent of cholesterol decomposition by Proactinomyces spp.

ORGANISM	NUMBER OF STRAINS EXAMINED	PER CENT DECOMPOSITION
P. coeliacus	9	19.3–35.0
P. globerulus	1	32.5
P. agrestis	15	11.0–31.5
P. erythropolis	145	22.0–41.7
P. crystallophagus	1	28.3
P. convolutus	1	27.8
P. actinomorphus	1	27.8
P. minimus	1	23.1
P. rubropertinctus	1	17.7
P. polychromogenes	1	5.5
P. aquosus	21	17.4–36.3
P. restrictus	1	31.6
M. lacticola	3	8.3–33.0

Extent of cholesterol decomposition by Proactinomyces species

A strictly accurate comparison of the cholesterol-decomposing powers of the organisms is impracticable owing to the insoluble nature of the sterol, and the consequent difficulty of presenting an identical surface area to attack in each case. The method described, however, gives an indication of the comparative activities of the various organisms which is sufficient for the present purpose.

For each strain, 100 ml mineral salt solution together with 0.05 g cholesterol and 0.5 g $CaCO_3$ in a 500 ml conical flask was sterilised by steaming, and inoculated from a 24-hour agar slope culture of the appropriate organism. The flasks were incubated at 25° for 2 months. After this time each flask was extracted thoroughly with light petroleum (40°–50°), and the extracts in each case made up to 100 ml. with light petroleum. 1.0 ml of the extract was treated, in a 15 ml graduated centrifuge tube, with 4.0 ml of a 0.2 per cent solution of digitonin in

95 per cent alcohol, and the mixture evaporated to dryness. The solid residue was extracted three times with boiling light petroleum, and then dissolved in 1 ml pyridine. 12 ml ether were added and the mixture stirred with a glass rod. Digitonin separated and was centrifuged off. The supernatant liquid was decanted and evaporated to dryness, the residual solid being taken up in 5.0 ml chloroform and transferred to a 10-ml stoppered measuring cylinder. 0.5 mg cholesterol dissolved in chloroform was measured into a second cylinder, and 1.0 ml acetic anhydride and 0.1 ml conc. H_2SO_4 added to each. The solutions were thoroughly mixed and allowed to stand at 25° for 15 minutes. The green colours which had developed were then compared in a colorimeter. In table 3 the results are expressed as percentage decomposition for each individual organism.

The cholesterol-decomposing power seems to be but little affected by repeated subculture. A number of strains of *P. erythropolis* has been cultured over a period of six years on nutrient agar and on synthetic media with little or no decrease in this property. There have, however, been occasional instances of quite vigorous sterol decomposers becoming completely inactive in this respect for no apparent reason. In such cases all attempts to induce reactivation have been unsuccessful.

SUMMARY

1. Cholesterol breakdown in soils has been shown to result from the activities of species of the genus *Proactinomyces*, and in particular of *P. erythropolis* Gray and Thornton. Other bacteria, moulds and actinomycetes have been found to be inactive.

2. Two new species of *Proactinomyces* are described, *P. aquosus* and *P. restrictus*.

3. A survey of the distribution of *Proactinomyces* species in soils indicated their relative abundance in fertile areas. Under conditions of water-logging, extreme acidity, and lack of aeration, they were present only infrequently.

4. In the presence of $CaCO_3$ to prevent acid accumulation, the average decomposition of cholesterol in mineral salt solution by *Proactinomyces* species during two months incubation at 25° was approximately 30 per cent.

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