BACTERIOSTATIC AND BACTERIOLYTIC PROPERTIES OF ACTINOMYCETES

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Investigations on the antimicrobial properties of actinomycetes can be divided into two groups: (a) those dealing with the bacteriostatic and bactericidal activities of these organisms, and (b) those concerned primarily with their bacteriolytic properties. In both cases, the possible practical applications have been kept in mind. The hope of isolating specific antibacterial substances potentially useful for the control of infectious diseases of man, animals, or plants has been general. Interest in the lytic agents is dominated by the possible use of bacterial lysates for vaccination purposes.

HISTORICAL

Since the literature concerning the antibacterial action of actinomycetes (and other microorganisms) has recently been fully reviewed by Waksman (1941), it will be sufficient to recall here that antibiotic properties are widely distributed among these organisms, as shown by the work of Nakhimovskaia (1937), Krassilnikov and Koreniako (1939), Alexopoulos (1941), Waksman, Woodruff and Horning (1941) and Welsch (1942). At the present time, two powerful antimicrobial agents have been obtained from cultures of actinomycetes. The first was designated as actinomycin; it was obtained from Actinomyces antibioticus (Waksman and Woodruff, 1940a), and has been purified and crystallized (Waksman and Woodruff, 1940b, Waksman and Tischler, 1942). The second, designated as streptothricin, was obtained from a strain of Actinomyces lavendulae (Waksman and Woodruff, 1942). The bacteriolytic properties of actinomycetes, with which the present paper is mostly concerned, are less well understood than their bacteriostatic activities.

The credit for having first recognized the ability of actinomycetes to destroy microbial cells is generally given to Gasperini (1890), who observed, in the course of his classical researches on Streptothrix foersteri Cohn, that the filaments of this organism may destroy the cell-membrane of several bacteria and fungi. The first experimental study of the bacteriolytic action of actinomycetes, however, did not begin until 30 years later. Lieske (1921) not only described the antagonistic effects of actinomycetes on Staphylococcus aureus, but also reported the dissolution of various dead or living bacteria, incorporated in water-agar, by actinomycetes streaked on the surface of this medium.

In the course of investigations on bacteriophage, Gratia and Rhodes (1924)

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nated group (Duche, 1924–1931) to the discovery, quite independently of Lieske, of the bacteriolytic action of actinomycetes; it was found that these organisms are able to dissolve many heat-killed or living pathogenic bacteria suspended in water. The lysates, although much less toxic than the nontreated suspensions, were very good antigens. Accordingly, Gratia (1930–1934) employed these “mycolysates” as a therapeutic agent in several infectious diseases of man. Rosenthal (1925) also observed the lysis of Corynebacterium diphtheriae by an actinomycete contaminating the Loeffler’s medium plate.

These cases of bacteriolytic action of actinomycetes are closely related to the type of activity exhibited by a species of Actinomyces belonging to the A. albus group, which has been extensively studied by the writer (Welsch, 1936–1939, 1937, 1938, 1941). A summary of the antibacterial properties of this organism follows.

Actinomyces G grows readily in liquid or agar mineral media containing heat-killed or chemically killed gram-negative or gram-positive bacteria, or living gram-positive bacteria. The bacterial suspensions become clarified after a few hours in the case of heat-killed gram-negative bacteria and only after 2 to 3 days in the case of either heat-killed or living gram-positive organisms. It is essential that the bacterial suspensions be inoculated with a sporulating broth-culture of Actinomyces G and that a small amount of the culture medium be transferred together with the organism. The antagonist grows very scantily in suspensions of living gram-negative bacteria where it produces no lysis at all. It dissolves killed bacteria suspended in nutrient-broth but does not affect living bacteria to any extent under those conditions (Welsch, 1936–1939).

Sterile filtrates of broth-cultures of Actinomyces G, obtained after sporulation, were designated as “actinomycetin” (Welsch, 1937); they dissolved in a few hours suspensions of heat-killed gram-negative bacteria and in 24 hours those of heat-killed gram-positive bacteria. They had no action, however, on most living gram-positive bacteria, although partial lysis of Klebsiella pneumoniae, Streptococcus hemolyticus and Staphylococcus aureus was obtained. They had no action on any living gram-negative organism so far tested.

The properties of the lytic agent are those of a protein (Welsch, 1936–1939) and of an enzyme (Welsch, 1938). Concentrated preparations of the lytic agent have been obtained (Welsch, 1941), but, though active upon killed bacteria, they were inactive when tested on most living organisms.

By the use of the cross-streak method, it has been shown that Actinomyces G has a bacteriostatic effect against many gram-positive bacteria but not against any of the gram-negative organisms examined (Welsch, 1942).

Crude actinomycetin (Welsch, 1937) has no bacteriostatic activity, but con-

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[1] The identity of this organism with any one member of the highly heterogeneous A. albus group (Duche, 1934) has not yet been established. The organism will, therefore, be designated in the present paper as Actinomyces G, in homage to Dr. A. Gratia who was one of the first to undertake a systematic study of the occurrence of bacteriolytic organisms in nature.
centrated preparations, added to nutrient-agar, prevented the growth of several gram-positive bacteria (Welsch, 1941). Ether-extracts of actinomycin had a notable bactericidal action on gram-positive bacteria suspended in an inorganic medium; this action was greatly reduced in the ordinary complex culture-media. The active agent appeared to be a lipoid, probably a fatty acid. Part of it originated from the culture-medium, since similarly active extracts were obtained from sterile media; another part of it was, undoubtedly, produced as a result of the growth of the Actinomyces (Welsch, 1941). "Actinomycin B" produced by Actinomyces antibioticus is, presumably, of a similar nature (Waksman and Woodruff, 1940b).

Bacteriolysis of living bacteria by Actinomyces G was visualized as a two-step reaction: first, the susceptible cells are killed by the selectively bactericidal lipoid; second, those dead cells are then dissolved by the bacteriolytic enzyme (which alone is responsible for the lysis of heat-killed bacteria). The phenomenon does not take place in complex culture-media, since the bactericidal action of the lipoid is greatly impaired under those conditions, and the presence of living Actinomyces is generally necessary, since free lipoid should be secreted in the susceptible suspension (Welsch, 1941).

Another type of bacteriolysis by actinomycetes, especially by Actinomyces violaceus, was reported recently by Krassilnikov and Koreniako (1939) and by Kriss (1940). The active principle involved differs notably from the lytic agent of actinomycin, since it is relatively thermostable, resistant to ultra-violet radiations, easily adsorbed on various substrates and most abundantly produced in synthetic media. Like the lytic agent of actinomycin, it is soluble in water, insoluble in organic solvents, and less active in acid media. This agent was said to resemble egg-white lysozyme (Fleming, 1922); however, the justification of this assumption may be questioned. The nature of the active principle remains wholly unknown, whereas lysozyme has been purified and crystallized (Meyer et al., 1936; Roberts, 1937; Abraham, 1939). The bacterial constituent acted upon by the new agent is not known, whereas the component susceptible to lysozyme has been isolated (Epstein and Chain, 1940) and shown to be a polysaccharide. Staphylococci are the organisms most susceptible to the new agent which has no selective action on Micrococcus lysodeikticus; the latter is the best test-organism for lysozyme, which has virtually no action on staphylococci (Thompson and Khorazo, 1935). Lysozyme is further resistant to acidification, even at a high temperature (Thompson, 1941).

CULTURES AND METHODS

Source of cultures studied. Altogether, 275 strains of actinomycetes were used. These may be divided into two groups: first, 111 cultures taken at random from a series of 250 strains freshly isolated from various soils by Mrs. E. S. Horning, in this laboratory; second, 164 strains, including representatives of the genera Actinomyces, Proactinomyces and Micromonospora, isolated from various soils, marine material, potatoes, lake-mud, etc., were obtained from the type culture
collection of the N. J. Agr. Exp. Station. The latter strains had been kept in
the collection for periods of time ranging from a few months to more than
25 years.

Cultivation of the actinomycetes. Stock-cultures were maintained on slants of
beef-extract-peptone-agar and of modified Czapek’s-agar. Spore-suspensions in
sterile tap-water were obtained from such slants for the inoculation of all experi-
mental cultures. The actinomycetes to be tested for bacteriolytic activity were
grown at 30°C. in 25 ml. portions of broth. Cultures on nutrient-agar plates
or on starch-tryptone-agar in Blake bottles were used in the tests for bacterio-
static activity.

Test-organisms. A strain of Escherichia coli, isolated by the writer from the
urine in a case of cystitis, was used as a typical representative of gram-negative
organisms. A strain of Staphylococcus aureus, isolated from a carbuncle, was
used as a typical representative gram-positive organism. A collection strain
of Bacillus subtilis and one of Sarcina lutea were widely used in the tests of
bacteriostatic activity. A strain of Micrococcus lysodeikticus, originally ob-
tained from Dr. Fleming, was used for lysozyme tests. In addition, a number
of bacterial strains of various origin were used and will be listed in the descrip-
tion of the corresponding experiments.

The stock-cultures of the test organisms were maintained on beef-extract-
peptone-agar slants or on serum-agar (pneumococci, streptococci, Neisseria,
corynebacteria). Suspensions of the organisms in sterile tap-water were made
from 24- or 48-hour-old slants for the inoculation of all experimental cultures.

The bacteria to be tested were grown for 24 or 48 hours at 30° or 37° on 25 ml.
beef-extract-peptone-agar or serum-agar in Blake bottles. The cells were then
aseptically suspended in 5 ml. of sterile mineral salt solution (Czapek without
sugar). In experiments with living test-organisms, this heavy bacterial suspen-
sion was used directly after suitable dilution. In experiments with heat-killed
test-organisms, the bacterial suspension was first inactivated either at 65° for
30 minutes or in a boiling water-bath for five minutes, then suitably diluted.

Tests for lysis by growing actinomycetes. Three slightly different methods
were used, and records made after 18, 24, 48, and 72 hours incubation, at 30°C.

(a) The heavy suspension of test-organisms, either fresh or heated, was diluted
with sterile mineral solution until a turbidity equivalent to that of 10⁶ cells per
ml. was obtained. The Pulfrich-Zeiss nephelometer was used for standardiza-
tion (Welsch, 1938). The dilute bacterial suspension was aseptically distributed
(4.5 ml. portions) in test-tubes and each tube inoculated with 0.5 ml. of a
broth-culture of the actinomycetes. Evidence of lysis was sought by direct
examination or by nephelometric measurements. A slightly different procedure
was used in the tests with living organisms. The mineral solution was distrib-
uted in 250 ml. Erlenmeyer flasks (25 ml. portions), sterilized, inoculated
with 0.5 ml. of a broth-culture of the actinomycetes, and incubated at 30° for 48

Bacto-tryptone, 10 gm.; corn starch, 5 gm.; NaCl, 0.2 gm.; K₂HPO₄, 0.2 gm.; bacto-
agar, 10 gm.; Tap-water, 1000 ml.
hours. Thereafter, a suitable amount of the heavy bacterial suspension was aseptically added to each flask to obtain the desired turbidity. After further incubation, the extent of lysis was determined.

(b) The heavy bacterial suspension was suitably diluted in melted and cooled soft (0.5%) synthetic agar. This was next distributed in test-tubes, in 5 ml. portions, and allowed to solidify. Each tube was inoculated with 0.2 ml. of a broth-culture of the actinomycetes and incubated. Development of the actinomycetes on the surface, the presence of a clarification zone underneath, its depth and intensity, and the presence of alternately clear and dark rings were recorded.

(c) The heavy suspension was suitably diluted in melted and cooled synthetic agar (1.5%); this agar was then poured into Petri dishes, in 15–20 ml. portions, and allowed to solidify. Each plate was inoculated with four different actinomycetes, streaks about 2 cm. in length being made. After incubation at 30°, growth of the actinomycetes, formation and width of clear zones around the colonies determined.

Tests for lysis by soluble products of actinomycetes. For this purpose, either liquid or solid medium was used. The heavy suspension of fresh or heat-killed bacteria was suitably diluted in M/15 phosphate buffer of pH 8.0; the dilute suspension was then aseptically distributed in test-tubes (4.5 ml. portions) and warmed to 38° in a water-bath. To each of several tubes was then added 0.5 ml. of a paper- or Seitz-filtrate of a broth-culture of the actinomycetes previously warmed to 38°C.; at this moment, the time was recorded with a stop-watch. The mixture was kept at 38° in a water-bath; samples were removed from time to time, rapidly cooled in ice, and examined in the nephelometer. With heat-killed gram-negative organisms, samples were examined during a period of 20 to 120 minutes and the mathematical relations described by the writer were used to express the activity in "mycolytic units" (Welsch, 1938). With heat-killed gram-positive bacteria and with living microorganisms, the examination of the samples was extended through a period of 24 to 48 hours. In each case, bacterial suspensions without filtrate and bacterial suspensions with boiled filtrate added were used as controls. When a solid medium was used, bacterial-agar plates were prepared as described above. Fragments of sterile agar were taken in the vicinity of the actinomycetes grown on various solid media and transferred to the bacterial-agar medium. After incubation at 37°, presence and width of the clarification zone surrounding the bits of agar were recorded.

Test for bacteriostatic action of growing actinomycetes. The actinomycetes to be tested were streaked across a plate of nutrient-agar and incubated for 48 hours at 30°C. A loopful of a suspension of each test-organism in tap-water was streaked at a right angle from the periphery of the plate toward the culture of the actinomycetes. Three test-organisms were generally used on every single plate. After further incubation for 24 hours at 30° or 37°, the presence and the width of a sterile zone in the proximity of the Actinomyces were recorded.

Test for bacteriostatic action of soluble products from actinomycetes. Paper-filtrates from broth-cultures as well as extracts from tryptone-starch agar-
cultures were tested. The extracts were obtained by shaking the cultures vigorously in 25 ml. of sterile tap-water, allowing to stand for two or more hours and filtering aseptically through paper. To test the activity, varying quantities of the filtrates were diluted in 10 ml. nutrient-agar, which was poured into a Petri dish and allowed to solidify. The test-organisms, four per plate, were streaked on the treated medium, tap-water suspensions of the bacteria from 24-hour-old slants being used. Occurrence of normal or reduced growth or absence of growth was recorded by comparison with the type of growth obtained on the control medium.

EXPERIMENTAL RESULTS

Type of bacteriolytic activity produced by actinomycetes

From a series of 250 cultures of actinomycetes, freshly isolated from various soils, 111 strains were taken at random, inoculated on living-staphylococcus mineral-agar and incubated for 6 days. At the end of this period, 39 strains had produced a clarification of the surrounding medium, whereas 72 cultures were inactive. For more detailed study, 42 cultures were selected from the above 111; they included 21 of the most active, six of the least active and 15 of the inactive strains. Actinomyces G was added to the series for the purpose of comparison; in all tests, this organism was as active as, or more active than, any of the strains studied.

The actinomycetes were inoculated in nutrient-broth and examined for bacteriolytic action on heat-killed E. coli and S. aureus, as well as on living E. coli and S. aureus. With each of these four substrates, the three methods above described were employed. On the basis of the results obtained, the strains were arbitrarily classified as highly active, moderately active or inactive. A final classification of the strains, on the basis of their action on each substrate, was next made by a careful comparison of the results recorded in the three different tests. Thus, organisms falling in the same activity-group in all three tests or in two of the three tests were finally classified in that group. Organisms falling in a different group in each test were classified in the middle group, i.e. as moderately active. At last, a statistical comparison of the ability of actinomycetes to attack the different bacterial substrates was made by the four-fold table and “chi-square” method (Fisher, 1930; Pearl, 1930).

The tests in soft mineral-agar were made with transfers from two-day-old cultures of the actinomycetes; when killed bacteria were used, they were inactivated in a boiling water-bath for five minutes. Tests in mineral agar were made with transfers from eight-day-old broth-cultures of the actinomycetes and tests in mineral-solution were made with transfers of eleven-day-old cultures; when killed bacteria were used in these two tests, they were inactivated at 65°C for 30 minutes.

Before testing for lysis of living bacteria, it was thought desirable to demonstrate that the test-organisms survived long enough in the basic mineral solution to permit experiments extending over a period of three days. For this purpose, suspensions of S. aureus and E. coli in mineral-solution were incubated at 30°
for four days and colony-counts by the plate method made daily. The number of viable cells in suspension was calculated from the average number of colonies counted on five replicate plates. It was found that the test-organisms survived well within the limits of time needed for the experiments.

Growth of all actinomycetes tested was obtained on heat-killed S. aureus and E. coli as well as on living S. aureus; on the contrary, only a few strains grew, and very scantily, on living E. coli.

With heat-killed suspensions in soft-mineral-agar, evidence of lysis was observed within 18 hours after inoculation with some of the actinomycetes; with other strains, however, lysis was not observed until after incubation for two days. The zone of lysis was always very sharp and, after three days, its depth varied from six to 20 mm. In many cases, there was a second sharp zone of partial lysis, five to 25 mm. deep, under the clear zone. With living S. aureus in soft-mineral-agar, evidence of lysis after 18 hours was observed only with a few strains but was commonly found after 48 hours; the depth of the clear zone, after three days, varied from five to 20 mm. The presence of a zone of partial lysis underneath the region of complete clarification was seldom observed, but the production of clear zones alternating with darker rings was of common occurrence. The phenomenon was highly dynamic, since the position of the deeper dark bands changed progressively, whereas the dark rings closest to the surface gradually faded away and eventually disappeared. This phenomenon, which is probably related to the, as yet unexplained, Liesegang's rings, was never observed with heat-killed bacteria. This suggests that it is the result of some biological activity of the test-organism.

In mineral-agar, evidence of lysis was generally not observed before 48 hours. The clear zone had a width of two to 12 mm. with heat-killed bacteria, and of two to 10 mm. with living S. aureus. In this last case, the production of dark rings was occasionally noted, but this phenomenon was less frequent and less conspicuous than in soft-agar.

In mineral-solution, a decrease of turbidity of the heat-killed suspensions was observed after a few hours' incubation, in a small number of cases. With most of the active organisms, however, evidence of lysis was obtained only after 24 to 48 hours; after that time, the effect produced varied from a partial clarification to a complete clearing. In the case of living S. aureus, the actinomycetes were incubated in mineral-solution for 48 hours before the introduction of susceptible cells; evidence of lysis was generally found after 18 or 24 hours; the lysis remained partial or, most often, became complete after 48 hours.

Not a single strain of the many actinomycetes tested was found able to produce lysis of living E. coli by any of the methods used.

The distribution of the different types of bacteriolytic properties of actinomycetes is indicated in table 1. This table shows that about the same number of strains fell into each of the three activity-groups, whether the test-organism used was heat-killed E. coli, heat-killed S. aureus or living S. aureus. The results do not show, however, whether the organisms placed in corresponding groups for their action on different substrates are the same or not. The fourfold
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Tables demonstrate, respectively, the relation between activity of actinomycetes on heat-killed *E. coli* and on heat-killed *S. aureus* (table 2), and between their action on heat-killed *S. aureus* and on living *S. aureus* (table 3). It may be seen from these tables that most organisms capable of lysing one substrate are also able to dissolve the others. The statistical significance of the results may be ascertained by the ‘‘chi-square’’ method, which shows, in effect, that the experimental data are clearly opposed to the hypothesis of independence of the properties tabulated.

### TABLE 1

**Lytic action of actinomycetes on various bacterial substrates**

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>ACTIVITY</th>
<th>NUMBER OF STRAINS ACCORDING TO ACTIVITY AND METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soft-agar</td>
</tr>
<tr>
<td>Heat-killed <em>E. coli</em></td>
<td>++</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Heat-killed <em>S. aureus</em></td>
<td>++</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Living <em>S. aureus</em></td>
<td>++</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Living <em>E. coli</em></td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>42</td>
</tr>
</tbody>
</table>

*In soft agar: ++ = clear zone 3-10 mm. deep in 18 hours; 14-20 mm. in 3 days; + = zone appearing after 48 hours; 5-12 mm. deep in 3 days; 0 = no clarification in 3 days.
In agar: ++ = clear zone 3 mm. in 2 days; 7-12 mm. in 3 days; + = Zone smaller than 6 mm. wide in 3 days; 0 = no clarification in 3 days.
In solution: ++ = complete clarification in 2 days or less; + = partial lysis in 2 days; 0 = no lysis in 3 days.
† In soft agar: ++ = lysis noticeable after 18 hours; zone 10-20 mm. deep in 3 days; + = lysis noticeable after 48 hours; zone 4-10 mm. deep in 3 days; 0 = no lysis in 3 days.
In agar: ++ = zone 4-10 mm. wide in 3 days; + = zone smaller than 4 mm. in 3 days; 0 = no lysis in 3 days.
In solution: ++ = complete clearing in 2 days or less; + = partial lysis in 2 days; 0 = no lysis in 3 days.
‡ In soft agar: ++ = lysis noticeable in 18 hours; zone 10-20 mm. in 3 days; + = lysis noticeable in 48 hours; zone 4-10 mm. in 3 days; 0 = no lysis in 3 days.
In agar: ++ = zone 3-10 mm. in 3 days; + = zone 1-3 mm. in 3 days; 0 = no lysis in 3 days.
In solution: ++ = complete lysis in 2 days or less; + = partial lysis in 2 days; 0 = no lysis in 3 days.
In the absence of a pure preparation of the lytic agent, it is, of course, impossible to know, by direct experiment, whether a single lytic principle is responsible for the action observed on the three different bacterial substrates used. The statistical evidence brought forward indicates that there is no reason, in the absence of contrary experimental data, to assume that different specific lytic agents are responsible for the action of any strain on different bacterial substrates.

**TABLE 2**
Fourfold table showing the relation between bacteriolytic action on heat-killed E. coli and on heat-killed S. aureus

<table>
<thead>
<tr>
<th>LYSIS OF E. COLI</th>
<th>LYSIS OF S. AUREUS</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Total.............</td>
<td>29</td>
<td>13</td>
</tr>
</tbody>
</table>

Chi square = \( \frac{(26.10 - 3.3)^2}{29.13.29.13} \) = 18.61.

\( P < 0.01. \)

* + = high or moderate bacteriolytic activity; 0 = no lytic activity.

**TABLE 3**
Fourfold table showing the relation between bacteriolytic action on heat-killed S. aureus and on living S. aureus

<table>
<thead>
<tr>
<th>LYSIS OF LIVING S. AUREUS</th>
<th>LYSIS OF HEAT-KILLED S. AUREUS</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Total.............</td>
<td>29</td>
<td>13</td>
</tr>
</tbody>
</table>

Chi square = \( \frac{(26.11 - 3.2)^2}{28.14.29.13} \) = 22.28.

\( P < 0.01. \)

The bacteriolytic activity of seven-day-old broth-filtrates of the actinomycetes was next studied by the liquid-medium method, using heat-killed and fresh suspensions of both E. coli and S. aureus. The distribution of lytic properties against heat-killed bacteria was found to be somewhat less common than the distribution of activity associated with the growth of the organisms. On the other hand, lytic activity of filtrates against living S. aureus was always slight and was far from common; no action was found on living E. coli (table 4). Since indirect evidence has been given that the same lytic agent is responsible for the dissolution of all three susceptible bacterial substrates, the apparent
discrepancy between the action of filtrates on heat-killed or living organisms requires explanation. It may be attributed to quantitative differences, the concentration of the active principle in the filtrates being sufficient to act on the more susceptible heat-killed bacteria but too low to affect the more resistant living organisms. Concentrated preparations of actinomycetin, however, were shown to have no lytic action on living organisms (Welsch, 1941). Another explanation is that the lytic agent acts only on dead bacteria and that actinomycetes able to dissolve living organisms produce not only the lytic principle but also a bacteriotoxic substance. This second hypothesis has been discussed in connection with the study of Actinomyces G (Welsch, 1941), and evidence that growth-inhibiting properties of actinomycetes are significantly associated with their ability to lyse living S. aureus will be given later.

<table>
<thead>
<tr>
<th>SUSCEPTIBLE ORGANISMS</th>
<th>NUMBER OF FILTRATES OF DIFFERENT ORGANISMS AND THEIR ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>++*</td>
</tr>
<tr>
<td>Heat-killed E. coli</td>
<td>11</td>
</tr>
<tr>
<td>Heat-killed S. aureus</td>
<td>11</td>
</tr>
<tr>
<td>Living S. aureus</td>
<td>4</td>
</tr>
<tr>
<td>Living E. coli</td>
<td>0</td>
</tr>
</tbody>
</table>

* ++ = highly active; i.e. 25-200 mycolytic units per ml. on heat-killed E. coli; turbidity of heat-killed S. aureus suspensions reduced to 50% or less of its original value in 2 days or less; turbidity of living S. aureus suspension reduced to 75% or less of its original value in 2 days.

+ = moderately active; i.e. less than 25 mycolytic units per ml. on heat-killed E. coli; turbidity of heat-killed S. aureus suspension reduced to at most 50% of its original value in 2 days; no action on living S. aureus.

0 = no lytic action.

The ability of actinomycetes to manifest "induced antagonism" (Schiller, 1914-1931) was also examined. For this purpose, the strains studied were grown on heat-killed and living suspensions of E. coli and S. aureus, in nitrogen-free mineral solution, with or without the addition of three per cent sucrose. After incubation at 30° for seven days, transfers from each of the eight cultures obtained with each Actinomyces were tested for bacteriolytic action on fresh or killed E. coli and S. aureus, suspended in nitrogen-free solution and in nitrogen-free soft agar. Transfers from seven-day-old cultures in broth were also used for comparison. In all cases, the activity of every strain tested was highest when grown in nutrient-broth; no "induced" action on E. coli was observed, transfers from cultures with bacteria being as inactive as those from broth-cultures. The same negative results were recorded when filtrates from the bacterial suspensions inoculated with the actinomycetes were examined for bacteriolytic activity, in comparison with filtrates from broth cultures.

Although the production of adaptive enzymes by bacteria is a well-known
PHENOMENON (Dubos, 1940), all attempts to demonstrate the formation of adaptive bacteriolyisins by Actinomyces G (Welsch, 1941) or by the actinomycetes used in the present study have failed. It may be concluded, therefore, that the theory of induced antagonism cannot be used for an interpretation of the facts observed with these organisms.

The claim of Krassilnikov and Koreniako (1939) and of Kriss (1940) that actinomycetes produce a lytic substance closely related to lysozyme has already been discussed from a theoretical point of view. It was thought desirable to obtain further experimental evidence on the possibility of production of true lysozyme by those organisms. For this purpose, advantage was taken of the high susceptibility of M. lysodeikticus (Fleming, 1922) to lysozyme of various origins (Roberts et al., 1938). Suspensions of this organism in mineral solution at 37° were dissolved in a few minutes by a 1/100 dilution of egg-white. Similar suspension, treated with paper-filtrates from four and eight-day-old broth and Czapek’s-cultures of the actinomycetes, were not clarified in two days. A reduction of turbidity was observed, however, with four filtrates having a similar action on suspensions of S. aureus. This action cannot be attributed to lysozyme since staphylococci are highly resistant to this agent (Thompson and Khorazo, 1935). The particular strain used in these experiments was unaffected after incubation at 37° for two hours in a 1/5 dilution of egg-white.

Suspensions of M. lysodeikticus were also inoculated with the different actinomycetes, but lysis was observed only after 48 hours and only with those strains that were able to dissolve fresh S. aureus within the same limits of time.

It may, therefore, be concluded that the production of true lysozyme by actinomycetes is yet to be demonstrated.

THE DISTRIBUTION OF BACTERIOLYTIC AND BACTERIOSTATIC PROPERTIES AMONG ACTINOMYCETES

A study was made of the distribution of bacteriolytic and bacteriostatic properties among 164 actinomycetes obtained from the New Jersey Type culture collection and of the relations between the two properties. The series of actinomycetes included representatives of the three genera Actinomyces, Proactinomyces, and Micromonospora, and consisted of 62 unidentified strains and of 102 identified organisms, representing at least 85 distinct species or varieties.

Since organisms able to dissolve one of the various susceptible bacterial substances used are generally capable of acting also on the others, the distribution of bacteriolytic properties among the actinomycetes was studied mainly by the use of living S. aureus. This bacterial substrate was previously shown to be the most resistant but also the most interesting.

The three methods of assay above described were used. The final classification of the tested strains into three groups (highly active, moderately active and inactive) was obtained, as shown above.

Broth-filtrates from eight-day-old cultures of the 24 most active organisms and of 43 of the less active forms were tested for lytic action on killed and fresh E. coli and S. aureus.

As shown in table 5, bacteriolytic properties are widespread among the actinomycetes, but the production of a soluble lytic agent, in a concentration sufficient
to permit detection, appears to be less widely distributed than bacteriolytic ability of the organisms themselves.

Available information on the wide distribution of bacteriostatic properties among soil actinomycetes merely tells us what proportion of active strains may be found in various soils. It does not indicate to what extent antibiotic activities are distributed among the different species or whether these activities are characteristic of certain species or of special strains. According to the state of confusion still existing in the classification of actinomycetes, an answer to those questions is not likely to be soon forthcoming. For practical purposes, the best approach to this problem is probably a systematic study of well-known culture-collection strains. The fungistatic action of 80 different species has already been studied (Alexopoulos, 1941).

Using the cross-streak method above described, it was found that, in addition to its various bacteriolytic abilities, Actinomyces G possesses the power of inhibiting the growth of several gram-positive bacteria. Inasmuch as such a property has been invoked to explain by a two-step mechanism the bacteriolysis of living organisms (Welsch, 1941), it was thought desirable to study the distribution of bacteriostatic ability among the organisms of the present series, and to compare this distribution to that of bacteriolytic properties.

The bacteriostatic properties of the 164 strains were examined by the cross-streak method. Three test-organisms were used: E. coli, S. aureus, and Bacillus subtilis.

Inhibition of E. coli was observed only in one case, namely, by a strain of Actinomyces lavendulae studied by Waksman and Woodruff (1942). Inhibition of S. aureus was invariably found to accompany inhibition of B. subtilis. The zone of inhibition of the coccus, however, was generally smaller than that of the bacillus. B. subtilis was inhibited by a number of actinomycetes and the length of the inhibition zone varied from 1 to 40 mm. It was found convenient to classify the organisms on the basis of their inhibitory action upon B. subtilis. Thus, actinomycetes producing an inhibition zone of 10 mm. or more were

TABLE 5

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>ACTIVITY ON LIVING S. AUREUS</th>
<th>TOTAL STRAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Number of strains*</td>
<td>24</td>
<td>54</td>
</tr>
<tr>
<td>Per cent of strains</td>
<td>14.63</td>
<td>32.93</td>
</tr>
<tr>
<td>Number of filtrates†</td>
<td>9</td>
<td>11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ACTIVITY ON HEAT-KILLED E. COLI AND S. AUREUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of filtrates†</td>
</tr>
</tbody>
</table>

* See table 1.
† See table 4.
grouped as highly active; those producing a zone of 1 to 9 mm. were termed moderately active; and a third group comprised all non-inhibiting strains.

Twenty-two highly active organisms, 33 moderately active strains and 12 inactive ones, or 67 strains in all, were tested for the production of soluble antibiotic substances. Tap-water extracts of 10-day-old cultures of these organisms grown on starch-tryptone-agar were obtained, as outlined above. Amounts of 1.0, 0.3, and 0.1 ml. of each filtrate respectively, were added to 10 ml. portions of melted and cooled nutrient-agar which was then plated and allowed to solidify. Four test-organisms were streaked on each prepared plate, namely, E. coli, S. aureus, S. lutea, and B. subtilis. Table 6 shows that bacteriostatic action is widely distributed among actinomycetes, but that only nine out of the 67 filtrates tested, had an antibiotic activity. Filtrates from five species, Actinomyces antibioticus (Waksman and Woodruff, 1940), A. lavendulae (Waksman and Woodruff, 1942), Proactinomyces sp.,* Actinomyces candidus, and Micromonospora sp. were active, inhibition of some of the test-organisms being observed.

* This organism was obtained from Dr. Gardner of Oxford.

TABLE 6
Distribution of bacteriostatic properties among actinomycetes

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>GROWTH-INHIBITION OF B. SUBTILIS</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>++*</td>
<td>+</td>
</tr>
<tr>
<td>Number of strains</td>
<td>22</td>
<td>54</td>
</tr>
<tr>
<td>Per cent of strains</td>
<td>13.42</td>
<td>32.93</td>
</tr>
<tr>
<td>Number of extracts</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

* ++* = highly active; i.e. inhibition zone 10 mm. long or more; for extracts, inhibition by 0.1 ml. per 10 ml. nutrient agar or less.
+ = moderately active; i.e. inhibition zone smaller than 10 mm.; for extracts, inhibition by 1 ml. or less, but by more than 0.1 ml. per 10 ml. nutrient agar.
0 = inactive.

TABLE 7
Fourfold table showing the relation between bacteriolytic and bacteriostatic properties of actinomycetes

<table>
<thead>
<tr>
<th>LYTIC ACTION</th>
<th>BACTERIOSTATIC ACTION</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>++*</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>57</td>
<td>21</td>
</tr>
<tr>
<td>0</td>
<td>19</td>
<td>67</td>
</tr>
<tr>
<td>Total........</td>
<td>76</td>
<td>88</td>
</tr>
</tbody>
</table>

Chi square = \[
\frac{[(57.67) - (19.21)]^2}{78.86 \cdot 76.88} = 42.76.
\]
P < 0.01.

* + = high or moderate activity; 0 = no activity.
obtained with less than 0.1 ml. filtrate per 10 ml. medium. Filtrates of four species, namely *Actinomyces roseus*, and three agar-liquefying actinomycetes probably identical with *Actinomyces violaceus* (ruber), were weakly active.

No relation was found between the taxonomic position of the organisms and possession of antibacterial properties; in several cases, two or more strains of the same species gave different results. Both bacteriostatic and bacteriolytic properties were found in representatives of the three families, namely, the Actinomycetaceae, Proactinomycetaceae, and Micromonosporaceae. A knowledge of the respective frequency of antibacterial properties in each of these families cannot be obtained at present, since a systematic morphological study of the strains used would be necessary to determine their exact taxonomic position.

Bacteriolytic actinomycetes were found in each of the three groups based on growth-inhibiting-power; however, they were much more numerous among the bacteriostatically active groups. This fact is brought out in the fourfold table (table 7) which, when tested by the "chi-square" method, shows that independence of bacteriolytic and bacteriostatic properties is highly improbable. The fact that bacteriolytic ability is significantly associated with growth-inhibiting power is in good agreement with the two-step theory proposed for the mechanism of lysis of living bacteria (Welsch, 1941).

*Action of Actinomyces G and its soluble products on a variety of gram-positive and gram-negative bacteria*

The different antibacterial properties previously recognized in *Actinomyces G* are thus shown to be commonly distributed among actinomycetes. Furthermore, the different properties are, most generally, associated in each active organism. Since *Actinomyces G* was in all respects (except for the bacteriostatic action of its filtrate) as active as any other organism studied, it was conveniently taken as a good representative of the kind of activity that may be described as activity of the "actinomycetin type."

Most of the experiments thus far published on the actinomycetin-type of bacteriolysis were conducted with a rather small number of test organisms. In order to find whether the gram-staining properties of the test organisms had a real significance in their susceptibility or resistance (Dubos, 1941), it was thought desirable to examine the action of *Actinomyces G* and its products on a large number of bacteria, heat-killed as well as living.

Altogether, 360 different strains of bacteria were used; they included 100 gram-negative organisms belonging to the genera *Brucella*, *Eberthella*, *Escherichia*, *Erwinia*, *Hemophilus*, *Neisseria*, *Pasteurella*, *Phytomonas*, *Pseudomonas*, *Salmonella* and *Serratia*, as well as 260 gram-positive organisms belonging to the genera *Bacillus*, *Corynebacterium*, *Diplococcus*, *Erysipelothrix*, *Phytomonas*, *Staphylococcus*, and *Streptococcus*.

When heat-killed, all the gram-negative organisms were susceptible not only to the action of growing *Actinomyces G* but also to the action of its active filtrate (actinomycetin); in the latter case, the assay values (Welsch, 1938) obtained by nephelometry, on various suspensions of equal turbidity varied slightly but were
all of the same order of magnitude. On the contrary, all living gram-negative organisms tested were found resistant, not only to actinomycetin but also to the action of the growing *Actinomyces* G. In the latter case, however, some evidence of lysis was observed with species of *Neisseria* and *Hemophilus*, but in both instances it was found that the bacteria were already dead in the suspension medium when the clarification was observed.

All heat-killed gram-positive organisms were more resistant to lysis by growing *Actinomyces* G and by actinomycetin than any of the heat-killed gram-negative ones. In these experiments, when spore-forming organisms were used, care was taken to inactivate the suspension before any appreciable sporulation had occurred.

With living gram-positive organisms, the results obtained were more complex. The action of the growing *Actinomyces* and of actinomycetin should be separately considered, the action on spore-formers and non-spore-formers being different. Growing *Actinomyces* G clarified the suspensions of all gram-positive bacteria studied, including those of spore-bearing organisms made from young, non-sporulated cultures; suspensions made from old cultures consisting mainly of spores, were unaffected. A non-sporulating variant of *B. megatherium* S 36 (den Dooren de Jong, 1931), was dissolved under the same conditions as typically non-sporing bacteria. Actinomycetin reduced the turbidity of the suspensions in mineral-solution of all strains of *Klebsiella pneumonieae* and *Streptococcus hemolyticus* examined. The same action was observed with a few strains of *Staphylococcus aureus* and with *Bacillus megatherium* S 36. With all other organisms suspended in mineral-solution it was impossible to obtain a constant action of the filtrate, although occasional lysis was noted with many of them. Better results were observed with many of the more resistant organisms by transferring sterile fragments of agar-medium from a culture of *Actinomyces* G on a bacterial-agar plate. Although the existence of a bacteriolytic action of actinomycetin on living gram-positive organisms may be considered as certain, the phenomenon is not fully understood as yet.

**SUMMARY AND CONCLUSIONS**

1. Bacteriolytic activities against killed bacteria and against living gram-positive bacteria are widely distributed among the actinomycetes. No lytic action was observed, however, against living gram-negative organisms.

2. Ability to dissolve heat-killed gram-negative and gram-positive bacteria is significantly associated with ability to dissolve living gram-positive organisms. This indicates that the same lytic principle is involved in the action on the three different substrates.

3. Sterile filtrates of many cultures of actinomycetes examined were able to dissolve heat-killed gram-negative bacteria as well as heat-killed gram-positive organisms. They were, on the contrary, less active, and less frequently so, against living gram-positive bacteria. No lytic action was ever observed against living gram-negative bacteria.

4. Ability to inhibit the growth of gram-positive bacteria, especially of spore-
bearing organisms, is widely distributed among the actinomycetes. Similar action against gram-negative bacteria was found to be, on the contrary, quite exceptional.

(5) Growth-inhibiting properties appear to be significantly associated with bacteriolytic action upon living gram-positive bacteria.

(6) The group of various antibacterial properties recognized in *Actinomyces* G, which may be designated as the “actinomycetin type” of activity, is commonly found among actinomycetes.

(7) The actinomycetin type of activity was found in representatives of the three genera: *Actinomyces*, *Proactinomyces* and *Micromonospora*. No relation was observed between the taxonomic position of the antagonists and their activity.

(8) No evidence was found for the production of adaptive lysins by actinomycetes grown in association with bacteria.

(9) No evidence was found for the production of true lysozyme by actinomycetes.

(10) The significance of the gram-staining properties in relation to susceptibility or resistance of bacteria to the actinomycetin type of activity was demonstrated by the examination of a large number of strains.

It is a pleasure to acknowledge here gratefully much help and guidance obtained from Dr. S. A. Waksman during the performance of this work. His kind interest in these investigations, and his daily advice have been most helpful in the realization of these aims. The writer is also deeply indebted to the Belgian American Educational Foundation for the grant of three successive annual Fellowships which enabled him to continue his researches for two years at The Rockefeller Institute for Medical Research at Princeton, and for one year at the New Jersey Agricultural Experiment Station, Rutgers University, at New Brunswick, N. J.

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