ACTINOMYCES ANTIBIOTICUS, A NEW SOIL ORGANISM ANTAGONISTIC TO PATHOGENIC AND NON-PATHOGENIC BACTERIA[1]

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In a search for microörganisms antagonistic to bacteria, especially to the gram-negative ones, the bacterial-agar plate method was used (Waksman and Woodruff, 1940a). This consists in adding a suspension of washed living bacteria to 1.5 per cent sterile washed agar containing 0.5 per cent NaCl and 0.5 per cent K_2HPO_4. To a fresh field or garden soil, or one previously enriched several times with washed centrifuged suspensions of the bacteria in question, is added sterile tap water, to give a series of dilutions; 1 ml. of each dilution is placed in a sterile plate and the bacterial agar added. If the soil contains specific antagonists, their colonies are usually surrounded by clear zones, in which the cells added to the agar have been killed.

By the use of the foregoing method, using suspensions of *Escherichia coli* or *Aerobacter aerogenes*,[2] several antagonistic organisms were isolated, comprising both bacteria and actinomycetes. One species of *Actinomyces* proved to be particularly active against a great variety of bacteria and fungi. It was found to produce a specific bacteriostatic and bactericidal substance,

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[2] The reason for using gram-negative bacteria for enrichment purposes rather than gram-positive forms becomes evident when one considers the fact that most of the selective bacteriostatic and bactericidal substances so far known to be produced by microörganisms are more active against the second than against the first. It is very easy to pick up antagonists active against *Sarcina lutea*, for example, but it is more difficult to obtain one active against *E. coli*. The latter may, of course, be far more effective against *S. lutea* than against *E. coli*.

which was designated as actinomycin (Waksman and Woodruff, 1940a). The organism is a chromogenic form producing a dark-brown to black pigment on protein- and peptone-containing media, but it is distinct in its physiology from the chromogenic species previously described. It is recognized as a new species, for which the name *Actinomyces antibioticus* is proposed.

In order to obtain the active substance, the culture was at first grown in a one per cent tryptone broth for 6 days at 37°C. The paper filtrate of the culture had an inhibiting effect on various gram-positive bacteria: *Sarcina lutea* and *Bacillus mycoides* were inhibited by 5 and 10 ml. of the filtrate, respectively, per liter of liquid or solid medium. The growth of *E. coli* was not inhibited, however, by the addition of 200 ml. of filtrate per liter of medium.

The active substance produced by *A. antibioticus* in liquid media is completely removed by charcoal; it resists 100°C. for 30 minutes; it is only partly removed by passage through a Seitz filter. It is soluble in ether, ethyl alcohol, carbon bisulfide, acetone, and chloroform. Ethyl ether is the best reagent for removing the substance from the medium. The addition to the above medium of a small amount of starch, mineral salts, and agar increased considerably the growth of the organism. The medium finally adopted consisted of 5.0 grams starch, 10.0 grams tryptone, 2.0 grams K_2HPO_4, 2.0 grams NaCl, 15.0 grams agar, and 1000.0 ml. distilled water. Forty-ml. portions of this medium were placed in Blake bottles, sterilized, inoculated with a spore suspension of the *Actinomyces*, and incubated for 7 to 9 days at 28°C. The contents of the bottles were combined and extracted three times with ether, thereby removing virtually all of the active substance. The solvent was evaporated, and the residue was weighed and taken up with alcohol. If the alcohol was allowed to evaporate and the residue was taken up with water, a yellow to orange-colored turbid solution was obtained. It was found best, however, to keep the substance in the form of an alcoholic solution (5 mg. per 1 ml.). When required, the solution was diluted with water, to give the required concentrations. The separation of the actinomycin into the A and B fractions was

brought about by the use of petrol ether, only the B fraction being soluble in this reagent. Actinomycin A was found to be highly inhibitive for various gram-positive bacteria; gram-negative organisms were less sensitive, much larger concentrations being required for their complete inhibition (Waksman and Woodruff, 1940b).

The following method was used in testing the bacteriostatic action of actinomycin: the alcoholic solution was diluted with distilled water (1:4) to give one milligram of the substance per 1 ml.; second, third, and fourth dilutions with distilled water were prepared, giving 0.01, 0.001, and 0.0001 mgm. of the substance per 1 ml. of water. Two series of tests were made, one against the more resistant and the other against the more sensitive bacteria. For the first, one and 2 ml. portions of the lowest dilution (1 mgm. per 1 ml.) were added to 10 ml. portions of nutrient agar; the agar was allowed to solidify in the plates and was inoculated with three gram-negative test bacteria, namely, *E. coli*, *Aerobacter aerogenes*, and an intermediary type; in some cases, *Brucella abortus* was used. The three higher dilutions were also added to 10 ml. portions of nutrient agar and the plates inoculated with three gram-positive bacteria, namely, *Sarcina lutea*, *Bacillus mycoides*, and *Bacillus subtilis*. The first group of plates was incubated at 37°C., and the second at 28°C. A record of bacterial growth was made after 24 and 48 hours. By this method, it was possible to demonstrate that there is a marked difference, among the various bacteria, in their sensitivity to actinomycin (Figs. 1 and 2).

The effect of the nitrogen source upon the production of the active substance by *A. antibioticus* is brought out in tables 1, 2, and 3. Large amounts of actinomycin are produced on a variety of media, with organic and inorganic sources of nitrogen. The yield and activity are highest on the tryptone media. On tyrosine and tryptophane media the organism grew only poorly, producing no pigment and little active substance. The gram-negative bacteria were inhibited only by the preparations obtained on the peptone, tryptone, and phenylalanine media. The lower activity of some of the crude preparations was undoubtedly

due to the higher concentrations of extraneous material accompanying the actinomycin (Waksman and Woodruff, 1940b). By the use of phosphate buffers, the tryptone medium was adjusted

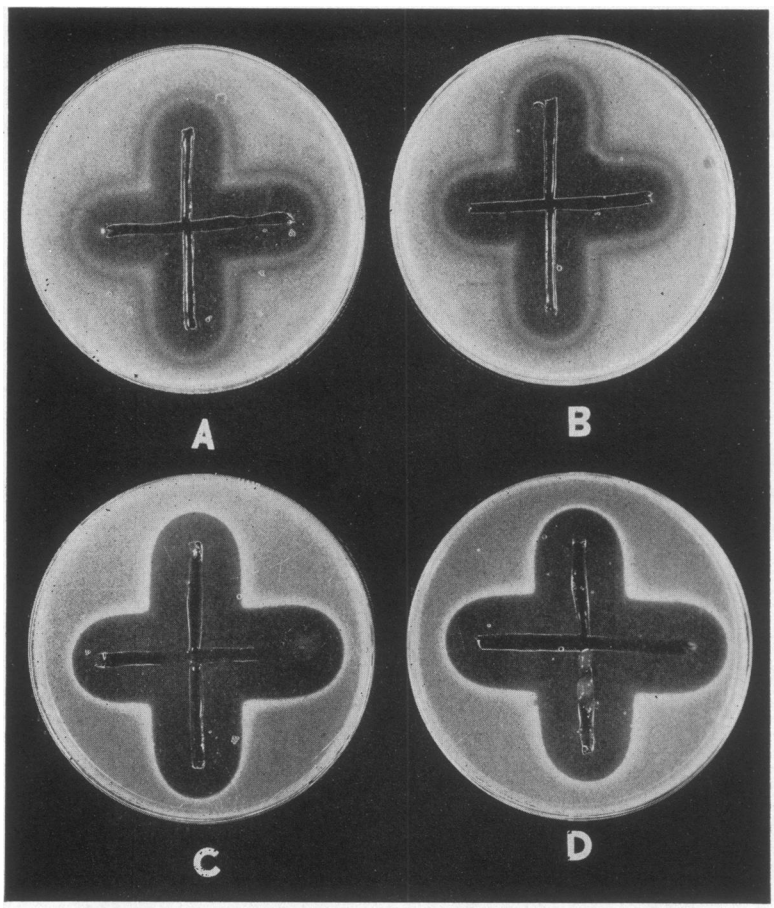

FIG. 1. BACTERIOSTATIC ACTION OF ACTINOMYCIN AGAINST TWO GRAM-POSITIVE BACTERIA.
A–B, *Bac. mycoides;* C–D, *Sarcina lutea.*

to different pH values (4.0–9.0), in order to determine the influence of reaction upon the production of actinomycin. The highest yield and activity were obtained at pH 7, decreasing above and below that point. Actinomycin A was found to be thermo-

stable at neutral or slightly acid reactions $\left(\frac{N}{28}\ HCl\right)$. At higher acidity $\left(\frac{N}{14}\ HCl\right)$, reduction in activity began at boiling tempera-

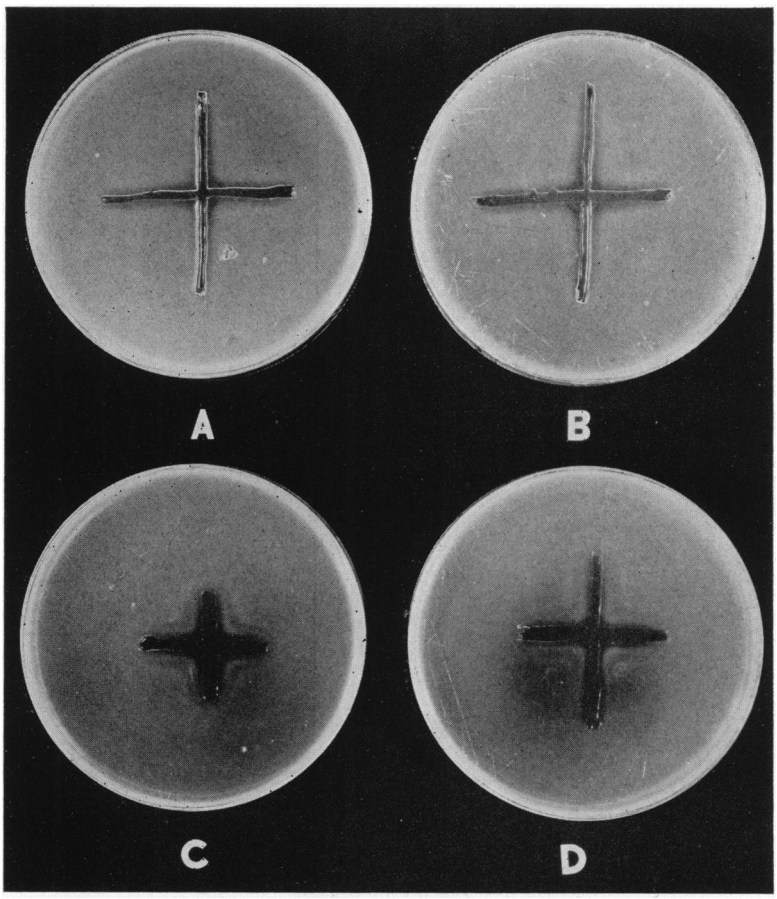

FIG. 2. BACTERIOSTATIC ACTION OF ACTINOMYCIN AGAINST TWO GRAM-NEGATIVE BACTERIA.
A–B, *E. coli;* C–D, *Azot. Beijerinckii.*

ture; there was a loss of 25 per cent activity in $\frac{N}{4}$ to $\frac{N}{2}$ solutions when kept at 100°C. for 30 minutes. An alkaline reaction brought about a change in the color of the solution from bright orange

TABLE 1

*Production of a bacteriostatic substance by A. antibioticus grown on various media**

MEDIUM NUMBER	STARCH ADDED	NITROGEN SOURCE		YIELD PER LITER
		Nature	Per cent	
	per cent			*mgm.*
1	0.5	Asparagine	0.1	8
2	1.0	Asparagine	0.1	49
3	2.0	Asparagine	0.1	84
4	2.0	Asparagine	0.2	110
5	0.5	Alanine	1.0	20
6	0.5	Phenylalanine	0.45	68
7	0.5	Peptone	1.0	66
8	0.0	Peptone	1.0	36
9	0.5	Tryptone	1.0	126
10	0.0	Tryptone	1.0	70

* A basal medium was used, containing 1.5 per cent agar, 0.2 per cent K_2HPO_4 and 0.2 per cent NaCl.

TABLE 2

Activity of a bacteriostatic substance produced by A. antibioticus grown on different media

MEDIUM NUMBER*	TEST ORGANISM AND MILLIGRAMS OF ACTIVE SUBSTANCE PER LITER OF AGAR														
	A. aerogenes		Intermediate		*E. coli*		*S. lutea*			*B. mycoides*			*B. subtilis*		
	100	200	100	200	100	200	1	.1	.01	1	.1	.01	1	.1	.01
1	3†	3	3	3	2	1	0	0	3	0	3	3	0	3	3
2	3	3	3	3	3	3	0	0	3	0	3	3	0	3	3
3	3	3	3	3	3	3	0	2	3	0	3	3	0	3	3
4	3	3	3	3	3	3	0	3	3	0	3	3	0	3	3
5	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
6	3	3	3	3	2	0	0	0	3	0	3	3	0	1	3
7	3	3	3	3	2	0	0	0	3	0	0	3	0	0	3
8	3	3	3	2	1	0	0	0	3	0	2	3	0	0	3
9	3	3	3	3	1	0	0	0	3	0	0	3	0	0	3
10	3	3	3	1	1	0	0	0	3	0	0	3	0	0	3

* See table 1.

† 0 = no growth; 1 = trace of growth; 2 = limited growth; 3 = good growth.

to brown. Reduction in activity began at $\frac{N}{28}$, especially at 100°C.; when the solution was brought to $\frac{N}{14}$ with NaOH, the

activity was rapidly destroyed. Some of this activity was restored upon neutralization of the solution.

A. antibioticus is strictly aerobic. It produced the active substance only when grown in shallow layers of medium. In deep layers, growth and activity were considerably reduced. The introduction of a small amount of agar (0.25 per cent) improved considerably the medium for the growth of the organism.

A. antibioticus produces a brown to black pigment on peptone and protein media. Beijerinck (1900) suggested that the formation of this type of pigment is due to the production of a quinone.

TABLE 3

Effect of inorganic and organic nitrogen sources upon the yield of actinomycin

	NITROGEN SOURCE			
	Nitrate*		Tryptone†	
	Incubation			
	7 days	15 days	5 days	14 days
Total yield, mgm./l.	26	80	57	87
Yield of actinomycin A, mgm./l.	13	39	48	63
Activity of actinomycin, mgm./l.‡				
0.1	+	+	+	+
0.025	0	0	0	0

* 0.2 per cent $NaNO_3$, 1.5 per cent starch, 0.2 per cent K_2HPO_4, 0.2 per cent NaCl, 0.25 per cent agar.

† 1.0 per cent tryptone, 0.5 per cent starch, 0.2 per cent K_2HPO_4, 0.2 per cent NaCl, 0.25 per cent agar.

‡ Against gram-positive test bacteria: + = active, 0 = no activity.

He believed that, because of this capacity, actinomycetes play an important rôle in the formation of black humus in soils. The quinone was looked upon as a derivative of some constituent of the protein molecule; in media containing asparagine, nitrate or ammonium salts, only traces of quinone or none at all were found. The action of the enzyme tyrosinase upon tyrosin was thought to result in the formation of nitrogen-containing phenol derivatives and quinones (Raper, 1932); these are known to act as strong oxidizing agents and produce black pigments from the proteins. Since *A. antibioticus* produces no pigment, or only

a faint yellowish pigment, when grown on media containing nitrate and amino acids, the reaction mechanism must be considered as distinct from that postulated by Beijerinck. The fact that actinomycin A, of a deep orange-red color, is produced alike on starch-nitrate and on peptone-containing media shows that there is no relation between the formation of this substance and the action of tyrosinase, or the formation of the black pigment on protein media. As has been pointed out, the organism grows only poorly on tyrosin without the production of a black pigment.

Other investigators (Borodulina, 1935; Nakhimovskaia, 1937; Waksman and Foster, 1937), who studied the formation by ac-

TABLE 4

Formation of active substances by different chromogenic soil actinomycetes

NATURE OF ORGANISM	YIELD OF SUBSTANCE	NATURE OF SUBSTANCE	BACTERIOSTATIC PROPERTIES, AMOUNT PER LITER PREVENTING THE GROWTH OF		
			S. lutea	*B. mycoides*	*B. subtilis*
	mgm./l.		*mgm.*	*mgm.*	*mgm.*
A. olivochromogenus	3	Oily	10	0*	0*
A. pheochromogenus	27	Oily	1	10	10
A. purpeochromogenus	22	Oily	0	0	0
A. viridochromogenus	450	Oily	0	0	0
A. antibioticus	100–150	Crystalline	0.01	0.1	0.1

* 0 = No bacteriostatic action even by 10 mgm. per liter.

tinomycetes of bacteriostatic and bactericidal substances (not, however, the actinomycin type of compound), could not find any correlation between pigment production by actinomycetes on protein-containing media and antagonistic action. In order to determine whether the formation of actinomycin is characteristic of *A. antibioticus* or of the group of chromogenic actinomycetes as a whole, several species producing dark pigments on protein media were selected and grown on the tryptone-starch medium, for 7 to 10 days, at 28°C. The cultures were then extracted with ether. Only oily extracts were obtained, not at all characteristic of the typical actinomycin produced by *A. antibioticus*, as brought out in table 4. These results show that the various chromogenic

actinomycetes do not produce any active substance of the actinomycin type. At best, the preparations had only very low bacteriostatic activity.

It is to be recalled, in this connection, that the formation of bacteriostatic and bactericidal substances by various actinomycetes has long been recognized. In 1890, Gasperini, one of the earliest students of this group of organisms, asserted that "the Streptothrix develops habitually in a spontaneous manner upon the surface of bacteria and fungi, upon which it lives to a limited extent in the form of a parasite, due to the fact that its mycelium possesses the capacity to digest the membrane or casing of these lower fungi." Rosenthal (1925), seeking for organisms effective against diphtheria of the pharynx, tested various antagonistic bacteria without success. He finally succeeded in isolating from the air a species of *Actinomyces*, which he designated as the true biological antagonist of Loeffler's organism. He inoculated the actinomyces in several spots of an agar plate, the surface of which was covered with an emulsion of diphtheria bacteria. At the end of two days, the colonies of the actinomyces on the plate were surrounded by large transparent zones; the rest of the plate was covered with the growth of the diphtheria organism. The actinomyces produced a lytic substance which diffused through the agar and dissolved the diphtheria bacteria.

The work of Gratia and Dath (1924–1926), Welsch (1937–39, 1940), Waksman and Foster (1937), and of various Russian investigators (Borodulina, 1935; Kriss, 1940; Nakhimovskaia, 1937), definitely established the wide occurrence of antagonistic actinomycetes in nature.

Several substances were indicated; these were designated as actinomycetin (Welsch, 1937–1939) and lysozyme (Kriss, 1940). The first was thermolabile and insoluble in alcohol and in acetone; the second was also insoluble in alcohol, in benzene, and in chloroform, but was said to be thermostable. Actinomycin, on the other hand, is thermostable and is readily soluble in ether, alcohol, acetone, and chloroform. Its specific chemical nature and its great effectiveness against bacteria and fungi definitely point to distinct chemical and biological differences from the other prepa-

rations. Actinomycin A and B have been crystallized and their chemical natures studied, as will be brought out in a later publication.

A comparison was made of the bacteriostatic properties of actinomycin and of active preparations obtained from *Pseudomonas aeruginosa*, namely, pyocyanase and pyocyanin. It was found (table 5) that actinomycin A partly inhibits the growth of *S. lutea*, even in concentrations as low as 1:100,000,000 (0.01 mgm. added per liter of agar). A dilution of 1:10,000,000 completely inhibits the growth not only of this organism but also that of other gram-positive bacteria (*Bacillus subtilis* and *B. mycoides*).

TABLE 5

Bacteriostatic effect of different microbial preparations on the growth of various bacteria

MGM. ADDED PER LITER OF AGAR	ACTINOMYCIN A						ACTINOMYCIN B			PYOCYANIN			PYOCYANASE		
	E. coli	*A. aerogenes*	*P. aeruginosa*	*B. mycoides*	*B. subtilis*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*
200.0	1*	3	1				3	0	0	3	0	0	3	0	0
100.0	3	3	3				3	0	0	3	0	0	3	0	0
10.0							3	1	3	3	1	3	3	0	2
1.0							3	3	3	3	3	3	3	3	3
0.1				0	0	0									
0.01				3	3	1									

* 3 = good growth; 2 = fair growth; 1 = trace of growth; 0 = no growth.

Actinomycin B has comparatively little inhibiting effect upon the growth of these bacteria, and is thus comparable to pyocyanin. Pyocyanase is somewhat more active bacteriostatically.

Bactericidal properties of actinomycin. For bactericidal studies, washed suspensions were prepared of *E. coli* and *B. abortus*, grown on agar media, using sterile tap water. Three different dilutions of these suspensions were treated with varying amounts of actinomycin. After 48 hours' incubation at 37°C., tests were made of the viability of the organisms and their ability to reduce methylene blue. The results presented in table 6 show that the addition of 1 mgm. actinomycin to the highest dilution of the

suspension of *E. coli* cells was sufficient to kill all the cells. A lower concentration of actinomycin (0.1 mgm.) was sufficient to

TABLE 6

Bactericidal effect of actinomycin upon E. coli and B. abortus

CELL DILUTION	ACTINOMYCIN*	E. COLI		B. ABORTUS	
		Growth on agar	Methylene blue reduction	Growth on agar	Methylene blue reduction
	mgm.				
1:0	0	+	+		
1:10	0	+	+		
1:100	0	+	0		
1:0	0.1	+	+		
1:10	0.1	+	+	+	+
1:100	0.1	+	0	+	+
1:1000	0.1			0	0
1:0	1.0†	+	+		
1:10	1.0†	+	0	0	?
1:100	1.0†	0	0	0	0
1:1000	1.0†			0	0

* To 10 cc. of bacterial suspension.

† 0.5 for *B. abortus*.

TABLE 7

Bactericidal action of actinomycin against three test organisms

ORGANISM	ACTINOMYCIN	NUMBERS OF VIABLE BACTERIAL CELLS, AFTER HOURS INCUBATION AT 37°		
		0	2	26
	mgm.			
E. coli	0	123,000,000	95,000,000	2,190,000
	0.1	123,000,000	71,000,000	330,000
	0.5	123,000,000	58,000,000	<10,000
B. abortus	0	18,000,000,000		23,750,000,000
	0.5	18,000,000,000		345,000,000
	2.0	18,000,000,000		143,000
S. lutea	0	161,000,000	275,000,000	191,000,000
	0.1	161,000,000	10,000,000	2,900,000
	0.5	161,000,000	10,000	<10,000

destroy the methylene blue reduction power. Similar results were obtained with *B. abortus*.

The rate of bactericidal action of actinomycin is brought out in table 7. One-half milligram actinomycin brought about complete destruction of all the *E. coli* cells in 26 hours; 0.1 mgm. reduced considerably the number of cells. In the case of *B. abortus*, the tests were made on broth cultures inoculated with the organism and incubated at 37° for 5 hours; actinomycin was then added. One-half milligram actinomycin reduced the number of cells by 98 per cent, whereas 2.0 milligrams brought about nearly complete destruction of all the cells.

It is interesting to note that though the two gram-positive bacteria are much more sensitive in their growth to the inhibiting action of actinomycin than are the gram-negative bacteria, the differences in the bactericidal properties of this substance against the various bacteria are not very marked. In another experiment, not reported here, a 10 cc. suspension of *B. abortus* cells was treated with 2 milligrams actinomycin. As a result, the number of cells was reduced from 70 billions to 33 millions in 17 hours. The addition of one more milligram of actinomycin resulted in the killing of all the remaining cells in 5 hours.

To study the effect of actinomycin upon the respiration of bacteria, a heavy suspension of *E. coli* cells was added to 20 ml. of a 1 per cent glucose solution, incubated at 37°C. for 64 hours, and titrated with N/50 NaOH solution. The control gave a titer of 3.2 per 10 ml.; the addition of 0.5 mgm. actinomycin reduced the titer to 0.45; 2 mgm. reduced it to 0. Twenty ml. portions of broth cultures were inoculated with *E. coli* and incubated at 28° for 92 hours; the air was passed above the medium and the CO_2 collected in standard $Ba(OH)_2$ solution and titrated. The uninoculated control medium gave 4.7 mgm. of carbon as CO_2; the *E. coli* culture gave 11.9 mgm.; the *E. coli* culture receiving actinomycin gave 5.7 mgm. with 0.5 mgm., and 4.85 mgm. with 2 mgm. actinomycin, the latter being just about the same as the control. It may be added here that actinomycin had no injurious effect on the action of several enzyme systems tested, including diastase, protease, and catalase.

The effect of actinomycin upon nitrogen fixation by *Azotobacter vinelandii* was studied. Although this organism is gram-

negative, it is more senstitive to actinomycin than certain other gram-negative bacteria, one mg. of actinomycin per liter of medium being sufficient to repress its development (Waksman and Woodruff, 1940b). Varying amounts of actinomycin A and B were added to a series of flasks containing nitrogen-free medium and inoculated with *Azotobacter*. The control cultures fixed, in 17 days, 8.6 mgm. nitrogen per 1 gm. mannitol. In the presence of 0.1 mgm. actinomycin A per 100 ml. of medium, there was some growth and 5.5 mgm. nitrogen was fixed in one duplicate, but no growth and no fixation occurred in the other duplicate.

TABLE 8

Fungistatic action of actinomycin

ORGANISM*	ACTINOMYCIN, PER 10 ML. AGAR	
	0.2 mgm.	1.0 mgm.
Rhizopus sp.	3	0
Trichoderma sp.	2	0
Penicillium sp.	0	0
Humicola sp.	2	1
Fusarium sp.	2	0
Aspergillus niger	1	0
Aspergillus candida	1	Trace
White yeast	1	0
Soil suspension	1†	0

* Incubation, 4 days at 28°C.

† Fungus growth.

Higher concentrations of actinomycin A completely prevented nitrogen fixation by the organism, although the cells of the inoculum remained alive. In the case of actinomycin B, higher concentrations also killed the cells of the inoculum.

Chemically, actinomycin A was found to contain 12.8 per cent nitrogen, none of which is in a free amino position, as well as a quinone group which makes up 10 to 12 per cent of the molecule. It is known (Cooper and Mason, 1927; Morgan and Cooper, 1921, 1924) that quinones possess bactericidal properties. However. there is a marked difference in the action of actinomycin and quinones: in the case of the latter it is similar for both gram-posi-

TABLE 9

Effect of actinomycin upon the microbiological population of three natural materials

NATURE OF MATERIAL	DILUTION OF MATERIAL	ACTINOMYCIN ADDED	COLONIES ON PLATE	TYPES OF BACTERIA ON PLATE
		mgm./10 ml. agar		
Air-dry soil	1,000	0	Numerous*	Largely gram-positive, many spore formers
	1,000	0.01	Fewer	Gram-negative rods
	1,000	0.10	96	Gram-negative rods
	1,000	1.00	0	
	10,000	0	Numerous*	Large gram-positive organisms
	10,000	0.01	Few	Gram-negative rods
	10,000	0.10	13	Gram-negative rods
	10,000	1.00	0	
Fresh soil	1,000	0	Numerous†	Largely gram-positive organisms
	1,000	0.01	Fewer	Gram-negative bacteria
	1,000	0.10	Few	Gram-negative bacteria
	1,000	1.00	0	
	10,000	0	Numerous†	Largely gram-positive organisms
	10,000	0.01	Few	Gram-negative bacteria
	10,000	0.10	0	
	10,000	1.00	0	
Fresh milk	10	0	Numerous	Gram-positive and gram-negative bacteria
	10	0.01	Numerous	Gram-negative bacteria
	10	0.10	Fewer	Gram-negative bacteria
	10	1.00	43	Gram-negative bacteria
	100	0	790	Gram-positive and gram-negative bacteria
	100	0.01	346	Gram-negative bacteria
	100	0.10	251	Gram-negative bacteria
	100	1.00	1	Gram-negative bacteria
Old milk	1,000	0	Numerous	Gram-positive‡ and gram-negative bacteria
	1,000	0.10	Numerous	Gram-negative rods
	1,000	1.00	216	Gram-negative rods
	10,000	0	Numerous	Gram-positive and gram-negative bacteria
	10,000	0.10	Fewer	Gram-negative rods
	10,000	1.00	26	Gram-negative rods

* Number of bacteria in original soil, 900,000 per 1 gram, developing on nutrient agar.

† Number of bacteria in original soil, 1,300,000 per 1 gram, developing on nutrient agar.

‡ Cocci, diplococci and rods.

TABLE 9—*Concluded*

NATURE OF MATERIAL	DILUTION OF MATERIAL	ACTINOMYCIN ADDED	COLONIES ON PLATE	TYPES OF BACTERIA ON PLATE
		mgm./10 ml. agar		
Fresh sewage	1,000	0	1,248	Mostly gram-negative bacteria
	1,000	0.01	1,172	Gram-negative rods
	1,000	0.10	1,131	Gram-negative rods
	1,000	1.00	121	Gram-negative rods
Old sewage	10,000	0	2,950	Mostly gram-negative bacteria
	10,000	0.01	2,480	Gram-negative rods
	10,000	0.10	954	Gram-negative rods
	10,000	1.00	217	Gram-negative rods

tive and gram-negative bacteria, whereas gram-positive bacteria are far more sensitive to the bacteriostatic action of actinomycin than gram-negative bacteria.

Fungistatic properties of actinomycin. The fungistatic action of actinomycin is brought out in table 8. Certain fungi, like *Penicillium* sp., were found to more sensitive than others, like *Humicola* sp. The inoculation of an agar plate containing 1 mgm. actinomycin per 10 ml. of medium with a loopful of a heavy suspension of soil in water gave no growth at all, either of fungi or of bacteria. The bacterial and fungus populations of the soil were found (Waksman and Woodruff, 1940c) to be very sensitive to the action of actinomycin.

Selective bacteriostatic action of actinomycin. The markedly greater sensitivity of some bacteria to actinomycin than of others can be utilized for the purpose of demonstrating and isolating certain specific organisms from a mixed population. In order to throw further light upon this phenomenon, three natural materials were used, namely, soil, milk, and sewage. Two kinds of soil, air-dry and fresh, as well as two forms of the other materials, namely fresh and old, were used. The results reported in table 9 show that the addition of 0.1 mgm. actinomycin to 10 ml. agar is sufficient to repress the great majority of soil organisms capable of growing on the plate, including all gram-positive types; the addition of 1 mgm. per 10 ml. agar inhibited completely the

total soil population. The particular sample of milk and especially that of the sewage were found to be much richer in gram-negative bacteria than was the soil. This is largely the reason why the addition of 0.1 mgm. actinomycin to 10 ml. agar hardly reduced the population of these two substrates; however, 1 mgm. actinomycin brought about a marked but not complete reduction in the bacterial population. The use of actinomycin in concentrations of 0.1 to 1.0 mg. per 10 ml. agar thus offers interesting possibilities for the demonstration and isolation of specific gram-negative bacteria in milk and in sewage.

Toxicity of actinomycin to animals. Actinomycin was found to be highly toxic to animals, when injected into the blood, muscle, or peritoneal cavity. Ten micrograms of the substance per 20 grams of body weight of animal were sufficient to kill, in the case of mice, and 0.5 to 1.0 mgm. per 2.5 kgm. in the case of fowls.[3]

DESCRIPTION OF THE ORGANISM

Actinomyces antibioticus, n. sp.

Morphology. Spore-bearing hyphae produced in the form of straight aerial mycelium. The sporophores are arranged in clusters; no spirals formed. The spores are nearly spherical to somewhat elliptical.

Growth on various media

Gelatin. Dark brown growth on surface, with patches of grey aerial mycelium. Dark pigment produced, which gradually diffuses into the unliquefied part of gelatin. Liquefaction of gelatin at first very slow, later becoming rapid.

Potato plug. Folded, brown-colored growth, with a thin black ring on plug, fading into a bluish tinge. No aerial mycelium.

Carrot plug. Cream-colored to faint brownish growth. No aerial mycelium. No pigment.

Litmus milk. Thick, brownish ring on surface of milk. Mouse grey aerial mycelium with greenish tinge; growth becomes brown,

[3] The authors are indebted to Dr. H. J. Metzger, of the Animal Husbandry Department of the New Jersey Station, and to Mr. H. J. Robinson, of the Merck Institute, for making the toxicity tests.

especially in drier portions adhering to glass. No reaction change, no coagulation of milk, no clearing; whitish sediment at bottom of tube. Old cultures—heavy growth ring on surface of milk, heavy precipitation on bottom; liquid brownish to black in upper portion.

Czapek's agar. Thin, whitish growth. Thin, grey aerial mycelium.

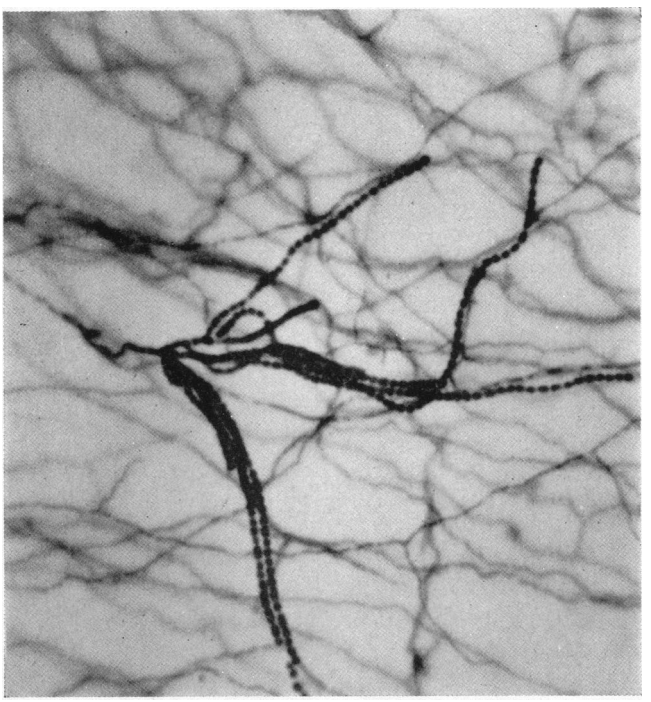

FIG. 3. *A. antibioticus*, SHOWING VEGETATIVE MYCELIUM AND SPORULATING HYPHAE.

Peptone media. Production of dark pigment at early stage of growth is very characteristic. Growth brownish, thin, with yellowish-grey to yellowish-green aerial mycelium.

Odor production. Very characteristic soil odor.

Antagonistic properties. Has a marked antagonistic effect on gram-positive and gram-negative bacteria, much more on the former than on the latter, as well as on actinomycetes. It is also active against fungi, which vary in degree of sensitivity.

Occurrence. Found in soil. Isolated on *E. coli* washed agar plate, using living cells of *E. coli* as the only source of available nutrients.

Figure 3 illustrates the vegetative growth of *A. antibioticus* and the manner of spore-formation.

SUMMARY

A species of *Actinomyces*, possessing strong bacteriostatic and bactericidal properties was isolated from the soil. This organism was found to belong to the chromogenic types of actinomycetes, producing brown to black pigments on peptone and protein media; it is described as a new species, under the name of *Actinomyces antibioticus*.

An active substance was isolated from cultures of this organism, by the use of solvents, including ethyl ether, petrol ether, ethyl alcohol and chloroform. The active substance was separated into two crystalline fractions, designated as actinomycin A and actinomycin B. The first fraction was found to be highly bacteriostatic, whereas the second had little bacteriostatic action but was often strongly bactericidal. Actinomycin A was found to possess bacteriostatic properties against all bacteria tested. Although gram-positive bacteria are much more sensitive to this substance than are gram-negative organisms, there was found a marked variation in the degree of sensitivity among the various bacteria within each of these two groups.

Both actinomycin A and B prevented the development of *Azotobacter* in concentrations of 1:1,000,000, and inhibited nitrogen fixation by this organism in artificial culture media.

Fungi were also found to be sensitive to actinomycin, the degree of sensitivity varying with the nature of the organisms.

When added to agar media for plating natural materials, like soil, milk, and sewage, actinomycin exerted a highly selective action upon the different organisms comprising the natural populations. It is suggested that this selective action offers interesting possibilities for demonstrating the presence of, and for isolating specific bacteria belonging to, the gram-negative groups by the use of this substance.

Actinomycin is highly toxic to animals, when injected intravenously, intraperitoneally, or intramuscularly.

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