MECHANISM OF INHIBITION OF FUNGI IN AGAR BY ACTINOMYCETES

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
SU-CHAN HSU

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ABSTRACT

MECHANISM OF INHIBITION OF FUNGI IN AGAR
BY ACTINOMYCETES

by Su-Chan Hsu

Experiments were designed to study the nature of the inhibition of fungi in agar by actinomycetes. Specifically, the possibility was tested that inhibition, in some cases, might be due to nutrient competition rather than due to antibiotics.

Twenty unidentified actinomycete isolates were tested for antagonism to Glomerella cingulata and Mucor ramannianus in agar media. All 20 actinomycetes inhibited M. ramannianus, and 18 inhibited G. cingulata. Some inhibition zones produced by the actinomycetes were shown to contain inhibitory substances. Agar disks from inhibition zones, or paper disks placed beneath inhibition zones, of 9 actinomycete isolates made new inhibition zones when transferred to fresh, seeded agar. By the same methods, antibiotics could not be demonstrated in inhibition zones of the other 11 isolates. Further, antibiotics were

produced in liquid cultures by 8 of the same 9 isolates which produced them in agar media, and no antibiotics were detected in liquid cultures of the other 11 isolates.

Conidia of G. cingulata, which do not require exogenous nutrients for germination, germinated in cultures of non-antibiotic producing actinomycetes but failed to germinate in cultures of antibiotic-producing actinomycetes. Sizes of inhibition zones produced by non-antibiotic producing actinomycetes against M. ramannianus, which needs exogenous nutrients for germination, were decreased when the concentration of nutrients in the medium was increased. However, zone sizes produced by antibiotic-producing actinomycetes did not decrease when the medium contained increasing concentration of nutrients.

When agar media containing glucose (0.2%) and glutamic acid (0.2%) were streaked with 4 isolates of actinomycetes, these compounds were rapidly utilized adjacent to the actinomycete colonies. About 80% of the glucose and glutamic acid were lost from the agar near the actinomycete colonies after 6 days incubation. Conidia of M. ramannianus germinated about 80% in a solution of glucose and glutamic

acid equal to that used in the agar medium, and germinated about 45% in 20% of the original concentration of nutrients.

Agar was leached by allowing sterilized distilled water to run slowly for 7 days through a groove cut in the agar. A clear inhibition zone developed on either side of the groove when conidia of M. ramannianus or G. cingulata were sprayed on the agar surface and water was again allowed to run through the groove for 3 more days. More than 90% of the glucose and amino acids had been leached from the agar.

These results indicate that while some antagonistic actinomycetes inhibit development of fungi in agar by production of antibiotics, others do so by means of nutrient competition.

MECHANISM OF INHIBITION OF FUNGI IN AGAR BY ACTINOMYCETES

by

Su-Chan Hsu

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1968

ACKNOWLEDGMENTS

Deep appreciation is expressed to Dr. J. L. Lockwood for his guidance and encouragement throughout this study, and special thanks to him for assistance during the course of this investigation and in preparation of the manuscript.

Thanks are also due to Dr. J. E. Cantlon, Dr. M. L. Lacy and Dr. R. P. Scheffer for their critical evaluation of the manuscript.

I would also like to extend my appreciation to Mr. P. G. Coleman for photographs.

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MECHANISM OF INHIBITION OF FUNGI IN AGAR BY ACTINOMYCETES

INTRODUCTION

Many actinomycetes can produce antibiotics which inhibit fungi in artificial media. Some inhibitory microorganisms do not appear to produce antibiotics, but until now experimental evidence implicating factors other than antibiotics was not available to explain inhibition in agar media.

The widespread fungistasis occurring in natural soil has recently been interpreted as due to a deficiency of nutrients required by fungal spores for germination in the soil (9, 10, 11). Ko and Lockwood (9) showed that failure of fungal spores to germinate on agar discs incubated on soil was correlated with rapid loss of nutrients from the agar discs. Moreover, agar disks could be made fungistatic by leaching them with sterile water. This suggested the possibility that the inhibition zones produced by microorganisms in agar might, in some cases, be due to

depletion of nutrients adjacent to the antagonistic colony. The purpose of this study was to investigate the mechanism of the inhibition of fungi by actinomycetes in agar, and particularly to determine if nutrient depletion might be a mechanism of antagonism.

LITERATURE REVIEW

When several microbes are growing in the same culture medium, growth of some will be repressed in proximity to other microbes, whereas others will continue to grow. This is usually attributed to the production by some organisms of inhibitory substances which diffuse through the medium and repress to growth of others at some distance away (2, 3, 4, 5, 25). Porter (18) in an early paper speculated that antagonisms displayed by fungi in mixed cultures are due either to exhaustion of nutrients or to the formation of detrimental products. Florey et al. (5) indicated that an organism which shows antagonism on solid media will often not produce any detectable inhibitory substances in a liquid medium containing the same nutrient materials. He speculated that in some cases this was due to the lack of ample aeration in liquid cultures, but in other cases the reason for the phenomenon was obscure. Some other suggestions for the mechanism of microbial antagonism include change in the pH reaction in the medium and enzyme action (4, 5, 17, 18, 23).

In numerous agar platings of mixed microbial populations from natural substrates, the antagonism of fungi by actinomycetes, rather than by fungi, has been more frequently observed (4, 20, 23), but not all actinomycetes can inhibit fungi. In one study, 43 to 51% of the actinomycetes tested by the agar streak method were active against one or more fungi. However, heated culture filtrates from the antagonistic cultures, when tested by the diffusion procedure, showed that only 43% had inhibitory properties (20, 24). Stessel et al. (21) studied the occurrence in soil of actinomycetes active against plant pathogenic fungi. Of 21 isolates of actinomycetes inhibitory to Glomerella cingulata, only 16 produced an antibiotic substances in the liquid media. Nakhimovskaia (16) also found that of 47 isolates of actinomycetes possessing antagonistic properties in agar media only 27 secreted antibiotic substances into the medium. Meredith (13) in a survey of the distribution of organisms antagonistic to Fusarium oxysporum cubense in Jamaican soil, found 17 actinomycetes antagonistic to this fungus. Of these only 8 produced inhibitory substances in the medium.

Inhibition of fungi in liquid media may also occur without the presence of demonstrable antibiotic substances. No antibiotics could be demonstrated when washed cells of bacteria or Streptomyces spp. inhibited the germination of spores of test fungi in buffer solutions or in water in the absence of added nutrients (10).

In several studies, when antagonistic cultures were tested for the presence of antibiotics using paper or thin layer chromatography, some cultures did not show the presence of antibiotics (7, 8, 28).

Many reports have indicated that the composition of the culture medium is important to the production of antibiotics by antagonistic microorganisms. The formulations of some of these media have been summarized by Waksman and others (3, 4, 19, 23). Many of these media have contained materials of natural origin, such as soybean meal and other seed meals (1), peptone and other digests (2). Despite much effort in developing these media, many antagonistic organisms still failed to produce antibiotics.

Recent information suggests that failure of fungal spore germination in soil is due to depletion of nutrients by microbial activity rather than to the presence of

inhibitory substances (9, 10, 11). The inhibition of spore germination on agar placed in contact with soil may be caused by the diffusion of nutrients from the agar rather than by the diffusion of fungistatic materials into agar. Agar disks supplemented with glucose or amino acids showed a rapid loss of these nutrients to soil by diffusion. Moreover, agar disks could be made fungistatic by leaching them with sterile water (9, 11).

MATERIALS AND METHODS

Preparation of actinomycetes and fungi.--Twenty unidentified actinomycete isolates from soil were maintained on potato-dextrose agar. Glomerella cingulata (Ston.) Spauld. & Schrenk and Mucor ramannianus Möller were used as test fungi; Conidia of G. cingulata do not require exogenous nutrients, whereas conidia of M. ramannianus require exogenous nutrients for germination. Both were maintained on potato-dextrose agar. Conidia were obtained by washing the surface of the agar slants with sterilized distilled water.

Assays of inhibition zones.--Tests for inhibitory activity were made in petri dishes on peptone agar (per liter: peptone, 2 or 5 g; agar, 20 g) or peptone-glucose agar (per liter: peptone, 2 or 5 g; glucose, 2 or 5 g; agar, 20 g). Actinomycetes were streaked on the media, and incubated at 24 C for 2 or 3 days. Then spore suspensions of test fungi, G. cingulata or M. ramannianus, were sprayed onto the agar surface with a modified Stansly's spray apparatus (8). Approximately 250,000 spores were sprayed onto each plate. After 3 days, the inhibition zones were measured.

The presence of antibiotics in the inhibition zones was tested by 3 methods: (a) Agar disks, 7 mm in diam., were cut from inhibition zones and transferred to peptone agar or peptone-glucose agar, or to petri dishes without agar, and allowed to incubate without supplying additional inoculum of the test fungus. Presence or absence of growth of the test fungus was observed; (b) Agar disks, 7 mm in diam., were cut from inhibition zones and transferred to peptone agar or peptone-glucose agar. Immediately or several hours later, a spore suspension of the same test fungus was sprayed onto the agar surface. The presence or absence of new inhibition zones was observed; (c) Sterile paper disks, 13 mm in diam., were placed beneath the agar of the inhibition zones and kept for 10-15 days to allow any inhibitory substances to diffuse into the paper disks. The disks were then transferred to peptone agar or peptone-glucose agar, and the test fungus was sprayed on the agar surface. The development of any new inhibition zones was observed.

Assays for lysis.--Actinomycetes were assayed for ability to lyse the mycelia of G. cingulata. Mycelia were prepared by mixing a suspension containing approximately

20,000 conidia per ml of water with warm (42 C) peptone agar at the rate of 1 volume of spore suspension to 5 volumes of agar. The agar was then poured in petri dishes and incubated for 2-3 days. Actinomycete isolates were then streaked on the surface of the agar. At 10, 25, and 30 days, the lytic zones were measured and the lysed mycelia was observed under the microscope. After 30 days, agar disks 7 mm in diam. were cut from lytic zones and transferred to peptone or peptone-glucose agar. A spore suspension of G. cingulata was sprayed onto the surface of the agar to test for the presence of diffusible inhibitory substances. Other disks were transferred to peptone agar containing growing mycelium of G. cingulata to test for diffusible lytic substances.

Detection of antibiotics.--Actinomycetes were cultured in 125 ml Erlenmyer flasks containing 20 ml liquid medium per flask. The media were (a) Difco-Bacto nutrient broth, 6 g; glucose, 5 g; NaCl, 5 g; $ZnSO_4$ $7H_2O$, 0.01 g; $CaCO_3$, 3.5 g; distilled water, 1000 ml. (b) Peptone, 5 g; glucose, 5 g; distilled water 1000 ml. Actinomycete isolates were grown, in duplicate, in these shaken media for 3 days at 24 C. Sterilized Millipore filters (0.22 μ or

0.44 μ) were used to obtain sterile culture filtrates. Five ml of each filtrate were applied to 13 mm. diam. paper disks by applying 0.1 ml at a time then alternately air-drying the disks. Assays for antibiotic activity were carried out by the agar diffusion method (5, 15). The paper disks impregnated with culture filtrates were placed on the surface of 0.5% peptone agar. The test fungus, G. cingulata, was then sprayed on the agar surface. The assay plates were incubated at 24 C for 3 days, when the zones of inhibition were observed.

Utilization of nutrients in agar by actinomycetes. --An agar medium was prepared in 0.1 M phosphate buffer (pH 7) and contained glucose, 2 g; glutamic acid, 2 g; agar, 25 g; distilled water, 1000 ml. This medium supported good growth of the test fungi and actinomycetes. Two layers of agar were prepared. The bottom layer of 5 ml was poured and distributed smoothly and evenly in the petri plate. Four selected isolates of actinomycetes were streaked on the agar surface. On the 1st, 2nd, 4th, and 6th days, 6 agar disks, 7 mm in diam., from the top layer of agar near the actinomycete colonies were removed and melted in 3 ml glass-distilled water. Glucose was assayed using the

Glucostat reagent (Worthington Biochemical Corporation). The reagent was used according to the manufacturer's directions. The tubes were read in a colorimeter at 400 mμ. Glucose at concentrations of 20, 40 and 80 μ/ml was used as a standard.

Glutamic acid was assayed by melting 3 disks in 6 ml glass-distilled water, using the method of Moore and Stein (14). Glutamic acid at concentrations of 4, 8 and 16 ug/ml was used as a standard.

RESULTS

Inhibition characteristics of actinomycetes.--To determine which actinomycetes were capable of producing inhibition zones in agar, 20 unidentified actinomycete isolates were streaked on peptone-glucose agar or peptone agar using *Glomerella cingulata* or *Mucor ramannianus* as the test fungus. Each isolate was tested in duplicate in each experiment, and at least 3 experiments were done. Of the 20 actinomycetes, 18 made inhibition zones (3-19 mm) against *G. cingulata* (Table 1) and 20 made inhibition zones (7-19 mm) against *M. ramannianus* (Table 2).

To determine whether the agar in the inhibition zones contained antibiotics, additional tests were made. For *G. cingulata*, agar disks from the original inhibition zones of 9 isolates (1A, 2A, 4A, 6A, 7A, 10A, 16A, 24A and 28A) made new inhibition zones when transferred to fresh agar plates seeded with *G. cingulata* (Table 1, Fig. 1). New inhibition zones were also produced by these isolates when paper disks placed beneath the original inhibition zones were transferred to fresh seeded agar. When agar

TABLE 1. Inhibition characteristics of actinomycetes against Glomerella cingulata in agar tests

Actinomycete isolates	Inhibition zone (mm)[a]	Inhibition characteristics of agar from inhibition zones			
		New inhibition zones (mm)		Growth of test fungus	
		Agar[b]	Paper disks[c]	Agar[d]	Glass[e]
1A	18	8	9	-	-
2A	18	8	9	-	-
3A	13	0	0	+	+
4A	18	9	10	-	-
5A	0		0		
6A	15	10	10	-	-
7A	18	6	4	-	-
8A	15	0	0	+	+
9A	3	0	0	+	+
10A	19	10	10	-	-
12A	4	0	0	+	+
13A	4	0	0	+	+
15A	3	0	0	+	+
16A	12	5	7	-	-
17A	13	0	0	+	+
18A	4	0	0	+	+
19A	10	0	0	+	+
24A	15	8	8	-	-
26A	0		0		
28A	19	13	10	-	-

[a]Actinomycetes were streaked on the agar. After 3 days, conidia of the test fungus were sprayed on the agar surface.

[b]Agar disks from inhibition zones were transferred to agar subsequently sprayed with conidia of G. cingulata.

[c]Paper disks were placed beneath the inhibition zones, then transferred to agar seeded with G. cingulata.

[d]Agar disks from inhibition zones were transferred to unseeded agar; -=No growth of test fungus. +=Growth of test fungus.

[e]Agar disks from inhibition zones were transferred to petri dishes without agar; -=No growth of test fungus. +=Growth of test fungus.

TABLE 2. Inhibition characteristics of actinomycetes against *Mucor ramannianus* in agar tests

Actinomycete isolates	Inhibition zone (mm)[a]	Inhibition characteristics of agar from inhibition zones			
		New inhibition zones (mm)		Growth of test fungus	
		Agar[b]	Paper disks[c]	Agar[d]	Glass[e]
1A	19	7	7	-	-
2A	18	7	8	-	-
3A	16	0	0	+	+
4A	19	8	8	-	-
5A	7	0	0	+	+
6A	18	7	6	-	-
7A	14	7	5	-	-
8A	16	0	0	+	+
9A	7	0	0	+	+
10A	19	10	8	-	-
12A	7	0	0	+	+
13A	8	0	0	+	+
15A	7	0	0	+	+
16A	18	5	4	-	-
17A	13	0	0	+	+
18A	11	0	0	+	+
19A	13	0	0	+	+
24A	14	9	6	-	-
26A	8	0	0	+	+
28A	17	7	10	-	-

[a]Actinomycetes were streaked on the agar. After 3 days, conidia of the test fungus were sprayed on the agar surface.

[b]Agar disks from inhibition zones were transferred to agar subsequently sprayed with conidia of *M. ramannianus*.

[c]Paper disks were placed beneath the inhibition zones, then transferred to agar seeded with *M. ramannianus*.

[d]Agar disks from inhibition zones were transferred to unseeded agar; -=No growth of test fungus. +=Growth of test fungus.

[e]Agar disks from inhibition zones were transferred to petri dishes without agar; -=No growth of test fungus. +=Growth of test fungus.

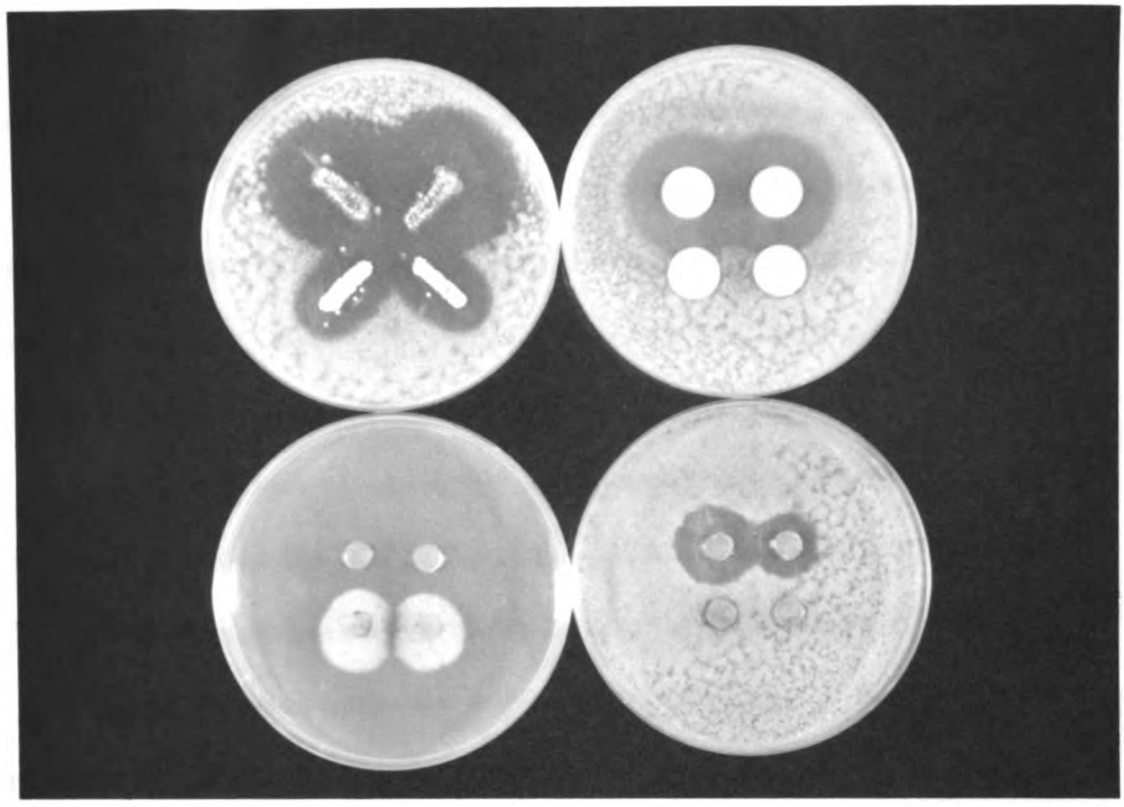

Fig. 1. Inhibition characteristics of actinomycetes against Glomerella cingulata. Above-left: The top 2 streaks, 1A and 4A, produced antibiotics, the bottom 2 streaks, 3A and 8A, were nonantibiotic-producing actinomycetes. Above-right: Paper disks placed beneath the corresponding original inhibition zones were transferred to fresh seeded agar. Below-left: agar disks from corresponding inhibition zones were transferred to unseeded agar. Below-right: Agar disks from corresponding inhibition zones were transferred to seeded agar.

disks from the original inhibition zones were transferred to fresh agar or petri dishes without agar, the test fungus failed to grow from disks of the same 9 isolates. These results showed that diffusible inhibitory substances were produced by 9 of the actinomycetes.

Agar disks from zones produced by the other 9 antagonistic isolates (3A, 8A, 9A, 12A, 13A, 15A, 17A, 18A and 19A) did not make new inhibition zones when transferred to new agar plates seeded with G. cingulata. Nor were new inhibition zones produced by these 9 isolates when paper disks placed beneath the original inhibition zones were transferred to fresh seeded agar. Agar from zones produced by these isolates supported growth of the test fungus when disks were transferred to fresh agar or to petri dishes without agar. These results indicated that no diffusible inhibitory substances were produced by these actinomycetes and that the inhibition zones were caused by some other factor.

The size of inhibition zones containing inhibitory substances ranged from 12-19 mm, with a mean of 17 mm. The size of inhibition zones without inhibitory substances ranged from 3-15 mm, with a mean of 8 mm.

In the case of M. rammannianus, agar disks or paper disks from inhibition zones of 9 isolates (1A, 2A, 4A, 6A, 7A, 10A, 16A, 24A and 28A) made new inhibition zones, when transferred to new agar plates seeded with M. ramannianus (Table 2). The test fungus did not grow on the agar disks from inhibition zones of the same 9 isolates transferred to fresh agar; the same results were obtained even when a drop of 0.2% of glucose solution was added to the agar disks. The results are the same as those with G. cingulata and indicated further that the actinomycetes produced antibiotics in the medium.

Agar disks or paper disks from inhibition zones of the other 11 isolates (3A, 5A, 8A, 9A, 12A, 13A, 15A, 17A, 18A, 19A and 26A) did not produce new inhibition zones when transferred to new agar plates seeded with M. ramannianus. Agar disks from the original inhibition zones of the same 11 isolaes supported growth of the test fungus when disks were placed on fresh agar in petri dishes, or in a petri dish without agar when a drop of glucose solution was added. From these tests it was concluded that the actinomycete isolates included two kinds of antagonistic cultures, one producing inhibitory substances, and the other not producing inhibitory substances.

The size of inhibition zones containing inhibitory substances ranged from 14-19 mm, the mean being 17 mm. The size of inhibition zones without inhibitory substances ranged from 7-16 mm with a mean of 11 mm.

Effect of pH on inhibition zones.--To determine if pH changes in the agar could account for the inhibition zones of non-antibiotic producing isolates, the 20 actinomycete isolates were streaked on unbuffered peptone agar (pH 6.9) and on peptone agar containing 0.1 M phosphate buffer (pH 7) and incubated for 5 days. A spore suspension of the test fungus, G. cingulata, was then sprayed on the plates. After 3 days, the sizes of the inhibition zones, pH of the agar in zones and pH of the agar outside of the zones (growth area) were measured (Table 3). In peptone agar either with or without buffer, the pH of the inhibition zones and the corresponding growth area did not differ appreciably. The pH of agar without buffer increased from 6.9 to 8.5 both in inhibition zones and in the growth area. Since G. cingulata grew well even at pH 8.5, the inhibition zones could not be attributed to an unfavorable, elevated pH of the medium. This was confirmed in tests with buffered agar, in which the pH remained essentially unchanged both

TABLE 3. Effect on pH on inhibition zones of Glomerella cingulata

Actinomycete isolates	No Buffer, pH 6.9			0.1 M PO Buffer, pH 7		
	Zone size (mm)	pH[a]		Zone size (mm)	pH[a]	
		Zone	Growth area[b]		Zone	Growth area[b]
1A	17	7.2	8.0	15	6.9	6.9
2A	15	8.2	8.0	14	6.9	6.9
3A	17	8.1	8.0	17	6.9	6.9
4A	18	8.1	8.0	17	6.9	6.9
5A	0		8.4	0		6.8
6A	17	8.4	8.5	17	6.9	6.9
7A	16	7.8	8.0	13	6.9	6.9
8A	14	7.6	8.0	10	6.9	6.9
9A	3	7.6	7.5	2	7.0	7.0
10A	19	7.8	7.9	16	7.0	7.0
12A	5	8.2	8.2	3	7.0	7.0
13A	5	8.1	8.2	3	7.0	7.0
15A	2	8.3	8.3	2	6.9	6.9
16A	16	8.1	8.1	1	6.9	6.9
17A	17	8.2	8.1	1	7.0	7.0
18A	3	7.9	8.0	3	7.1	7.1
19A	13	7.9	8.0	1	7.1	7.1
24A	19	8.1	8.1	16	7.1	7.1
26A	0		8.1	0		6.9
28A	19	8.2	8.1	13	6.9	6.9

[a]pH was measured by placing electrodes on the agar surface after the cultures had grown for five days.

[b]Area outside of the inhibition zones where the test fungus grew.

in the inhibition zones and in the growth area. Zone sizes for the same isolates were similar in both buffered and unbuffered agar with the exception of isolates 16A, 17A and 19A, which produced much smaller zones in the buffered agar. Similar small zones were produced by isolates 16A, 17A and 19A in agar buffered with 0.1 or 0.01 M phosphate buffer at either pH 7.0 or 8.0. In all tests, these 3 isolates grew poorly on buffered agar, whereas they grew normally on unbuffered agar. Therefore, the aberrant results with these isolates appear to be due to poor growth of the actinomycetes on buffered agar rather than to any effect of pH.

Lysis characteristics of actinomycetes.--The 20 actinomycete isolates were tested for their ability to lyse G. cingulata mycelium. Thirteen isolates (1A, 2A, 3A, 4A, 6A, 7A, 8A, 10A, 16A, 17A, 19A, 24A and 28A) produced lytic zones after 10 days incubation (Table 4). The size of lytic zones ranged from 3-7 mm and the mycelium in these zones was less dense when examined with the microscope. After 25 days, the size of lytic zones produced by the 13 isolates increased and 3 additional isolates (9A, 12A and 13A) made small lytic zones. After 30 days, 2 additional actinomycetes made lytic zones. The mycelium in the lytic zones produced

TABLE 4. Lysis of *Glomerella cingulata* by actinomycetes in agar

Actino-mycete isolates	Zone diameter (mm)			Degree of lysis[a]	Effect of transferred lysis zone	
	10 days	25 days	30 days		Lysis	Inhibition zone (mm)
1A	3	12	14	+	0	8
2A	6	12	15	+	0	8
3A	6	6	8	+	0	0
4A	6	7	10	+	0	6
5A	0	0	0	-	0	0
6A	4	8	13	+	0	7
7A	5	12	15	+	0	3
8A	7	8	12	++	0	0
9A	0	3	4	+	0	0
10A	5	12	15	++	0	8
12A	0	2	6	+	0	0
13A	0	3	8	+	0	0
15A	0	0	0	-	0	0
16A	3	7	10	+	0	6
17A	3	7	8	+	0	0
18A	0	0	2	+	0	0
19A	4	9	12	+	0	0
24A	6	6	12	+	0	6
26A	0	0	2	+	0	0
28A	8	9	15	+	0	8

[a] - = No lysis of mycelium. + = Partial lysis. ++ = Complete lysis.

by 16 isolates (1A, 2A, 3A, 4A, 6A, 7A, 9A, 12A, 13A, 16A, 17A, 18A, 19A, 24A, 26A, and 28A) was partially lysed, whereas mycelia in the zones produced by isolates 8A and 10A were completely lysed. Agar disks from lytic zones of these 18 actinomycetes were transferred to fresh test fungal cultures, but none made new lytic zones. Agar disks from lytic zones were also tested for the presence of antibiotics by transferring them to fresh, seeded peptone agar. Once again, the same 9 isolates (1A, 2A, 4A, 6A, 7A, 10A, 16A, 24A, and 28A) which produced transferable inhibition zones also made such zones when disks from lytic zones were transferred to new, seeded agar. However, the other 9 isolates (3A, 8A, 9A, 12A, 13A, 17A, 18A, 19A, and 26A) which caused lysis of G. cingulata mycelium demonstrated no antibiotics in the agar when tested this way. Therefore, the lytic zones produced by these 9 isolates must be caused by factors other than antibiotics or other diffusable lytic factors.

At 30 days, the sizes of lytic zones containing antibiotics ranged from 10-15 mm, with the average, 12 mm. The sizes of lytic zones without antibiotics ranged from 2-12 mm, and averaged 7 mm.

Antibiotic production in liquid media.--To further test whether antibiotics were produced only by the previously designated actinomycete isolates, antibiotic production was tested in 2 kinds of liquid media. Sixteen isolates of actinomycetes, 9 of which produced diffusible inhibitory substances in agar and 7 of which did not produce inhibitory substances, were tested. Sterile culture filtrates of 8 isolates (1A, 2A, 4A, 6A, 10A, 16A, 24A, and 28A) contained antibiotic substances (Table 5). Filtrates of the other 8 isolates (3A, 5A, 7A, 8A, 9A, 12A, 13A, and 18A) contained no detectable inhibitory substances. The 8 isolates producing antibiotics in liquid media were the same as those producing transferable inhibition zones on agar, whereas with one exception the other isolates did not produce transferable inhibition zones on agar plates. This isolate, 7A, grew poorly in the liquid media, and this may account for its failure to produce antibiotics in these tests. Thus, with this exception, the results confirm those of tests done in agar.

Relation of antibiotic production to inhibition of fungal spore germination in actinomycete cultures.--To further investigate the inhibitory cultures that were unable

TABLE 5. Production of antibiotics by actinomycetes in liquid media

Actinomycete isolates	Medium A[a]	Medium B[b]
1A	+[c]	+
2A	+	+
3A	-	-
4A	+	+
5A	-	-
6A	+	+
7A	-	-
8A	-	-
9A	-	-
10A	+	+
12A	-	-
13A	-	-
16A	+	+
18A	-	-
24A	+	+
28A	+	+
Control	-	-

[a]Nutrient broth 6g, glucose 5g, NaCl 5g, Zn SO_4 $7H_2O$ 0.01g, $CaCO_3$ 3.5g, distilled watter 1000 ml.

[b]Peptone 5g, glucose 5g, distilled water 1000 ml.

[c]+ = Production of antibiotics; - = no production of antibiotics.

to produce inhibitory substances, 4 isolates of actinomycetes were selected. Isolates 1A and 4A produced inhibitory substances, and 3A and 8A did not produce detectable inhibitory substances. Conidia of G. cingulata, which do not require exogenous nutrients for germination, were incubated directly in 3-day-old cultures of these actinomycetes grown in liquid media. Conidia of G. cingulata were introduced directly into the actinomycete cultures and incubated for 14 hours, when germination was determined. A low level of germination, 11% and 18%, occurred in cultures of isolates 1A and 4A, respectively (Fig. 1). These results were not significantly altered when potato broth was added at the time conidia were introduced. This confirmed that these cultures produce some inhibitory substances. By contrast, a high level of germination, 77% and 72%, occurred in cultures of 3A and 8A, respectively. When potato broth was added to these cultures, 98% of G. cingulata conidia germinated, indicating that actinomycetes cultures 3A and 8A did not contain any inhibitory substances (Fig. 2). The results are consistent with the view that no inhibitory substances inhibitory to the test fungi are produced by actinomycete isolates 3A and 8A.

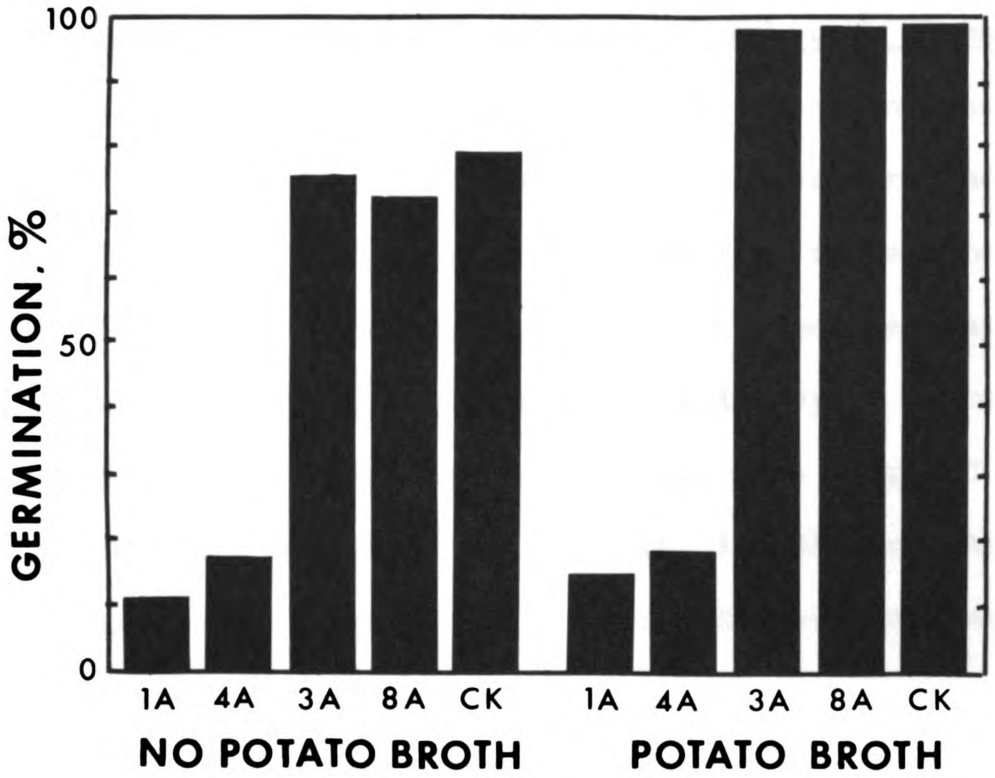

Fig. 2. Germination of Glomerella cingulata conidia in actinomycete cultures with and without added potato broth. Isolates 1A and 4A produced inhibitory substances; isolates 3A and 8A did not produce inhibitory substances. Data are the mean values of three experiments. In each experiment 150-180 spores were counted in each of 10 microscopic fields.

Effect of nutrient enrichment on inhibition zones.--If isolates such as 3A and 8A produce inhibition zones as results of exhaustion of nutrients from the agar surrounding the actinomycete colonies, agar containing increasing amounts of nutrients should give inhibition zones decreasing in size. To test this possibility, 9 different concentrations (0.2-4.0%) of peptone and glucose were prepared in agar. The 4 representative isolates (1A, 3A, 4A, and 8A) of actinomycetes were streaked on the agar media and incubated for 3 days. Conidia of the test fungus, M. ramanneanus, a species requiring nutrients for germination, were sprayed on the agar surface. After 3 days, inhibition zones were measured. The size of inhibition zones produced by isolates 3A and 8A gradually decreased as the concentration of nutrients increased in the medium (Fig. 3, 4). Agar disks from these inhibition zones produced no new inhibition zones when transferred to fresh, seeded agar, and the test fungus grew when similar agar disks were transferred to unseeded agar. The size of inhibition zones produced by isolates 1A and 4A did not decrease as the concentration of nutrients increased in the medium. Agar disks from these inhibition zones produced new inhibition zones when transferred to fresh seeded

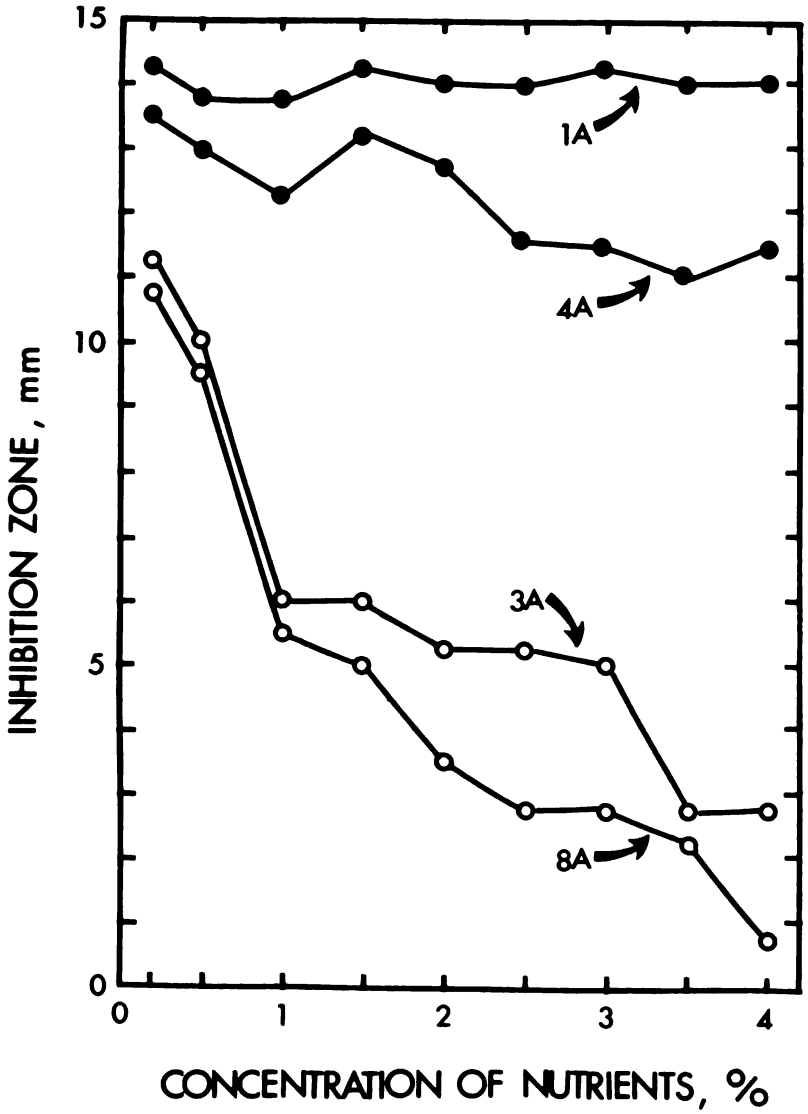

Fig. 3. Effect of concentration of glucose and peptone on size of inhibition zones produced by antibiotic and non-antibiotic actinomycetes. Actinomycetes 1A and 4A were antibiotic producers. 3A and 8A were non-antibiotic producers.

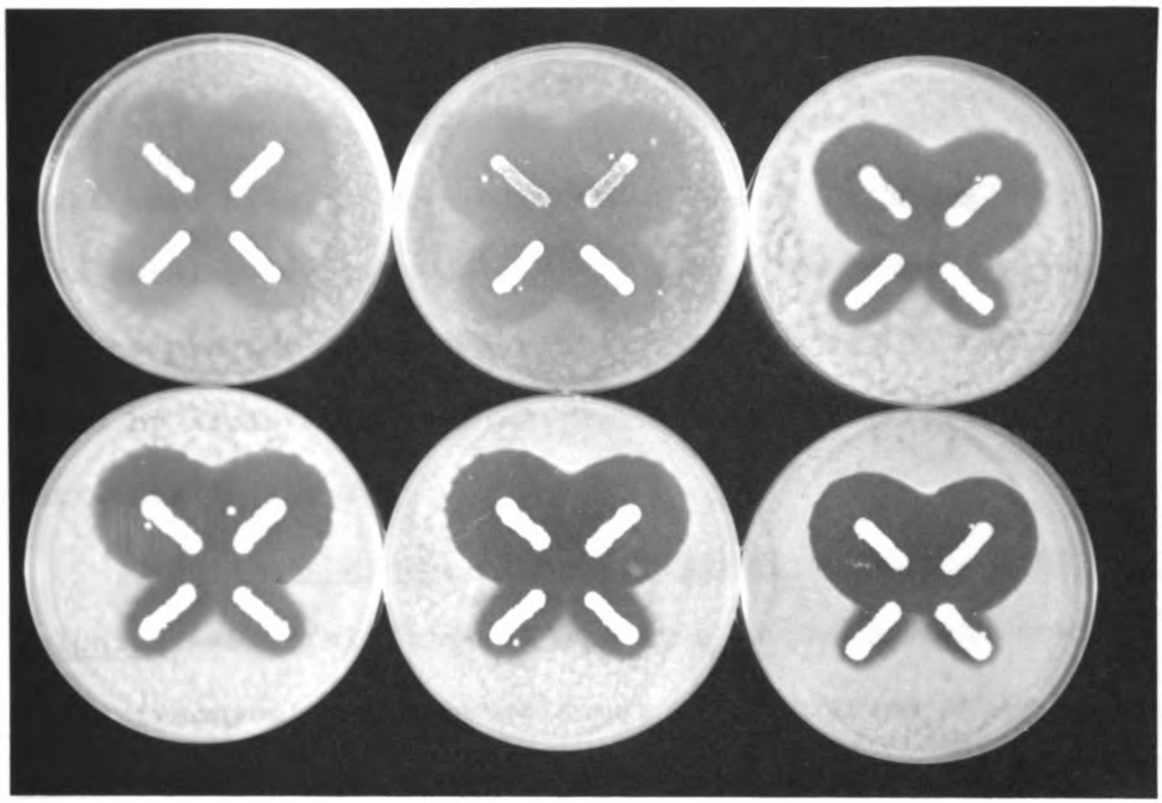

Fig. 4. Effect of nutrient concentration on size of inhibition zones produced by actinomycetes. The concentration of peptone and glucose increased from above-left to below-right. The top 2 isolates in each petri dish were antibiotic-producing actinomycetes (1A and 4A); the bottom 2 isolates in each petri dish were non-antibiotic-producing actinomycetes (3A and 8A).

agar, and the test fungus did not grow when similar agar disks were transferred to unseeded agar. The same results were obtained in two additional tests. Therefore, these results provide further evidence that inhibition zones produced by isolates 3A and 8A were caused by depletion of nutrients in the agar.

Nutrient status of agar adjacent to actinomycete colonies.--Several lines of evidence indicated that some of the actinomycetes inhibited fungi by some means other than antibiotic production, probably depriving the medium of nutrients. Therefore, the nutrient status of agar near the actinomycete colonies was investigated. To determine the rate at which nutrients were utilized by actinomycetes from agar, glucose (0.2%) and glutamic acid (0.2%) were used as the sole ingredients in the agar. Actinomycete isolates 1A, 3A, 4A, and 8A and the test fungus, M. ramannianus, grew well on this medium. Glucose was rapidly lost from the agar within 7 mm of all of the actinomycete streaks; 60-70% was lost by the second day, and 80-90% was lost by the 6th day (Fig. 5). Glutamic acid was also rapidly utilized by the 4 actinomycetes. More than three-fourths of the glutamic acid was lost by the 6th day (Fig. 6). As before, agar

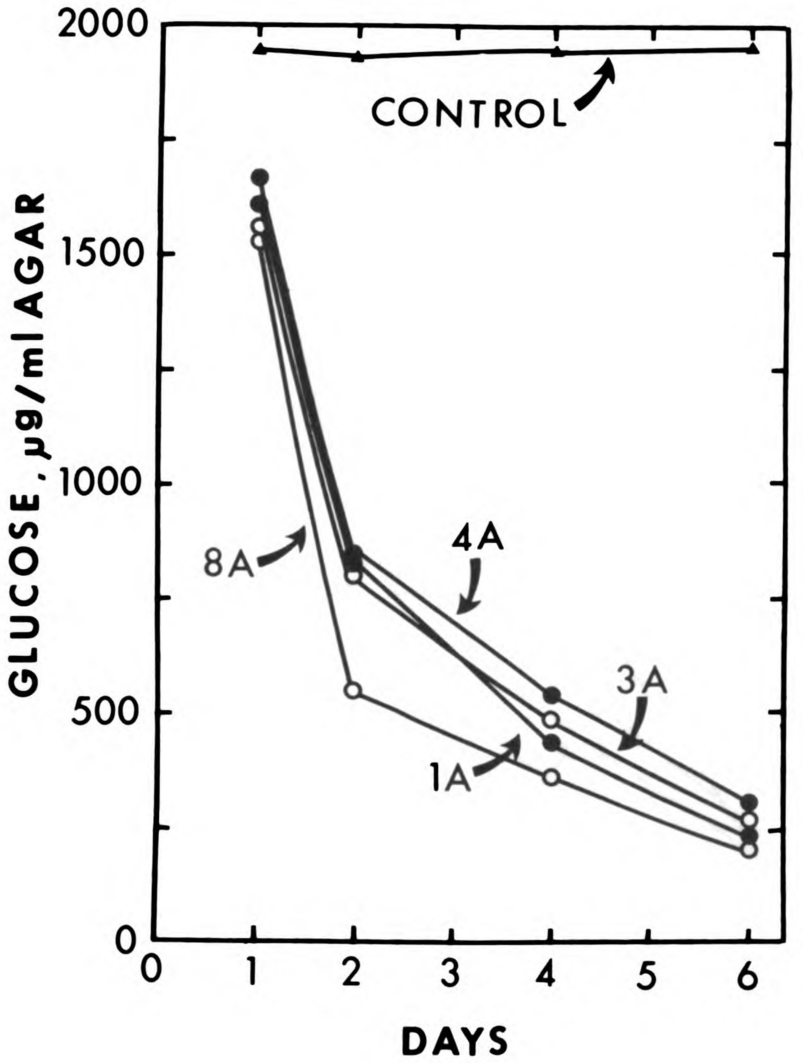

Fig. 5. Loss of glucose from 7 mm diam. agar disks immediately adjacent to actinomycetes. Agar containing glucose 0.2% and glutamic acid 0.2% was used. Actinomycetes 1A and 4A were antibiotic producers; 3A and 8A were non-antibiotic producers. In the control, no actinomycetes were streaked on the agar.

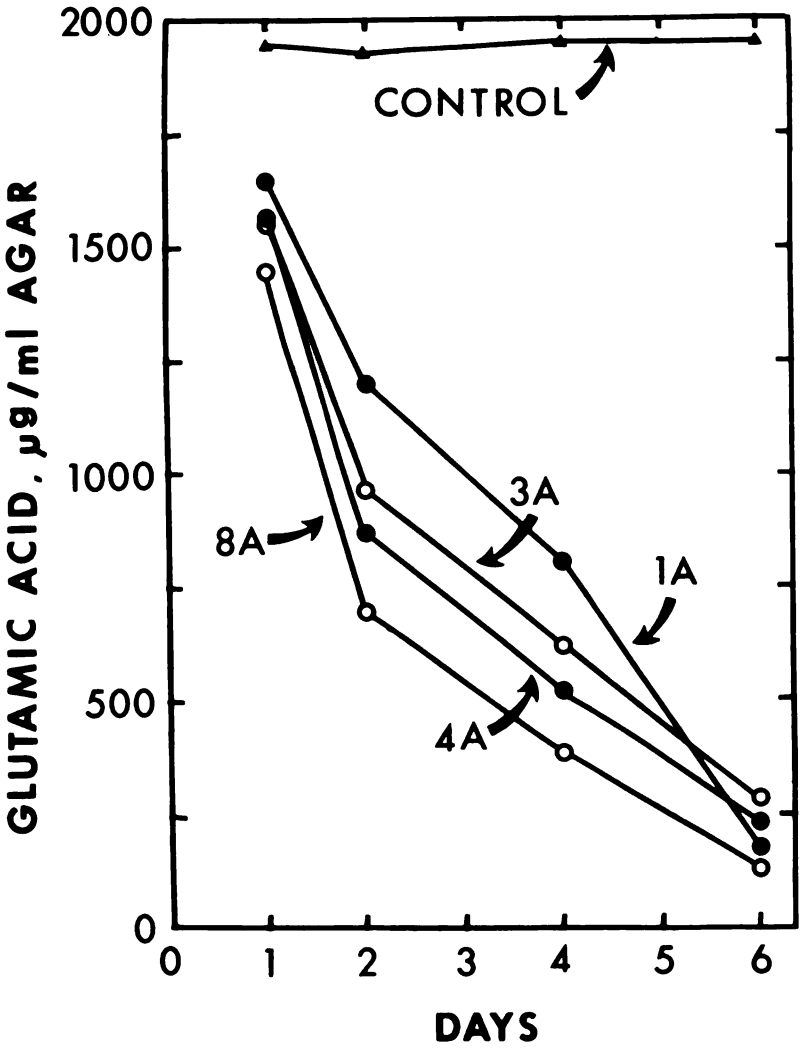

Fig. 6. Loss of glutamic acid from 7 mm diam. agar disks immediately adjacent to actinomycetes. Agar containing glucose 0.2% and glutamic acid 0.2% was used. Actinomycetes 1A and 4A were antibiotic producers; 3A and 8A were non-antibiotic producers. In the control, no actinomycetes were streaked on the agar.

disks from the area adjacent to isolates 1A or 4A, when transferred to fresh seeded agar, made new inhibition zones, but agar disks from the area adjacent to isolate 3A or 8A, when transferred to fresh seeded agar, did not make new inhibition zones.

Experiments were done to determine the effect of amount of glucose and glutamic acid on germination of conidia of the test fungus, M. ramannianus. Two levels of each compound were used, a "low" amount (250 μg/ml of each compound), and a "high" level (2000 μg/ml). The low level approximates the amount of each compound remaining in the agar after the 5th day, whereas the high level corresponds to the amount originally in the medium. The compounds were prepared in the following mineral salt solution: $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4$ $7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$ $7H_2O$, 0.01 g; water, 1000 ml. The low level of glucose and glutamic acid supported about 45% germination of Mucor spores, and the high level of these compound supported about 80% germination. The relatively lower amount of germination occurring in the low concentration of nutrients may explain the occurrence of the inhibition zone. Moreover, the dynamic depletion of nutrients would continue after germination of

conidia, and the germ tubes would be exposed to a more and more depleted environment. Mycelia would not be expected to develop in such conditions. Observation of conidia of test fungi in inhibition zones supports this view; although some germination occurred, the germ tubes were unable to develop.

To determine directly whether exhaustion of nutrients can cause inhibition zones on agar, the following experiment was devised. A special petri dish was prepared with the cover fitted with an inlet tube and the bottom with an outlet tube. The inlet tube was connected with sterilized plastic tubing to a separatory funnel containing sterilized distilled water. The outlet tube was connected with sterilized plastic tubing to a sterilized flask. The entire system was autoclaved and maintained in a sterile condition. Twenty ml of 0.5% peptone-glucoes agar was poured into the special petri dish. A 10 mm wide strip of agar was removed from the plate, and replaced with a small amount of agar to seal the 2 semicircles of agar. The dish was tilted slightly and sterilized distilled water was allowed to drip into one end of the groove and run out the other end into the sterile flask. The rate of water flow

into the agar plate was about 0.5-0.8 ml a minute and continued for 7 days. At this time conidia of M. ramannianus or G. cingulata were sprayed on the agar surface, and water was again allowed to run through the system for 3 more days. At this time clear inhibition zones had developed adjacent to the groove (Fig. 7). The inhibition zones were about 10 mm wide at each side. The spores of the test fungi in the inhibition zones had germinated poorly. When agar disks from these inhibition zones were transferred to fresh peptone agar, the conidia of both fungi germinated and developed mycelia. No contaminating microorganisms developed. Analysis of the agar in the zones showed that more than 90% of the glucose and amino acid were lost during the 10 day period of the experiment. Therefore, leaching of nutrients from agar can make inhibition zones which duplicate in every way those produced by actinomycetes. These results further indicate that the inhibition zones produced in agar by some actinomycetes, such as 3A and 8A, may be entirely due to the depletion of nutrients from the agar.

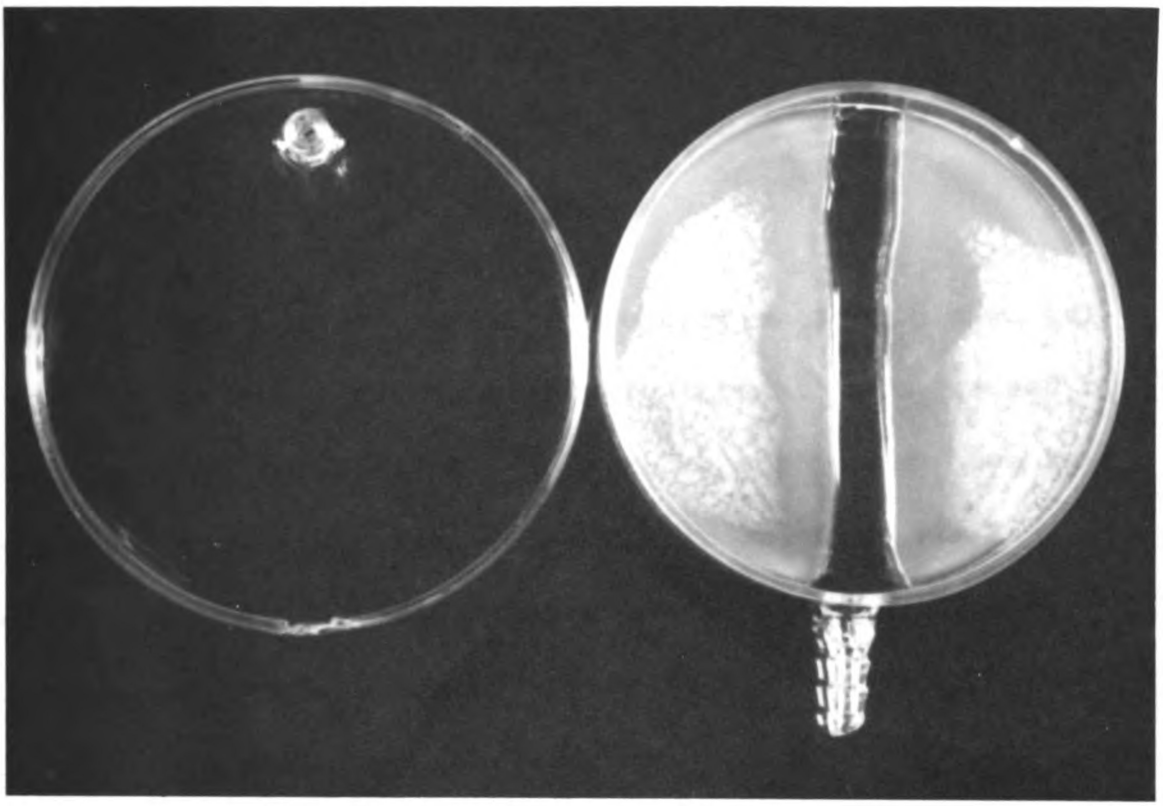

Fig. 7. Development of inhibition zones on leached agar. A special petri dish was designed to leach nutrients from peptone-glucose agar by allowing sterilized distilled water to run slowly through the groove between the two semicircles of agar. A clear inhibition zone developed following seeding with conidia of Glomerella cingulata.

DISCUSSION

Inhibition of fungal spore germination in agar cultures by actinomycetes is generally thought to be caused by production of antibiotics. Inhibitory substances have been extracted many times from antagonistic cultures (2, 3, 4, 8, 25, 26). All antibiotics tested so far are diffusible in agar and should be detectable by diffusion assays (7, 15, 24). In this research, agar disks from inhibition zones of 9 actinomycete isolates or paper disks placed beneath inhibition zones of these isolates, made new inhibition zones, when placed on fresh, seeded agar, thus demonstrating the presence of diffusible inhibitory substances. Eight of the same 9 isolates also produced diffusible antibiotics in liquid cultures. However, several findings indicated that 9 other actinomycete isolates that made inhibition zones in agar did not produce antibiotics. Agar disks from inhibition zones when transferred to fresh seeded agar did not produce new inhibition zones. Negative results were also obtained with paper disks placed beneath the inhibition zones.

Many reports indicate that some cultures that showed antagonism on agar media failed to produce antibiotics in liquid media (7, 17, 21, 22, 24, 27). This research also showed that certain actinomycetes which produced inhibition zones against fungi failed to produce detectable antibiotics in agar or liquid cultures. There are some reasons for considering the possibility that these actinomycete cultures may inhibit fungi by inducing a deficiency of nutrients in the agar. In this research, conidia of G. cingulata, which do not require exogenous nutrients, germinated when agar disks from the original inhibition zones were transferred to sterile petri dishes without agar. Conidia also germinated very well when incubated directly in 3-day old cultures of these actinomycetes grown in liquid media. Conidia of M. ramannianus, which required exogenous nutrients, germinated when nutrients were added to agar disks transferred from original inhibition zones.

Actinomycetes are able to utilize a variety of complex organic compounds. Carboyhydrates and nitrogen sources, including glucose and amino acids, are known to rapidly utilized (2, 18, 23, 25, 27). Evidence was obtained that

glucose and glutamic acid were rapidly lost from agar media adjacent to actinomycete colonies.

Results with leached agar showed that agar artificially deprived of nutrients failed to support fungal growth. Almost all nutrients were leached from adjacent agar when water was allowed to run slowly between 2 semicircles of agar. This area failed to support spore germination and mycelia growth. The inhibition zone which resulted closely resembled those produced by actinomycetes. Therefore, inhibition zones produced by certain actinomycetes may be due entirely to depletion of nutrients from the agar. The fact that agar in the inhibition zones produced by 2 antibiotic-producing actinomycetes was also rapidly deprived of nutrients raises a question as to the relative importance of antibiotics and nutrient depletion in production of inhibition zones by actinomycetes which produce antibiotics.

Although 18 actinomycetes lysed fungal mycelia, in no case did agar disks from lytic zones make new lytic zones on fresh fungal cultures. However, new inhibition zones were made by those isolates shown to produce antibiotics. Therefore, the production of lysis zones can not be

attributed to antibiotics or to any demonstrable diffusible lytic factor. The role of nutrient deprivation in inducing autolysis of fungi in soil was shown by Lloyd and Lockwood (12). In view of the ability of 4 of the actinomycetes to effectively deplete agar of nutrients, possibly lytic zones on agar result from nutrient deprivation.

These results also suggest the possibility that nutrient competion as an antagonistic mechanism in agar may occur not only in actinomycetes, but also in bacteria and fungi.

Much research in plant pathology has dealt with determination of numbers of antagonistic microorganisms in soils (6, 27). In work of this type, attempts are often made to correlate these populations with incidence of disease in certain soil types or following some kind of soil treatment. Assessment of number of antagonists and degree of antagonism are done in agar tests. It is usually stated or implied that these data provide a measure of the number of organisms capable of producing antibiotics active against a given pathogenic fungus. The results of this research suggest that interpretation of these kinds of data based on antibiotic production may be erroneous without further examination of the mechanism involved.

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