ANTIBIOTIC PRODUCTION IN THE GYMNOASCACEAE WITH
REFERENCE TO THE NITROGEN SOURCE

by

LUCILLE ANNETTE PASLAY

A. B., UNIVERSITY OF KANSAS, 1945

———

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Botany and Plant Pathology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1949

TABLE OF CONTENTS

INTRODUCTION

The substratum upon which a microorganism is grown influences the growth of that organism; enhancing the growth in some cases, retarding it in others and causing a variation in the metabolic products produced. The problem presented in this paper involved varying the source of nitrogen incorporated in a basic nutrient medium upon which the fungi were grown, in an attempt to determine the effect of the nitrogen source on the production of antibiotic substances.

REVIEW OF LITERATURE

An immense amount of research has been done in an effort to find the optimum substrata for the production of commercially important antibiotics. However, few workers have dealt with a basic medium in which one constituent was varied in order to ascertain the influence of that variable constituent on the production of antibiotics. There is a paucity of literature concerning the influence of the nitrogen source on the production of antibiotics. In many papers published on various phases of research in antibiotics, the substratum as an important factor has been mentioned repeatedly (2, 8, 9, 12, 17, 18, 19, 20). Usually this mention was based upon an observation which became evident when data of another sort had been compiled.

Reference to the importance of the nitrogen source was first made in 1945 when Rosenthal (13) stated that an unidentified mold from human hair produced a red pigment in the presence of an organic nitrogen source.

Only when this pigment was present did the organism show production of an
inhibitory substance. Hockenhull (6), in a preliminary study of the metabo-
lism of carbon, nitrogen, sulphur, and phosphorus by _Penicillium notatum_
Westling, as correlated with the production of penicillin, found that produc-
tion follows the general rate of absorption of carbon, nitrogen, and sulphur
into the mycelium. Decline in penicillin production was accelerated by the
addition of the ammonium radical, although apparently no pH change was pro-
duced. Brian, Curtis, Hemming, and McGowan (1) found the production of
viridan, an antibiotic from _Trichoderma viride_ Pers. ex. S. F. Gray, to
be optimum on a synthetic medium with an ammonium salt as the nitrogen
source. Stone, Patterson, and Farrell (15) studied the effect of adding
various adjuvants to synthetic media on penicillin production by _Penicillium
chrysogeneum_ Thom. They found that most of the amino acids had little effect.
L-leucine and certain sulphur-bearing amino acids were shown to increase the
yield, but not consistently. Aromatic compounds related to phenylacetic
acid and phenylethylamine stimulated production markedly. P-nitrophenylacetic
and p-aminophenylacetic acids increased antibiotic activity for _Bacillus
subtilis_ Cohn emend. Prazmowski and _Salmonella enteritidis_ Habs.

Dulaney (3), in studying the effect of the nitrogen source on the
production of streptomycin by _Streptomyces griseus_ (Krainsky) Waksman and
Henrici, incorporated in a basic medium both organic and inorganic nitrogen
sources. Compounds containing the ammonium radical were readily utilized
especially if calcium carbonate were added to maintain a relatively high pH.
In testing ammonium compounds the highest consistent yield, 150 micrograms
per milliliter, was obtained with di-basic ammonium phosphate. The pH
remained high in this medium without the addition of calcium carbonate.

The mold was unable to utilize the nitrate radical. If small amounts of corn steep solids, casein digest, or soybean meal were added to the di-basic ammonium phosphate medium production of streptomycin was greatly increased. Addition of amino acids singly to the medium gave no greater production of streptomycin than when ammonium phosphate was the only nitrogen source. Dulaney tested 33 organic nitrogen compounds as possible substitutes for ammonium phosphate. Six of the compounds, dl-alpha-alanine, beta-alanine, l-histidine HCl, l-arginine HCl, glycine, and l-proline supported yields of streptomycin in excess of 100 micrograms per milliliter. Yields of 800 micrograms per milliliter were obtained by the use of l-proline as the sole source of nitrogen. When used as a supplement to other nitrogen sources l-proline fails to increase streptomycin production. Compounds structurally related to l-proline were tested as substitutes, but none of them could replace this amino acid.

Woodruff and Ruger (21), in continuing the investigation of the l-proline medium, found that the extended fermentation time necessary for production of streptomycin on the proline medium as compared with the fermentation time on corn steep medium could be reduced by the addition of traces of di-basic ammonium phosphate. Excess ammonium-nitrogen caused reduction of the strep-tomycin yield, however. It is interesting to note from this work that inoculum grown on the proline medium is more satisfactory for streptomycin production than is an inoculum produced on the di-basic ammonium phosphate medium.

Emerson, Whiffen, Bohonos, and DeBoer (4) used four different media in testing a large number of actinomycetes and molds against five bacteria and six pathogenic fungi. As the nitrogen source, corn steep liquor was used in two of the media, in the third sodium nitrate, and in the fourth Difco

peptone. No medium proved generally superior to the others in antibiotic production. However, for any particular mold or actinomycete great differences were shown in the antibiotic yields produced on different culture media. In the media used in the experiment described above other constituents as well as the nitrogen source were varied.

Waksman and Bugie (16), in testing the antibiotics actinomycin and clavacin against Ceratostomella ulmi Buisman, found that these substances showed a strong inhibitory effect. This ability to inhibit could be overcome or reduced markedly by adding certain nutrients to the medium. They suggested that these results might have been due to the pyridoxene content, some other neutralizing effect, or the presence of peptone in the medium.

A portion of the paper written by Slagg and Fellows (14) led essentially to the conception of the problem described in this paper. In their work ammonium chloride, ammonium nitrate, sodium nitrate, and urea were used individually as the nitrogen source in a basic Czapek's solution. The six fungi used in this experiment had been found in previous tests to show inhibition against Ophiobolus graminis Sacc. when potato-dextrose agar was used as the culture medium. Antibiosis against O. graminis was altered greatly depending on the specific mold and the nitrogen source used. For instance, Aspergillus niger Tieghem, when grown in Czapek's solution with ammonium chloride, sodium nitrate, or potato-dextrose solution, developed products that completely inhibited O. graminis; only slight inhibition resulted when the mold was grown on ammonium nitrate or urea. In contrast, Gliocladium fimbriatum Gilman and Abbott produced substances that completely inhibited O. graminis when ammonium nitrate or urea was the nitrogen source, but not when the other compounds were used. This work shows that antibiotic

production is affected by the nitrogen source. Indication from this and other papers is that no single nitrogen source will produce universally optimum antibiotic yields, but that one or several nitrogen substrata tend to be optimum for the production of an antibiotic by a given organism.

MATERIALS AND METHODS

Choice of a basic medium in which to incorporate the different nitrogen sources was made by comparing growth of 20 different molds on basic substrata of modified Czapek's medium or modified Waksman's medium using several inorganic nitrogen compounds and peptone as the nitrogen sources. Growth was much better on the modified Waksman's medium. The formula for the basic medium was as follows:

distilled water	1000	cc
agar	20	g
glucose	16	g
KH_2PO_4	1	g
$MgSO_4 \cdot 7H_2O$	0.5	g
nitrogen source variable		

The amount of any given nitrogen source per liter was computed on the basis of the percentage of nitrogen in the compound as compared with the amount of nitrogen in peptone. Following the Waksman formula peptone was added at the rate of two grams per liter. Peptone contains about 15 percent nitrogen, hence a compound containing more than 15 percent nitrogen would be added at the rate of less than two grams per liter. For example, nicotine, containing 19.09 percent nitrogen, was added at the rate of 1.57 grams per liter. Thirty different nitrogen sources were chosen on the basis of their dissimilarity of composition and structure. The availability of the nitrogen in the molecular structure of the compounds was not considered.

Fellows' paper (5) on nitrogen utilization by Ophiobolus graminis was consulted carefully in choosing the sources. At least partial solubility in water was one of the criteria used in selecting the compounds. Compounds containing the cyanide radical were avoided because of their toxicity. In the case of 8-hydroxyquinoline, a component of the medium, presumably this compound, crystallized out on the surface of the agar and into the air. None of the five fungi grew on this medium. On cysteine HCl a very slight fungous growth appeared, but the substratum was unfavorable for growth of the test bacteria, except in the case of Bacillus subtilis when grown with the mold KSC 11-1-1. All other nitrogen substrata chosen supported growth of the organisms. Nitrogen sources stable at autoclave temperature were added to the basic medium before autoclaving. Jenkins filters were used to sterilize those nitrogen compounds that decomposed at autoclave temperature and these were added sterilely to the basic medium after it had been autoclaved.

The pH was checked by colorimetric estimation and adjusted before autoclaving, but not subsequently, to approximately pH 6.5. Sodium bicarbonate and lactic acid were used to make necessary pH adjustments. After the tests for antibiosis were completed some of the plates were flooded with brom-thymol blue to check any changes in the pH of the substrata caused by the growth of the organisms.

The fungi used were all lower Ascomycetes, members of the Gymnoascaceae, and for the most part were isolated from rat dung. Ascocarps in this family typically consist of a loose meshwork of hyphae enveloping the asci. Phylogenetically, the family lies just below the Eurotiaceae (Aspergillaceae) to which the genus Penicillium belongs. Four of the molds used as test organ-

isms belong to the genus <u>Arachniotus</u>. They will be designated in the remainder of the paper by culture numbers, KSC 30-1-6, KSC 11-1-1, KSC 3-2-8, and KSC 3-1-2. The fifth test mold, KSC 12-1-5, belonged to the genus <u>Gymnoascus</u>. Species determinations were not made on these molds. Preliminary experiments were run to test the antibiotic possibilities of 12 members of the Gymnoascaceae against four bacteria using peptone as the nitrogen source. The five test molds were selected from the data secured in this work.

Four bacteria were used in testing for antibiotic substances. <u>Salmonella pullorum</u> Rettger and <u>Escherichia coli</u> (Migula) Castellani and Chalmers, both gram negative, nonsporeforming rods; <u>Bacillus subtilis</u>, a gram positive, sporeforming rod; and <u>Micrococcus pyogenes</u> (Rosenback) Zopf. (Staphlococcus aureus), a gram positive coccus. <u>S. pullorum</u> is pathogenic to chickens. <u>B. subtilis</u> is usually considered nonpathogenic. <u>E. coli</u> is a normal intestinal organism which under certain conditions may be slightly pathogenic. <u>M. pyogenes</u> is a human pathogen causing severe suppurative processes. Septicemic infections by <u>M. pyogenes</u> have been reported (7). It is of interest to note that in the numerous tests for antibiosis carried on in recent years gram positive bacteria seem to be much more susceptible to the various antibiotic substances. Pure cultures of the bacteria were secured from the Department of Bacteriology at Kansas State College.

In making the tests, the fungi were grown separately in petri dishes on the basic Waksman's medium in which only the nitrogen source was varied. The fungi were allowed to grow at room temperature for 8 or 10 days depending on the rate of growth. Twenty-four hour cultures of the bacteria were streaked from the edge of the mold colony to the rim of the petri-dish. Each bacterium was streaked on a different side of the plate to avoid mix-

ing the different organisms, Plate I. After being streaked with bacteria, the plates were incubated at 37° C. for 24 hours, except for those on which the higher temperature caused the fungus to increase its rate of growth to such a degree that twelve-hour readings were necessary. The width of the zone of inhibition was recorded in millimeters. Although antibiotic effectiveness was evaluated exclusively by the width of the inhibition zone, there is a possibility that in some cases width of zone lacks correlation with the potentialities of the antibiotic. Growth of bacteria to the edge of the mold colony was considered a negative reading, even though the zone of inhibition might have been obscured by subsequent fungous growth.

The nitrogenous substrata were not usually amenable to the growth of the bacteria. It was found that they would grow on the media after meat infusion broth had been added to the nutrient broth in which they were being grown. The directions given by the American Association of Medical Milk Commissions, Inc. 1946 (10) were followed in the preparation of the infusion broth except that the tallow was not removed.

In early experiments, difficulty was encountered when an attempt was made to grow the fungi on liquid media and then test the presence of an antibiotic. When a small piece of blotter paper saturated with the liquid on which the mold was grown was placed on a nutrient agar plate with the test bacteria, negative results were obtained on all substrata and for all the molds. Consultation of the literature indicated that similar difficulties had been encountered by other workers. Waksman and Horning (17) ventured the idea that perhaps many molds do not produce antibiotic substances unless the organism to which they are antagonistic is present to stimulate production. A similar phenomenon is observed in certain members

of the Hyphomycetes in which nematode catching apparatus occurs only when nematodes are present. The stimulus in this latter instance, of course, results in a morphological rather than a physiological response.

DISCUSSION OF EXPERIMENTAL DATA AND CONCLUSIONS

Test mold KSC 3-1-2 showed the least antibiotic production of the molds tested. Of the 30 nitrogen substrata, on only 6 was any antibiosis evident. Allantoin, choline chloride, caffeine, nicotine, peptone, and tryptophane all gave zones of inhibition for M. pyogenes and B. subtilis. S. pullorum and E. coli were never inhibited, Table 3. The widest zones of inhibition occurred on the choline chloride medium and the narrowest upon the peptone medium. Flooding of some of the plates with brom-thymol blue showed an acidified region around the growth of E. coli. The remainder of the plate approximated the original pH. Evidently, therefore, antibiosis could not be attributed to the increased acidity of the medium.

The amount or appearance of growth seemed to have no correlation to the amount of antibiosis produced. The broadest diameter of the mold colony was consistently between 25-30 millimeters, except on the caffeine medium. On this medium growth never reached a diameter of over three millimeters and in some cases was restricted to the old inoculum. Antibiosis was present on the caffeine substratum. Two types of growth were quite evident. On peptone the fungus produced a small amount of white aerial mycelium and purple surface and subsurface growth. After an extended period of growth the substrata appeared black. Cleistothecia are grayish white and on petri-dish cultures are typically arranged in concentric circles. This type of growth occurred on

all media that gave positive results, except caffeine, and was also the most
common type of growth found on substrata where no antibiosis was recorded.
The second type of growth was characteristically nearly colorless and very
thin and diffuse. The diameter of this type of colony approximated that of
the other type. Negative results were obtained when the second type of
growth occurred. Difficulty was encountered in duplicating results with this
mold, so three complete runs with a check plate for each nitrogen substratum
were recorded before final averages were made.

KSC 3-2-8 showed almost consistent antibiosis for M. pyogenes and B.
subtilis. Negative results were obtained with S. pullorum and E. coli,
Table 3. Positive results for the former bacteria were obtained on all sub-
strata where growth occurred except in the case of caffeine where growth was
scanty and results on all bacteria were negative. Considering the influence
of the nitrogen source on the tendency of the mold to produce inhibition zones
against M. pyogenes and B. subtilis, four categories of inhibition could be
set up. On some of the nitrogen substrata a wide zone was present against
B. subtilis and a very narrow zone against M. pyogenes. On others the opposite
effect was produced; though M. pyogenes was strongly inhibited, B. subtilis
was not. A wide zone of inhibition was shown with B. subtilis, but none was
evident against M. pyogenes on nitrogen substrata placed in the third cate-
gory. On nitrogen substrata belonging in the fourth category, a comparable
zone was formed with both bacteria. The tabulated data are shown in Table 1.
This great variation in the amount of antibiosis might indicate the produc-
tion of two antibiotic substances--each active against a different bacterium.
This is particularly plausible when one considers the second and third cate-
gories. With this in mind it would seem that many of the nitrogen sources

Table 1. Nitrogen substrata on which KSC 3-2-8 produced wide zones of inhibition for M. pyogenes and B. subtilis, listed to show the influence of the nitrogen source on the amount of inhibition produced.

Categories of inhibition	Nitrogen substrata	Width of inhibition zone in mm. against	
		M. pyogenes	B. subtilis
Section I			
Wide zone of inhibition against B. subtilis; narrow zone for M. pyogenes	Dinitrobenzoic acid	3.0	12.5
	Tryptophane	5.5	12.0
	Urea	7.0	15.0
Section II			
Wide zone of inhibition against M. pyogenes; narrow zone against B. subtilis	l-tyrosine	21.5	1.0
Section III			
Wide zone of inhibition against B. subtilis; no inhibition against M. pyogenes	Hydroxyproline	0.0	14.0
	Taurine	0.0	2.5
Section IV			
Similar zone of inhibition against both bacteria	Ammonium chloride	12.0	11.0
	Ammonium nitrate	16.0	16.0
	Peptone	11.5	14.0
	Phenylalanine	12.5	12.5
	Sodium nitrate	12.0	16.0

(Table I, Section IV) are favorable for the production of both substances, while others are favorable for the production of either one or the other (Table 1, Sections I, II, III). This variation also might be explained as modification of a single antibiotic due to differing nitrogen sources.

On plates tested for changes in pH there was a definite zone of increased acidity. In a few cases, the cystine plates for example, the zone of inhibition corresponded to the acidified zone surrounding the mold colony. On most of the plates, however, changes in pH did not coincide with the width of the inhibition zones. Inhibition could not be attributed to increased acidity of the media.

An interesting phenomenon was observed in the inhibition zones formed against B. subtilis. With all but 2 of the first 20 nitrogen substrata tested showing inhibition, scattered bacterial colonies began to appear in the inhibition zones 48 to 72 hours after inoculation. This indicates that certain bacterial cells were resistant to the antibiotic. Since these isolated colonies probably arose from individual bacteria, the colonies were much too small to be visible in the earlier readings. A similar phenomenon has been observed when streptomycin was tested against M. pyogenes, E. coli and other bacteria (11).

In the case of KSC 3-2-8 the colonies usually ranged between 15-22 millimeters in diameter. There were several deviations from this, however. On the caffeine medium, growth was restricted to the old inoculum. On proline, growth was luxuriant--reaching 24-36 millimeters in diameter. Very little variation in the type of growth was noted. The mold produced a smooth gray mycelium which developed a definite apricot color with age. Cleistothecial formation was abundant on most media. As the cleistothecia matured the cul-

ture became burnt orange in color. This color was apparent on all of the nitrogen substrata except the amino acid, cystine. No fruiting occurred on the cystine medium and the colony became pale yellow instead of apricot. The only inhibition recorded against S. pullorum occurred on this substratum. With this mold, readings were easy to duplicate and quite definite.

Test mold KSC 30-1-6 showed antibiosis for M. pyogenes and B. subtilis and gave negative results for S. pullorum and E. coli. The four categories of inhibition set up in studying KSC 3-2-8 were not so clearly evident with this organism. One substratum, benzenesulphonamide, gave a very wide zone in the case of B. subtilis and a much narrower zone against M. pyogenes. Two, proline and dinitrobenzoic acid, gave zones of inhibition against B. subtilis while M. pyogenes was not inhibited. The other substrata on which wide zones of inhibition occurred showed comparable zones against both bacteria. On the ammonium chloride, ammonium nitrate, phenylalanine, tryptophane, and histidine HCL plates the zones of inhibition averaged at least 10 millimeters in width, Table 3.

Only one type of growth was common and diameters of the colonies varied between 15-22 millimeters. KSC 3-2-8 and KSC 30-1-6 are very difficult to distinguish since their microscopic and macroscopic characteristics were similar. As usual, the caffeine medium produced the least amount of mold growth.

In some cases, pH tests showed increased acidity of the media around the mold colony. These acidified regions did not correspond to the zones of inhibition. On many of the nitrogen media, the pH remained relatively constant except around the streaks of E. coli and B. subtilis. With this mold the latent development of resistant individuals of the B. subtilis strain within the inhibition zones also occurred. The phenomenon was present on fewer of

the plates than with KSC 3-2-8 and was not so marked. Results were consistent, distinct, and easily repeated with this mold.

Test mold KSC 11-1-1 produced zones of inhibition against all four bacteria on 13 of the 30 nitrogen sources. S. pullorum was inhibited on 14 of the substrata, M. pyogenes on 20, B. subtilis on 24, and E. coli on 18 of the substrata. Where the mold inhibited all four bacteria, the widest zones of inhibition occurred on the ammonium chloride, p-aminobenzoic acid, and phenylalanine media. Substrata on which the mold produced wide zones of inhibition for the different bacteria were quite varied. On the dinitrobenzoic acid, guanadine carbonate, and sodium nitrite media the mold produced no antibiotic substance affecting any of the test bacteria, Table 3.

Plates on which this mold had grown were acidified over their entire area. The colorimetric reaction indicated that the pH had dropped to somewhere below six. Results were very difficult to duplicate as the bacteria often failed to grow. This was particularly true with S. pullorum. It was thought at first that the antibiotic produced inhibited the growth of the bacteria over the entire plate. Later tests gave negative results when the mold was grown at one side of the plate instead of at the middle in an effort to get beyond the antibiotic's limit of diffusion and the plate was heavily inoculated with rapidly growing bacteria in an enriched medium. It seems possible that the zones of inhibition evident with test mold KSC 11-1-1 might be due to the change in acidity of the media rather than to the production of a substance or substances otherwise antibiotic. The application of greater numbers of bacteria in the enriched medium enabled the bacteria to grow through the highly acidified region to the edge of the mold colony. This idea seemed quite logical where a "no bacterial growth" reading was replaced

by a negative reading, but broke down completely in those cases which subsequently gave inhibition zones, even though the heaviest application of bacteria was next to the mold colony where the inhibition zone became apparent later. The mold evidently produced antibiotic substances, but very wide zones of inhibition might be attributable to the pH change of the medium.

Colonies ranged between 40-55 millimeters in diameter on most of the nitrogen substrata. This test mold grew profusely on media that the other molds found very difficult to utilize. The colonies, normally white when young, changed to bright yellow in a short period of time. Cleistothecia of this fungus at first yellow, became pink due to the maturation of ascospores. The colony often showed some green coloration because of the conidial heads. Variations from this type of growth were frequent, but seemed to have no influence on the production of antibiotics. Nor was there any correlation between fruiting and antibiotic production. The rapidity with which KSC 11-1-1 grew made it necessary to read the plates 12 hours after the bacteria had been applied.

Interesting results were obtained with test mold KSC 12-1-5. The first 20 nitrogen substrata upon which antibiotic activity of the mold was tested gave negative results except on the two substrata containing the ammonium radical, ammonium chloride and ammonium nitrate. On these substrata M. pyogenes and B. subtilis were inhibited, Table 3. Seven additional ammonium compounds were then tested to check the influence of the ammonium radical, Table 2. Only two ammonium compounds, ammonium acetate and ammonium formate, failed to show the expected results.

Growth on the peptone medium, Plate I, was smooth and white with an entire margin. Growth on most of the 30 nitrogen sources tested was similar to the growth on the peptone plate. The growth on most of the ammonium sub-

strata was whiter with extremely irregular margins resembling frost etching of glass. The name "snowflake" is used to describe this type of growth. A third type of growth, characterized by its fuzzy white aerial mycelium and entire margin was produced on the ammonium formate and ammonium acetate media. So closely correlated was the snowflake type of growth and antibiotic production that results could be predicted accurately in advance. Though none of the last 10 nitrogen substrata tested contained an ammonium radical, KSC 12-1-5 exhibited antibiosis against B. subtilis when grown on the tryptophane medium. Growth on the tryptophane medium was the fuzzy white type typical of the ammonium acetate and ammonium formate colonies. The pH tests on tryptophane plates showed an acidified region coinciding with the inhibition zone against the bacteria. Except for the fact that many of the plates showing this definite acidified zone gave negative results, this might explain the inhibition on the tryptophane plate.

EXPLANATION OF PLATE I

Characteristic growth of test mold KSC 12-1-5 on six nitrogen substrata.
Nitrogen substrata L to R, top row, ammonium formate, peptone; second row,
ammonium tartrate, ammonium sulphate; third row, ammonium nitrate, ammonium
oxalate. Bacterial streaks clockwise from the left hand side of the plate;
Micrococcus pyogenes, Bacillus subtilis, and Escherichia coli. Salmonella
pullorum was streaked on the lower quadrant of each plate and gave negative
readings on all plates. Growth for this bacterium was too light to photograph.
Note zones of inhibition against M. pyogenes and B. subtilis on the four
lower plates and the typical snowflake type of growth which could always be
correlated with antibiosis. Negative results were obtained on the ammonium
substrata that gave the fuzzy white type of growth pictured for ammonium
formate. Growth on most of the thirty nitrogen sources tested was similar
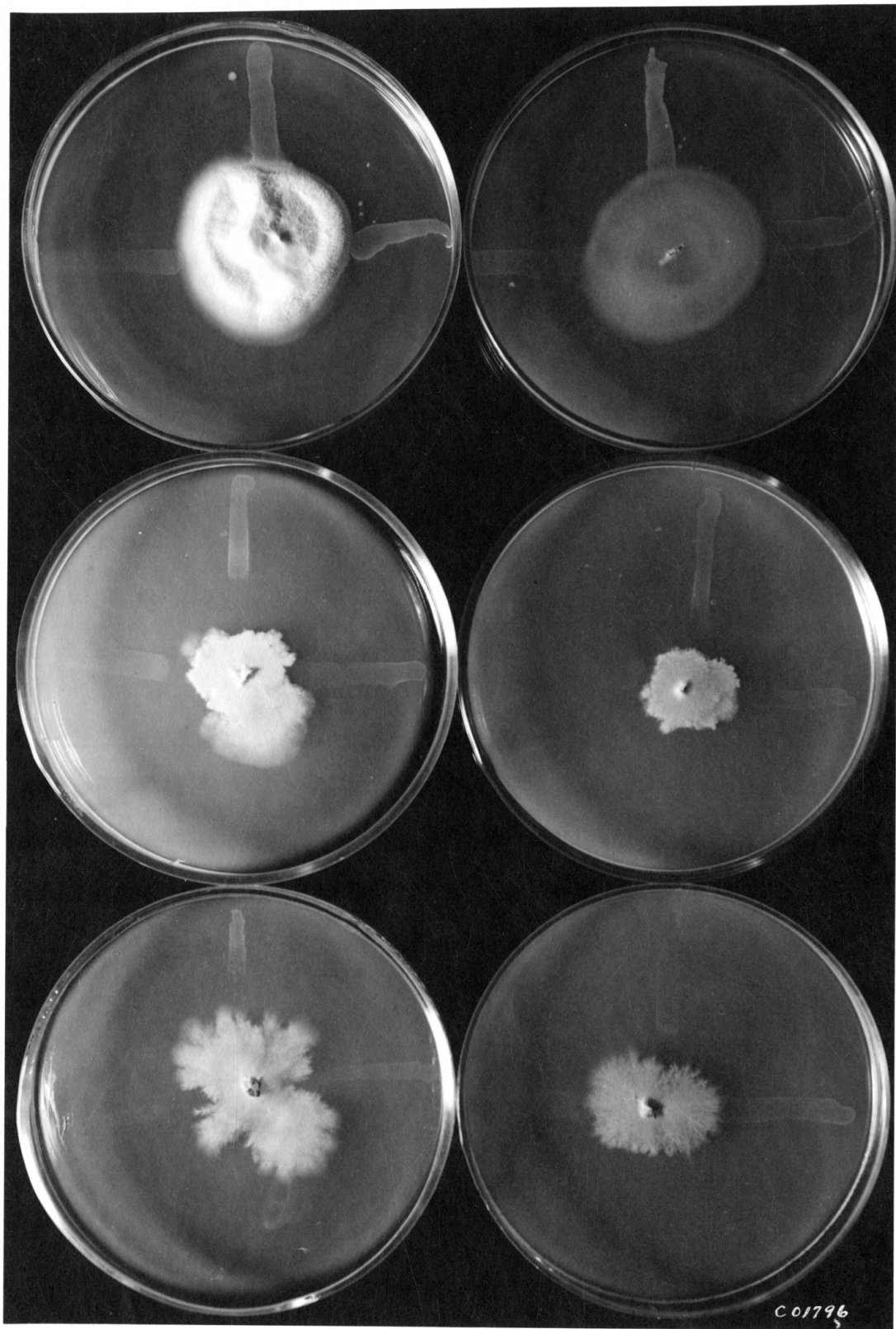to the smooth white growth found on the peptone plate.

PLATE I

C 01796

Table 2. Compiled data showing antibiotic tendencies of test mold
KSC 12-1-5 on compounds containing the ammonium radical.

Nitrogen source	Salmonella pullorum	Micrococcus pyogenes	Bacillus subtilus	Escherichia coli	Type of growth[4]
Ammonium thiocyanate	0[1]	nbg[2]	3.0[3]	0	snowflake
Ammonium tartrate	0	5.9	11.0	0	snowflake
Ammonium acetate	0	0	0	0	fuzzy-white
Ammonium formate	0	0	0	0	fuzzy-white
Ammonium chloride	0	3.0	11.0	0	snowflake
Ammonium nitrate	0	4.5	8.0	0	snowflake
Ammonium oxalate	0	0	8.5	0	snowflake
Ammonium sulphate	0	2.0	5.0	0	snowflake
Ammonium phosphate	0	2.0	3.0	0	snowflake
Peptone	0	0	0	0	smooth

1 no zone of inhibition present
2 no bacterial growth occurred
3 width of inhibition zone in millimeters
4 Plate I.

GENERAL CONCLUSIONS

Only one test mold, KSC 11-1-1, showed any tendency to inhibit all four
bacteria. KSC 3-2-8 and KSC 30-1-6 almost consistently produced substances
antibiotic against M. pyogenes and B. subtilis. The antibiotic effect of
KSC 3-1-2 and KSC 12-1-5 was limited to the same bacteria, but with these
inhibition was evident on only a few nitrogen substrata. With these members
of the Gymnoascaceae, positive results were obtained only when the bacteria

were streaked on the agar plate on which the mold was growing. The blotter paper method was never successful, although it works well in testing for penicillin production and also in the case of certain other antibiotics. It should be remembered that although width of inhibition zone has been used exclusively in evaluating the different nitrogen substrata tested in regard to their favorability for production of antibiotic substances, in some cases the excellence of the antibiotic substance probably cannot be measured by the distance through which it will diffuse; i.e., the width of the inhibition zones.

The pH changes in the media were not considered responsible for the zones of inhibition, except perhaps in the case of KSC 11-1-1 where bacteria failed to grow.

The effect of the nitrogen substrata on mold growth and colony characteristics provided an interesting incidental study. KSC 3-1-2, KSC 11-1-1, and KSC 12-1-5 responded markedly to the nitrogen source, while KSC 3-2-8 and KSC 30-1-6 did not. The test molds were all able to utilize a much wider range of nitrogen substrata than was expected. Caffeine consistently supported the smallest amount of growth. Through the literature there seems to be a tendency to purport the idea that conditions which are optimum for growth are not optimum for antibiotic production. While optimum conditions for growth were not considered in this experiment, the lack of an optimum or even amenable substrata for growth did not seem to influence antibiotic production. On all substrata where a wide zone of inhibition was recorded, growth had been good. With such substrata as caffeine, where growth was slower, no increase in antibiotic production was notable. The most interesting correlation between antibiotic production and type of mold growth was

with test mold KSC 12-1-5. Whenever the colonies were of the snowflake
type, antibiosis was evident.

Considering the three molds that gave fairly consistent antibiosis on
most substrata, ammonium nitrate, phenylalanine, and ammonium chloride pro-
duced the widest zones of inhibition. Substrata ranking very high against
individual bacteria or with individual molds were usually found among the
first ten when arranged according to the width of inhibition zones for all
the molds. Peptone, the check substratum, gave wide zones of inhibition,
but was excelled repeatedly by the above listed substrata and in the case
of individual molds by many others. Antibiosis in all but one instance was
evident with 12-1-5 only on media containing the ammonium radical. In several
of the papers discussed in the review of literature, ammonium compounds were
listed as favorable for antibiotic production. Results of this work would
indicate that the same was true for these molds also. Some compounds con-
taining the phenyl ring and dl-alpha- and beta-alanines were also mentioned.
Phenylalanine was one of the three compounds giving the most consistent
results with the test molds in this experiment. Increase in production was
not notable with most of the amino acids, except in a few cases where a wide
zone was produced against one of the bacterial test organisms by a specific
mold. L-proline reported by Dulaney (3) to give very high yields of strepto-
mycin with Streptomyces griseus showed no particular increases in production
for these test molds. Efforts to show correlation between the structural
formulae of the nitrogen substrata and the production of antibiotics failed
completely in all cases except with the ammonium radical and KSC 12-1-5.
With molds showing very limited production of antibiotic substances, it is
quite possible that favorable nitrogen substrata were not tested. It may

be concluded that certain nitrogen substrata were very amenable to antibiotic production by the organisms tested, several much more so than the control substratum, peptone. Further tests might indicate the advisability of using these substrata in screening various groups of organisms for antibiosis.

SUMMARY

1. The problem presented in this paper involved varying the source of nitrogen incorporated in the basic nutrient medium upon which test fungi were grown in an attempt to determine the effect of the nitrogen source on the production of antibiotic substances.

2. Thirty nitrogen sources were chosen primarily on the basis of their dissimilarity of composition and structure.

3. The five test molds, members of two genera, Arachniotus and Gymnoascus, belong to the family Gymnoascaceae. Antibiosis was tested for Bacillus subtilis, Micrococcus pyogenes, Escherichia coli, and Salmonella pullorum. A slightly modified streak plate method was used.

4. Four of the test molds inhibited M. pyogenes and B. subtilis, but not E. coli and S. pullorum - two of them on nearly all substrata, and two of them on only a few specific nitrogen sources. The fifth test mold produced evidence of antibiosis for all four of the bacteria on 13 of the 30 nitrogen substrata and zones against individual bacteria on many other substrata. Results suggest that more than one antibiotic substance was produced in several cases. Inhibition zones were obtained with these test molds only when the bacteria were streaked on the plates where the molds were growing

5. Correlation between pH changes in the media after growth of the fungi and the zones of inhibition was inconsistent, thus indicating that antibiosis probably could not be attributed to the increased acidity of the medium.

6. Considerable variation was evident in the type of mold growth on the different nitrogen substrata. The molds were able to utilize a much wider range of nitrogen sources for growth than was expected. Only in one case could antibiosis be definitely correlated to a specific type of growth.

7. Efforts to correlate the structural formulae of the nitrogen compounds with the production of antibiotic substances failed, except in the case of one test mold where in most cases the presence of the ammonium radical was closely allied with antibiotic production. With a few exceptions, the substrata on which all the molds produced wide zones of inhibition were quite similar. Ammonium nitrate, ammonium chloride, and phenylalanine gave the widest average zones of inhibition for all three molds where consistent evidence of antibiosis occurred. On these three substrata, the molds produced wider zones of inhibition than they did on peptone, the control substratum.

8. Some of the substrata on which wide zones of inhibition occurred might be used advantageously in screening molds for antibiotic potentialities. In the case of those organisms showing little or no evidence of antibiosis, it is possible that favorable nitrogen substrata were not tested.

Table 3. Compiled data for the five test molds showing occurrence and extent of inhibition zones on the 30 nitrogen substrata.

	Test molds																			
	Arachniotus KSC 3-1-2				Arachniotus KSC 3-2-8				Arachniotus KSC 30-1-6				Arachniotus KSC 11-1-1				Gymnoascus KSC 12-1-5			
	Test bacteria																			
	Salmonella pullorum	Micrococcus pyogenes	Bacillus subtilis	Escherichia coli	Salmonella pullorum	Micrococcus pyogenes	Bacillus subtilis	Escherichia coli	Salmonella pullorum	Micrococcus pyogenes	Bacillus subtilis	Escherichia coli	Salmonella pullorum	Micrococcus pyogenes	Bacillus subtilis	Escherichia coli	Salmonella pullorum	Micrococcus pyogenes	Bacillus subtilis	Escherichia coli
Allantoin	0[1]	1.5[2]	3.5	0	0	7.0	6.0	0	0	3.5	7.0	0	3.5	3.0	6.0	1.5	0	0	0	0
Ammonium chloride	0	0	0	0	0	12.0	11.0	0	0	17.0	17.0	0	16.0	17.5	9.0	6.0	0	3.0	11.0	0
Ammonium nitrate	0	0	0	0	0	16.0	16.0	0	0	12.5	14.0	0	7.0	6.0	6.0	8.5	0	4.5	8.0	0
Benezensul- phonamide	0	0	0	0	0	3.0	8.5	0	0	8.0	18.5	0	3.0	10.0	14.0	0	0	0	0	0
Betaine hydrochloride	0	0	0	0	0	3.0	6.5	0	0	3.0	6.5	0	0	0	*[4]	0	0	0	0	0
Caffeine	0	5.4	4.0	0	0	0	0	0	0	8.0	6.5	0	nbg	14.5	10.0	4.5	0	0	0	0
l-cysteine hy- drochloride	nbg[3]	nbg	nbg	nbg	nbg	nbg	nbg	nb	nbg	nbg	nbg	nbg	nbg	nbg	3.0	nbg	nbg	nbg	nbg	nbg
l-cystine	0	0	0	0	1.5	7.5	7.0	0	0	8.0	11.0	0	0	2.0	3.5	2.5	0	0	0	0
Choline chloride	0	4.0	3.5	0	0	7.0	9.0	0	0	9.5	8.5	0	6.0	9.0	9.0	2.0	0	0	0	0
3-5 Dinitroben- zoic acid	0	0	0	0	0	3.0	12.5	0	0	0	7.0	0	0	0	0	0	0	0	0	0
Guanadine carbonate	0	0	0	0	0	5.0	7.0	0	0	3.0	9.0	0	0	*	0	0	0	0	0	0
Hippuric acid	0	0	0	0	0	5.5	3.0	0	0	4.0	2.0	0	0	5.0	2.0	5.0	0	0	0	0
l-histidine monohydro- chloride	0	0	0	0	0	8.0	9.0	0	0	11.5	11.0	0	10.5	2.0	9.0	3.0	0	0	0	0
l-hydroxy- proline	0	0	0	0	0	0	14.0	0	0	9.5	9.0	0	0	3.0	2.0	0	0	0	0	0
8-hydroxy- quinoline	-[5]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
dl-methionine	0	0	0	0	0	5.0	8.0	0	0	3.5	8.0	0	0	nbg	0	0	0	0	0	0
Nicotine	0	0	0	0	0	4.0	6.0	0	0	5.0	6.5	0	7.5	7.0	8.0	4.5	0	0	0	0
Nicotinic acid	0	0	0	0	0	4.0	5.0	0	0	2.0	5.0	0	30.0	3.5	2.5	2.0	nbg	0	0	0
p-aminoben- zoic acid	0	0	0	0	0	3.0	7.0	0	0	0	4.0	0	8.0	10.5	12.0	9.0	0	0	0	0
Peptone	0	*	4.0	0	0	11.5	14.0	0	0	8.0	7.0	0	4.0	2.0	7.0	2.0	0	0	0	0
dl-phenyl- alanine	0	0	0	0	0	12.5	12.5	0	0	13.0	12.5	0	10.0	6.5	10.5	4.0	0	0	0	0
l-proline	0	0	0	0	0	nbg	8.0	0	0	0	9.0	0	0	12.0	6.0	0	nbg	0	0	0
Pyridine	0	0	0	0	0	7.0	10.5	0	0	5.0	8.0	0	nbg	0	37.0	0	0	0	0	0
Sodium nitrate	0	0	0	0	0	12.0	16.0	0	0	10.5	11.0	0	0	0	3.0	7.0	0	0	0	0
Sodium nitrite	0	0	0	0	0	4.0	2.5	0	0	5.0	7.0	0	0	0	0	0	0	0	0	0
l-taurine	0	0	0	0	0	0	2.5	0	0	4.5	7.0	0	0	nbg	2.0	0	0	0	0	0
dl-tryptophane	0	*	7.0	0	0	5.5	12.0	0	0	12.0	11.0	0	0	5.0	5.5	3.5	0	0	16.5	0
l-tyrosine	0	0	0	0	0	21.5	1.0	0	0	*	11.0	0	12.0	4.0	5.5	3.5	0	0	0	0
Urea	0	0	0	0	0	7.0	15.0	0	0	7.0	14.0	0	6.5	4.0	6.0	5.5	0	0	0	0
Uric acid	0	0	0	0	0	7.0	9.8	0	0	5.0	5.5	0	3.5	2.5	9.0	7.0	0	0	0	0

1 No antibiosis.
2 Millimeters of inhibition; i.e., area around mold colony through which bacteria would not grow.
3 No bacterial growth.
4 Growth of bacteria thinning near mold colony.
5. No growth of fungus or bacteria.

ACKNOWLEDGMENTS

The writer wishes to express her gratitude to Professor T. E. Brooks, major adviser, Department of Botany and Plant Pathology, for suggesting the problem and for his most helpful criticism and guidance. Sincere thanks are extended to Dr. F. C. Gates, Department of Botany and Plant Pathology, for his helpful suggestions. Appreciation is expressed to Miss Bettie Brass, Instructor, Department of Bacteriology, for supplying pure cultures of the test bacteria. The writer is also deeply grateful to Professor L. E. Melchers, Head of the Department of Botany and Plant Pathology, for his encouragement and interest.

LITERATURE CITED

1. Brian, P. N., P. J. Curtis, H. G. Hemming and J. C. McGowan.
   Production of viridin by pigment-forming strains of Trichoderma viride.
   Ann. Appl. Biol. 33(2): 190-200. 1946.

2. Cook, Lacy.
   An antibiotic for Aspergillus parasiticus. Nature (London) 153: 460.
   1944.

3. Dulaney, Eugene L.
   Observations on Streptomyces griseus II. Nitrogen sources for growth and
   streptomycin production. Jour. Bact. 56(3): 305-313. Sept., 1948.

4. Emerson, R. L., Alma Whiffen, Nestor Bohonos and D. De Boer.
   Studies on the production of antibiotics by actinomycetes and moulds.
   Jour. Bact. 52(3): 357-366. 1946.

5. Fellows, Hurley.
   Nitrogen utilization by Ophiobolus graminis. Jour. Agr. Res. 53(10):
   765-769. Nov., 1936.

6. Hockenhull, H. J. D.
   Studies in penicillin production by Penicillium notatum in surface cul-
   ture. 1. A preliminary study of the metabolism of carbon, nitrogen,
   sulphur, and phosphorus. Biochem. Jour. 40(3): 337-343. 1946.

7. Jordan, E. O. and W. Burrows.
   Textbook of Bacteriology. Philadelphia. W. B. Saunders Co. 308-310,
   370, 394, 509. 1945.

8. Konikova, A. S., R. M. Asarkh, E. I. Blimikova and i N. N. Dolvert.
   The biochemistry of the sporulating gramicidin-producing bacteria.
   Mikrobiologiia 13(4): 171-179. 1944.

9. Meredith, C. H.
   The antagonism of soil organisms to Fusarium oxysporum cubense.
   Phytopathology 34(4): 426-429. 1944.

10. Methods and Standards for the Production of Certified Milk, adopted by
    the American Association of Medical Milk Commissions, Inc. 1946.

11. Paine, Tom Fite and Maxwell Finland.
    Streptomycin-sensative, -dependent, and resistant bacteria. Science
    107(2771): 143-144. Feb., 1948.

12. Robbins, W. J., K. F. Kavanaugh and Annette H. Hervey.
    Production of antibiotic substances by basidiomycetes. Ann. N. Y. Acad.
    Sci. 48(2): 31-218. 1947.

13. Rosenthal, L.
    Antagonistic action of a red mould pigment against bacteria of the
    typhoid-paratyphoid, dysentery group. Science 102(2642): 176-177. 1945.

14. Slagg, C. M. and Hurley Fellows.
    Effects of certain soil fungi and their by-products on Ophiobolus graminis
    Sacc. Jour. Agr. Res. 75(11/12): 279-293. 1947.

15. Stone, R. W., H. T. Patterson, and M. A. Farrell.
    Adjuvants affecting penicillin yields on synthetic media. Jour. Bact.
    51(5): 598. 1946. Abstract.

16. Waksman, Selman A. and E. Bugie.
    Action of antibiotic substances upon Ceratostomella ulmi. Soc. Exp.
    Biol. and Med. Proc. 54(1): 79-82. 1943.

17. Waksman, Selman A., and E. S. Horning.
    Distribution of antagonistic fungi in nature and their antibiotic effect.
    Mycologia 35(1): 47-65. 1943.

18. Waksman, Selman A. and H. C. Reilly.
    Strain specificity and production of antibiotic substances III.
    Penicillium chrysogenium group. Nat. Acad. Sci. Proc. U. S. A. 30(5):
    99-105. 1944.

19. Waksman, Selman A., Albert Schatz and D. Reynolds.
    Production of antibiotics by actinomycetes. Ann. N. Y. Acad. Sci. 48(2):
    31-218. 1947.

20. Wiedling, S.
    The production of antibiotics by Penicillium sp. Bot. Notiser 1944:
    433-443. 1944.

21. Woodruff, H. Boyd and Myrle Ruger.
    Studies on the physiology of a streptomycin-producing strain of
    Streptomyces griseus on proline medium. Jour. Bact. 53(3): 315-321.
    Sept., 1948.