A modified agar overlay technique for the preliminary screening of antibiotic producing Streptomyces spp. has been developed and compared to the routinely used shake flask method and the unmodified agar overlay technique. The percentage of isolates exhibiting antimicrobial activities was found to be greater in the modified overlay technique.

Three types of inhibition were normally associated with the modified overlay technique. The first type included those plates of Streptomyces cultures with zones of inhibition not extending past the edges of the colonies of the Streptomyces (inhibition was just over the antibiotic producing organism). The second type showed inhibition zones which did extend past the edges of the Streptomyces colonies.
but did not show a steady and progressive increase of the zone of inhibition with time. The third type were those plates of *Streptomyces* isolates which demonstrated inhibition zones between the antibiotic producing organisms and the test organisms with the zones of inhibition increasing steadily and markedly with the time of assay. Antibiotics could not be extracted from types 1 and 2 but could always be extracted from type 3.

It was shown that the antibiotics extracted after 2 days of culture growth from the modified overlay plates were of a much higher concentration than those from the shake flask cultures or unmodified overlays extracted at 2 days also.

Antibiotics of crude concentrates from the cultivation medium of 38 cultures isolated from three different acidic soils were classified by a systematic paper chromatographic analysis. There were 51 antibiotics which could be classified according to this scheme, and no new classes or subclasses were found.
Diffusible Antimicrobial Agents Produced by *Streptomyces* from Acidic Soils: A Modified Overlay Method for Detecting Antimicrobial Activity and Partial Characterization of the Antimicrobial Agents

by

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A THESIS

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Typed by Lyndalu Sikes for Enefiok James Nkanga
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Also, I would like to thank the Government of the Cross-River State of Nigeria for paying my salary during the period of this research.

This work was supported by the National Institute of Health, Biomedical Sciences Support Grant RR07079 as awarded and administered through the Oregon State University Research Council and Grant 79-19280 from the National Science Foundation. The author would like to express his gratitude to these agencies for making this study possible.
This thesis is dedicated to my wife Quinta for her perseverance and concern during the period of this research and to my son IniObong who died during the most crucial part of this work and to all those that strengthened me emotionally during this great loss.
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I. INTRODUCTION

Streptomycetes as a group are considered as acid intolerant, and some investigators (9) have mentioned that the growth of streptomycetes is virtually inhibited in mineral soils having a pH value of 5.0 or less. Application of acid sensitivity was used as a method to suppress development of the potato scab organism *Streptomyces scabies* by lowering the pH in potato fields (29). As a result of the consideration that the proliferation of streptomycetes was restricted in acid soils, most of the antibiotics discovered during the antibiotic era that were produced by *Streptomyces* were usually isolated from neutral or alkaline soils.

Corke and Chase (11) examined streptomycete populations in acid, podzolic, forest soils and found that the numbers of streptomycetes that were isolated from the acidic soils varied with the isolation medium used and the pH of the medium. More recently, Hagedorn (17) found that the lowering of the pH of a selective isolation medium to the pH of the soil being examined increased the yield of streptomycetes from the acidic horizons when compared to
the same medium at neutral pH. He also showed that, even though
the greatest numbers of streptomycetes in the acid horizons developed
on a medium adjusted to the same pH as the soil, there was only a
certain proportion of the strains which could make the adjustment
from an acidic medium onto a neutral medium. These are collectively
called acidoduric streptomycetes. Hagedorn's results also pointed
out that the reason many acidoduric streptomycetes do not develop
when soils are first plated on a selective medium at neutral pH is
because they have a competitive disadvantage compared to the neutro-
philic streptomycetes and other bacteria, whereas at acid pH the
neutrophilic streptomycetes and most other bacteria are inhibited.

The primary objective of this investigation is to explore the
Streptomyces populations in three soil series which contain acidic
A and B horizons: Nekia, Marty, and Dixonville; and isolate as many
acidoduric streptomycetes as possible, test them to see whether they
do produce antimicrobial agents, and perform a partial characteriza-
tion of the antimicrobial agents by paper chromatography to see
whether or not they correspond to already existing groups.

Although many methods have been described for the detection
of antimicrobial activities of potentially antibiotic-producing isolates,
no one single method has been described which is a rapid, sensitive,
preliminary screening method in which inhibition could be detected with-
out preliminary concentration of the growth medium, and which simultan-
eously ensures that the inhibition is due to a diffusible product and not
just an artifact due to a process such as nutrient deprivation. The secondary objective of this investigation is to develop just such a method.
II. LITERATURE REVIEW

A. Detection of Antimicrobial Activity of Antagonist Organisms Against Test Organisms

The method employed most routinely for the preliminary screening of pure culture isolates for antimicrobial activities usually involves placing both a potential antagonist and the test organism on an agar surface a specified distance apart. The organisms grow towards each other, and the antagonist, if it is antimicrobially active, may cause inhibition of the test organism.

Johnson (21) placed the antagonist and the test organism on an agar medium opposite each other at the periphery of a petri dish. He used this method to study antagonisms of bacteria, fungi, and actinomycetes against Pythium arrhenomanes. Alexopoulos and Herrick (1) placed the antagonist on a medium at two locations 4 cm. apart, while the test organism was placed midway between the two in a study of the inhibition of Alternaria solani by actinomycetes. Other variations have been described (35, 45, 60).

The method by Alexopoulos and Herrick (1) and the modification by Johnson (21) are often inadequate because if neither of the organisms is a spreader and therefore cannot grow towards the other, any inhibition will not be apparent. Also, the physiological
requirements of one organism may be different from the other, and consequently they may not both grow on the same medium.

Because of these difficulties, other investigators have adopted the overlay method. The original overlay method was first described by Fleming (13). In his method, the antagonist organism to be tested for antibiotic production is plated in a single streak across one side of a culture plate of nutrient agar or other suitable medium. The culture is allowed to grow at an appropriate temperature for a suitable time. The plate is covered with a thin layer of agar which could maintain the growth of the presumably susceptible test organisms, and these are placed in streaks on the overlay at right angles to the antagonist when the agar has hardened. Many modifications of this method have been reported. Peterson (41) streaked the antagonist on an agar medium, and five days later 3 ml of an agar suspension containing the test organism was poured over the antagonist. He used this method to study the inhibitory effect of actinomycetes against Streptomyces scabies and Helminthosporium sativum. Since the medium for stimulating the production of antibiotics does not always support the growth of all the test organisms, this alternative method utilizes two different media, each suitable for the growth of the antagonist and test organism. The most important factors here are the time lag between the application of the test organism to the antagonist and the depth of
the overlay layer. Variations to this method have also been reported (40, 50, 59). Evidence of antagonism by this method, however, does not always indicate the production of some antimicrobial agent.

Hsu and Lockwood (20) obtained evidence that a clear zone of inhibition resulting between two cultures does not mean that an antibiotic substance is produced. There are indications in this work that zones of inhibition may result from nutrient deprivation. Also, 3 ml of agar is not enough to cover highly elevated aerial mycelia. If a larger volume is used as an alternative without the modification of the method, this might obscure positive results by the antibiotic not diffusing fast enough into the overlay. There is also evidence that inhibition can also be produced in response to the effect of the change in pH of the overlay layer by the underlying antagonist in the base layer (22). Therefore, it is mandatory to use a method which will demonstrate that any inhibition is actually caused by an antibiotic substance.

Many workers who are interested in isolation of a reasonable amount of an antimicrobial agent for chromatographic studies often choose the culture filtrate method after preliminary screening of the isolates has been completed. In this technique, a shake flask culture may be tested directly for antimicrobial activity after removing the potential antibiotic-producing organism. The filtrate
can then be examined by the broth-tube assay, the agar plate
dilution, or the cylinder plate method by Pratt and Dufrenoy (43)
and Waksman (54), the paper disc method by Loo et al. (30), or the
gradient plate technique of Szybalski (49).

The production of antibiotics in a liquid medium suffers from the
fact that the antimicrobial activity of newly isolated strains is
normally very low (12), and concentration of the filtrate must be
carried out every time in order to ascertain the maximum level of
production. In addition, the enzymatic breakdown of the anti-
microbial agent by the same organism that produced it
may obscure detection (16, 31, 39).

In summary, significant progress has been made in designing
methods for detecting antimicrobial activities of freshly isolated
presumable antibiotic producers. None of these methods have
successfully reduced the problems of discriminating between true
inhibition by an antibiotic substance and inhibition due to effect of
pH or nutrient deprivation, while simultaneously increasing the
sensitivity of detecting antimicrobial activity without the concen-
tration of the growth medium of the antibiotic-producing organism.
The method to be investigated in this project will attempt to do just
that while also making it possible to extract the antimicrobial
agents in aqueous form from the growth medium and subsequently
characterize them.
B. Classification of Antimicrobial Agents Produced by Streptomyces

Many classification schemes have been explored by workers in the field of antibiotic research to determine useful criteria for the differentiation of antibiotics into unique groups. In the different approaches to a classification system, many methods based on chromatographic analysis have been developed. Miyazaki et al. (37) grouped antibiotics according to their salting out chromatograms. They examined pure antibiotics and materials containing antibiotics by ascending paper chromatography. Concerning their irrigation solvents, in each case and with each antibiotic, distilled water and various concentrations of ammonium chloride (0.5, 1, 2, 3, 5, 10, 30 percent and saturated) were used. The $R_f$ values were then determined bioautographically (7). Antibiotics were then divided into four groups. In group A, $R_f$ value was not correlated with ammonium chloride concentration; they all showed identical $R_f$ values with all the irrigation solvents. Group B had $R_f$ value 0 in distilled water, and increasing $R_f$ values with increasing concentrations of ammonium chloride; group C had the highest $R_f$ value in distilled water, and lowering of the values with increasing concentration of solution of ammonium chloride; group D consisted of antibiotics which did not show movements in irrigation solvents, but an increased
tendency to form cones from the point of origin was observed with higher concentrations of irrigation solvents. Uri (52) added two new groups; E had an $R_f$ value of 0 in distilled water and an initial increase with rising concentrations of the irrigation solvents with the maximum being ($R_f = 1$) in 5 percent ammonium chloride after which a decrease occurred. Group F had an $R_f$ value of 0 in distilled water and an increasing $R_f$ with increasing concentration of irrigation solvent up to 3-5 percent; thereafter $R_f$ values decreased and $R_f = 0$ was associated with saturated ammonium chloride solution.

Miyazaki et al. (37) concluded that these groups reflected the chemical nature of the antibiotics in that the paper acted as an anionic resin adsorbing basic antibiotics, making it difficult to elute with water while neutral ammonium chloride could elute the antibiotic.

Another method which demonstrates the ionic character of the antibiotics, and which is used for classification is called pH chromatography (5). In this method, the chromatographic papers are buffered with McIlvain citrate-phosphate buffers of pH 2.2, 3, 4, 5, 6, 7, 8, and with phosphate buffers of pH 9 and 10. After drying, the strips are spotted with the extract to be analyzed, and all nine strips are chromatographed in the solvent which proved to be the most suitable by virtue of the solubility of the antibiotic in the solvent. The chromatograms are dried and detected bioautographically. The antibiotics are then divided into four groups: acidic, basic, neutral,
and amphoteric. Acidic antibiotics gave spots farther from the starting line in acidic pH range, the $R_f$ decreasing with increasing pH. Basic substances show the reverse behavior. The neutral antibiotics were unaffected by the pH. Betina and Nemec (8) added the amphoteric group in which the $R_f$ values increased in the range of a lower pH, and after reaching a certain maximum, began a diminishing trend.

Another way which also exploits the ionic character of the antibiotics for their classification is called systematic analysis (6). In this procedure, antibiotics are analyzed in four solvent systems and divided into five classes with fourteen subclasses. The four principal solvent systems include 1) water, 2) n-butanol saturated with water, 3) ethyl acetate saturated with water, and 4) benzene saturated with water. The polarity of the principal solvent systems decreases from system 1 to system 4. Solvent system 1 is water, which accepts or donates hydrogen and also forms hydrogen bridges with other molecules of water. n-Butanol is the main component of system 2 and accepts or donates hydrogen and forms hydrogen bridges with molecules of other compounds. Solvent system 3 contains ethyl acetate as its main component and can only accept hydrogen. Benzene, in system 4, has the least possibility of forming hydrogen bridges. The classification scheme according to Betina (6) is represented in Table 1).
Table 1. Classification of antibiotics into 5 classes and 14 subclasses according to their Rf in 4 principal solvent systems (6).

**PART A**

<table>
<thead>
<tr>
<th>Rf values in principal systems</th>
<th>CLASSES</th>
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<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Rf1a</td>
<td>&gt;0.00</td>
</tr>
<tr>
<td>Rf2b</td>
<td>0.00-0.30</td>
</tr>
<tr>
<td>Rf3c</td>
<td>0.00</td>
</tr>
<tr>
<td>Rf4d</td>
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**PART B**

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<tr>
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<td>Rf1</td>
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**PART C**

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<td>IIa</td>
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<tr>
<td>Rf1 &gt; Rf2 &gt; Rf3</td>
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### TABLE 1. Continued

#### PART D  \hspace{1cm} CLASS III

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<th>$R_f$ values</th>
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<th>Subclasses IIIb</th>
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<td>$R_{f4}$</td>
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#### PART E  \hspace{1cm} CLASS IV

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<th>Subclasses IVb</th>
<th>Subclasses IVc</th>
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<td>$&gt; R_{f1}$</td>
<td>$&gt; R_{f1}$</td>
<td>$&gt; R_{f1}$</td>
</tr>
<tr>
<td>$R_{f3}$</td>
<td>$&gt; R_{f1}$</td>
<td>0.00</td>
<td>$&gt; R_{f4}$</td>
</tr>
<tr>
<td>$R_{f4}$</td>
<td>0.00</td>
<td>0.00</td>
<td>0.05-0.60</td>
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#### PART F  \hspace{1cm} CLASS V

<table>
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<th>Subclasses Va</th>
<th>Subclasses Vb</th>
<th>Subclasses Vc</th>
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<tbody>
<tr>
<td>$R_{f1}$</td>
<td>$&gt; 0.60$</td>
<td>0.31-0.60</td>
<td>0.00-0.30</td>
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a water  
b n-butanol saturated with water  
c ethyl acetate saturated with water  
d benzene saturated with water
The advantage of this method over others lies in the fact that larger numbers of subclasses have been created for the greatly increasing number of antibiotics. The properties of species within the subclasses are comparatively constant in many representatives of a subclass, and they serve as a useful characteristic along with biological, physical, and other chemical tests in completely establishing the identity of a given antibiotic. This means species delineation within the subclass can easily be performed if this type of chromatographic method is used and the results compared to those already described. Also, discovery of a new group is possible if the antibiotic produced by a given isolate does not fit into any of the subclasses. The grouping according to Betina (6) expresses the ionic nature of the antibiotics analyzed since the ionic nature of the groups decreases from group Ia through Vc.

C. **Bioautographic Detection of Antimicrobial Activity of Spots on Chromatograms**

During the process of investigation of an unknown antibiotic, produced by a new isolate, the antibiotic is never present in a pure form and nothing is known about its chemical structure, hence, the most convenient method for detecting the active compound on the chromatogram is bioautography (7).

In the bioautographic detection of antibiotics on paper chromatograms, the chromatographic strips are placed on the surface of
large nutrient agar plates inoculated with microorganisms that are sensitive to the antibiotic being analyzed. After about 30 min, the strips may or may not be removed (depending upon the technique) and the plates then incubated and checked periodically for zones of inhibition. The antibiotics diffuse from their positions on chromatograms into the agar layer and inhibit the growth of the test organism. Zones of inhibition can be made more conspicuous when some indicators for the bacterial dehydrogenases (for example, tetrazolium chloride) are used (53). This method will also be adopted in detecting the antimicrobial activity during this research. However, removing the paper after it has been placed on the nutrient agar for a given length of time is necessary because it is possible that the antibiotic may not diffuse beyond the width of the paper and thus prevent visualization of the inhibitory zone.
III. MATERIALS AND METHODS

A. Determination of the pH of the Soil

The three soil series examined in this study were the Marty, Nekia, and Dixonville. The method used for the determination of the soil pH was the one described by Roberts et al. (46). From each sample, 20 g of soil was weighed into separate beakers, and 40 ml of water was added and stirred thoroughly to mix. The suspension was left to stand for 30 min and stirred twice at 10 and 20 min. The pH meter was standardized with a pH 7.0 buffer (Harleco, Gibbstown, N.J.). After the soil had settled to the bottom (approximately 30 min), the pH was read by placing the pH meter electrodes into the supernatant solution. The pH was recorded to the nearest 0.1 unit.

B. Isolation of Cultures from the Three Soil Series

The Marty (M) and Nekia (N) are both mountain soils that developed from quartz diorite and are under a Douglas-fir forest with a light, mixed shrub and fern understory. Both are highly leached, reddish brown in color, and strongly acidic. The Dixonville (D) is a terraced soil derived from weathered basaltic colluvium and is under a mixed fir and alder forest. This soil
series is highly leached, dark reddish-brown in color, moderately acidic, and is found on foothills bordering mountainous uplands. All three soil series are moderately extensive in west-central Oregon.

A 16 m² grid (4 m x 4 m) was marked out and divided into 16 subplots (each 1 m²) at one sampling site for each soil series. One core sample was collected from the approximate center of each subplot by using an Oakfield Orchard Auger (Oakfield Apparatus Co., Oakfield, Wis.) with extensions and a 7.5 cm diameter sampler. Samples were separately removed from both the A and B horizons of each subplot, (Nekia A horizon is 0 to 9 in. and B22t is 24 to 36 in., Marty All horizon is 0 to 6 in. and B1 is 16 to 31 in., Dixonville Al horizon is 0 to 5 in. and B2t is 13 to 27 in.), transported to the laboratory, and platings performed within 48 hr of sample collection. Samples were collected from each series three times at 4 week intervals, beginning Oct. 1, 1975.

The cores were removed from the samplers, and using sterile spatulas, the samples were cut open and a 50 g portion aseptically removed. The cores from all 16 subplots at each sampling site were treated in this fashion, placed in a sterile plastic bag, thoroughly shaken, and used for platings. A 50 g portion was then aseptically removed from each composite and suspended in 450 ml of sterile 0.5% peptone buffer. Each sample was then agitated in a Waring blender for 3 min at low speed, serial dilutions in 0.5% peptone buffer were made, and 0.1 ml portions of appropriate
dilutions were spread over the surface of a sterile medium in plastic petri dishes. The isolation was done on the starch-casein agar medium developed by Kuster and Williams (27), supplemented with the antibiotic cyclohexamide to inhibit fungi (agar, 18.0 g; potato starch, 10.0 g; casein vitamin-free amino acids, 0.30 g; KNO₃, 2.0 g; NaCl, 2.0 g; K₂HPO₄, 2.0 g; MgSO₄ · 7H₂O, 0.50 g; CaCO₃, 0.02 g; FeSO₄ · 7H₂O, 0.01 g; distilled water, 1 liter; cyclohexamide, 50 ìg/ml) (57). The pH of the medium was adjusted with 1 N HCl to that of the particular soil horizon being plated. The agar medium was autoclaved at 121 °C at 15 lb, 20 min, and the sterile cyclohexamide solution added to the agar which had been cooled to 47 °C to achieve a final concentration of 50 ìg/ml of agar. The plates were poured and incubated at 37 °C for 24 hr to check for sterility. Various dilutions of the soil suspension were inoculated onto the plates by spread-plating 0.1 ml of the soil suspension per plate, and each dilution was done in duplicate. Typical dilutions onto the plates were 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸, and all plates were incubated at 25 °C for 12 days. The presumptive characteristic of the Streptomyces group used to pick colonies for further testing was the identification of colonies which were cartilaginous in consistency with either smooth surface of substrate mycelium or with a loose powdery aerial mycelium on the agar surface (48). These colonies were re-streaked in the same isolation medium at the same pH to
obtain isolated colonies. If the isolates had the characteristic smell of damp soil (geosmin), they were saved for further testing.

C. Utilization of Carbon Compounds by Streptomyces

To be identified as belonging to the genus Streptomyces, the isolates obtained had to be tested for their ability to utilize carbon compounds according to the method of Pridham and Gottlieb (44).

Stock cultures of the isolates were maintained on yeast extract-malt extract agar slants (Bacto-yeast extract (Difco) 4.0 g, Bacto-malt extract (Difco) 10.0 g, Bacto-dextrose (Difco) 4.0 g, Bacto-agar 20.0 g), with the pH adjusted to that of the soil the isolate was obtained from. The utilization of carbon compounds was tested in the following basal medium:

\[
\begin{align*}
(NH_4)_2SO_4 & \quad 2.64 \text{ g} \\
KH_2PO_4 & \quad 2.38 \text{ g} \\
K_2HPO_4 & \quad 5.65 \text{ g} \\
MgSO_4 \cdot 7H_2O & \quad 1.00 \text{ g} \\
CuSO_4 \cdot 5H_2O & \quad 0.0064 \text{ g} \\
FeSO_4 \cdot 7H_2O & \quad 0.0011 \text{ g} \\
MnCl_2 \cdot 4H_2O & \quad 0.0079 \text{ g} \\
ZnSO_4 \cdot 7H_2O & \quad 0.0015 \text{ g} \\
Difco agar & \quad 15.00 \text{ g} \\
Distilled water & \quad 1,000 \text{ ml}
\end{align*}
\]
This basal medium was adjusted to the pH of the soil the isolate came from, tubed in 9-1/2 ml amounts, and autoclaved at 121 C, 15 lb. 20 min. After cooling to about 47 C, sterile aqueous solutions of the carbon compounds were added to give the proper concentrations. The carbon compounds used included sodium oxalate, dextrin, starch, dulcitol, inositol, maltose, D-xylose, L-arabinose, rhamnose, D-fructose, D-galactose, sucrose, maltose, lactose, raffinose, inulin, D-mannitol, D-sorbitol, sodium acetate, sodium citrate, sodium succinate, D-glucose, D-mannose, cellobiose, glycerol, phenol, o-cresol, p-cresol, sodium formate, and sodium tartrate. The carbohydrates, polyhydric alcohols, and DL-inositol were added to achieve a final concentration of 1.0 percent, the phenols at 0.1 percent and the sodium salts of the organic acids at 0.15 percent (44). Those materials sufficiently soluble in water were sterilized by filtering through millipore filter (type HA, 0.45 \( \mu \) pore size, Millipore Corp., Bedford, Mass.). Some compounds (sodium oxalate, dextrin, starch, dulcitol, DL inositol, and maltose) that were relatively insoluble were added directly to the basal medium in the proper concentration prior to tubing and sterilization. After the addition of the carbon sources, the tubes were slanted, allowed to solidify, and incubated at 37 C for 24 hr to determine sterility.
Inocula were prepared by growing the isolates in yeast extract-malt extract broth at room temperature for 10 days. The growth medium was centrifuged at 8000 g for 20 min, the supernatant poured out, and the remaining mycelia washed twice with sterile distilled water. A loopful of the sediment was used to inoculate the test medium. Controls consisting of the basal medium alone were always inoculated with each run. The presence or absence of growth were scored after 10 days at 27 C. The ability of the different carbon compounds to support growth was tested in duplicate tubes.

D. Development of the Modified Overlay Method

The modified overlay method was initially developed using *Streptomyces* N-156, an isolate from the Nekia soil which was pre-tested in liquid culture and found to produce an antibiotic active against *Bacillus subtilis* and *Staphylococcus aureus*. The medium used for the development of this method is described by Pridham et al. (45). The medium is made up of two parts: M-1 (corn steep liquor 10 ml, NaCl 5.0 g, distilled water 500 ml); and M-2 (glucose 10 g, agar 20 g, distilled water 500 ml). M-1 and M-2 were each separately adjusted to pH 5.6 (which is the pH of A horizon of Nekia where N-156 was obtained), and each was autoclaved separately (15 lb, 121 C for 20 min); after cooling both to 50 C, M-1 and M-2 were mixed and poured into petri plates at 15 ml per
plate. After the agar plates had hardened, they were incubated at 37°C for 24 hr to dry before use and then divided into two unequal sections (40 mm and 50 mm) by drawing a line with a marking pencil across the bottom of the plate. In developing the modified overlay method (see Figure 1), cultures were first inoculated from a slant into 100 ml of yeast extract-malt extract broth and grown at room temperature for 7 days while shaken at 150 rpm using a Labline Junior Orbit Shaker (Labline Instruments Inc., Melrose Park, Ill.). The cells were centrifuged out at 8000 x g for 20 min, washed twice with sterile distilled water, and resuspended with 6 ml sterile distilled water. The suspension was mixed thoroughly and diluted to give 10 mg dry weight mycelial suspension/ml. Dry weights of the washed cell preparations were determined by the method of Kominek (23). A portion of the cells were centrifuged, washed twice with sterile distilled water, and dried to a constant weight in tared aluminum dishes at 80°C in a vacuum oven. Ten duplicate plates marked day 2 through 20 at 2-day intervals were used, and each plate was inoculated by depositing 0.1 ml of mycelial suspension on the center of the smaller of the two sections. The mycelial suspension was then streaked across the entire area covered by the smaller section (40 mm), leaving the larger section (50 mm) untouched. The plates were then incubated at 27°C, and beginning on day 2 and every 2-day interval thereafter,
Figure 1. Representation of modified and unmodified overlay techniques: 1A, 2A, 3A show steps followed for the unmodified overlay techniques and 1B, 2B, 3B show the steps for the modified overlay. After steps 1A and 1B, the antagonist organism is allowed to grow for a suitable period of time before the overlay.
**Figure 1**

**UNMODIFIED OVERLAY**

1. **SINGLE STREAK**
2A. Overlay with 15 mls of agar suitable for the growth of the test organism and when solidified, follow step 3A.
3A. Streak the test organisms at right angles to the antagonist on the overlay reincubate for 24 hrs and look for zones of inhibition, can use more than 1 test organism per plate.

**MODIFIED OVERLAY**

1. **STREAK COVERING ENTIRE HALF OF PLATE**
2B. Overlay with 15 mls of agar suitable for the growth of the test organism, when solidified, reincubate for another 24 hrs and follow step 3B.
3B. Spread the test organism over the entire plate, one test organism per plate, reincubate for 24 hrs and look for inhibition.

1. **STREAK THE BASAL LAYER WITH ANTAGONIST**
a 15 ml overlay layer of agar was poured over the basal layer of each duplicate plate on the days marked, and the plates were re-incubated for an additional 24 hr at 27 C. Afterwards, 0.1 ml of a 24 hr culture of the appropriate indicator organism was spread over the entire plate (including the larger uninoculated section) and the plates then incubated at 37 C for 24 hr, after which they were observed for inhibitory zones.

E. Preliminary Screening: Comparison of the Modified Overlay to the Original Overlay and the Shake Flask-Disc Assay Methods

Six hundred Streptomyces cultures were isolated from the three soil series and the antibiotic production tested by the modified overlay method, original overlay method (13), and a combination of shake flask-disc assay method (45). The modified overlay method was as described (Section IIID). The medium used as a base medium for the inoculation of the antagonist for both overlay, modified, and original techniques was that of Pridham et al. (45) which was previously described. In the shake flask method, Streptomyces isolates were grown at 27 C in 250 ml flasks containing 100 ml of the medium reported by Pridham et al. (45) but without agar and using 0.1 ml of 10 mg/ml mycelial suspension. The pH of the medium was adjusted to that of the respective horizon of the soil series. Twenty ml of the samples were taken in two-day intervals, and the mycelium of each isolate
was separated from the medium by centrifugation at 18,000 x g. The supernatant was divided into two 10-ml samples and then freeze-dried, re-dissolved in 2 ml of 50% or 80% aqueous acetone, and centrifuged. For the paper disc assay, 20 ml of sterile agar was added to plastic petri dishes, and after the assay agar had hardened and dried overnight, 0.1 ml of the test organism containing a quantity of cells known to give an even lawn of growth was spread on top of the agar. Paper discs (Schleicher and Schuell Inc., Keene, N.H.) 740-E 6.35 mm diameter were impregnated with 0.02 ml of the concentrate, allowed to dry in a sterile dish, and then placed in the pre-inoculated agar and incubated at 37 C for 24 to 48 hr.

In the original overlay method, each Streptomyces isolate was prepared for inoculation as described for the modified overlay method, but instead of streaking the cells over the entire half section of the agar plate, a single streak was placed in the center of one half of the plate (see Figure 1). Beginning on day 2 and on 2-day intervals, 15 ml of overlay layer of agar were poured over the base layer, and the test organisms were streaked at right angles to the antagonist (Streptomyces) colonies as soon as the agar had hardened.

Six test organisms were used for sensitivity testing: Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Aspergillus niger and Saccharomyces cerevisiae (stock cultures, Department of Microbiology, Oregon State University, Corvallis). For the disc assay
testing, Difco brain heart infusion agar (BHI) was used for the four bacterial cultures, while the yeast and fungal cultures were grown on Difco potato dextrose agar. The same media were used in the overlay layers for the modified and unmodified overlay procedures.

For all three techniques, assay was done at 2-day intervals beginning day 2, and if still negative by day 10, the isolate was declared non-inhibitory against the test organisms.

For the unmodified overlay technique, two plates of the antagonist were normally made for each set of test organisms. One plate was overlaid with Difco brain heart infusion agar and *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris* placed in single streaks with spaces in between each streak at right angles to the antagonist. The other plate was overlaid with Difco potato dextrose agar and *Saccharomyces cerevisiae* and *Aspergillus niger* similarly streaked.

F. Secondary Screening: Kinetics of Antibiotic Production

Five isolates (D-75, N-139, N-141, N-145, N-156) were compared for their abilities to produce antibiotics using both the modified overlay and shake flask-disc assay procedures in the same medium at both the pH of isolation and neutral pH. The modified overlay technique was as described previously, but the antagonist
was streaked over half of the basal layer. Duplicate plates marked days 2, 4, 6, 8, 10, 12, and 14 were used and were each inoculated with 0.1 ml of 10 mg/ml of the cells. This was necessary to see whether any sudden changes in pH from that of isolation to neutral would affect the ability of the isolates to produce antimicrobial agents.

For the shake flask production, two flasks containing 150 ml of the same medium of Pridham et al. (45) (minus agar) were labelled as master flasks, and the pH of one adjusted to the pH of the soil that the isolate came from, while the other was adjusted to pH 7.0. For each master flask at each pH, seven other subsidiary flasks with the same volume of medium and labelled identically were also prepared. The seven subsidiary flasks were labelled days 2, 4, 6, 8, 10, 12, and 14. All were inoculated with 0.1 ml of 10 mg/ml of mycelial suspension. The mycelia was always blended to obtain a uniform suspension (23). For the modified overlay at the different days (as labelled on the plates), the plates were treated as described under the modified overlay method (Sec. IIID). One test organism most susceptible to the antibiotic as predetermined by former experiments was normally used to obtain data for the plot. The isolates for secondary screening were chosen for their ability to inhibit only one kind of organism such as gram-positive or gram-negative or fungi. In the shake flask method, 10 ml of the sample were removed from the master flasks beginning day 2 and at 2-day
intervals through day 14. The sample was centrifuged to sediment
the mycelia and the supernatant freeze-dried and re-suspended in
2 ml. of 50 or 80% aqueous acetone, depending on which of the
acetone concentrations showed the greatest activity during the
extraction and disc assay for antimicrobial activity in the pre-
liminary screening. The sample was then centrifuged at 18,000 x
g and assayed by paper disc method as described previously (Sec.
IIIE). At the same time, depending on the day the sample was
removed, 10 ml of a sample were also taken out from a subsidiary
flask that had the same day, same pH, and contained approximately
the same concentration of cells of the same isolate, and put in the
master flask to maintain a constant volume in those flasks.

G. Extraction of Antibiotic and Disc Assay for Antibiotic
    Activities of Isolates with Positive Inhibition
    in the Overlay Plates

After prominent inhibition zones had appeared in the overlay
plates, the antibiotic was extracted using the following procedure.
The inhibitory zone, including the area occupied underneath by
the antagonist, was cut out of the agar and the agar was then
transferred to a Waring blender and treated as in Figure 2.

For the antimicrobial activity disc assay, a battery of test
organisms representing gram-positive, gram-negative bacteria,
fungi, and yeasts were used. Test organisms included
10 plates showing marked inhibition

5 plates

5 plates

cut zones of inhibition and put in blender
cut zones of inhibition and put in blender

Add 50 ml of 50% aqueous (aq.) acetone
Add 50 mls of 100% acetone

blend at low speed for 3 min into a slurry and decant into centrifuge bottles, wash blender with 25 ml of 50% aq. acetone and add to centrifuge bottle, shake to mix, and centrifuge at 8000 x g
blend at low speed for 3 mins into a slurry and decant into centrifuge bottles, wash blender with 25 ml of 100% acetone and add to centrifuge bottle, shake to mix, and centrifuge at 8000 x g

lyophilize supernatant
lyophilize supernatant

redissolve in 50% aq. acetone (5 ml ) and centrifuge 18,000 g
redissolve in 80% aq. acetone (5 ml ) and centrifuge 18,000 g

* disc assay (.02 ml/disc)
* disc assay (.02 ml/disc)

* The one showing greater diameter of inhibition is used for chromatographic studies.

Figure 2. Extraction of antibiotics from overlay plates.
Staphylococcus spp., Bacillus subtilis, Streptococcus pyogenes, Corynebacterium xerosis, Corynebacterium diptheriae, Staphylococcus aureus (enterotoxin A producer), Streptococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella paratyphi, Vibrio parahemolyticus, Proteus vulgaris, Shigella sonnei, Brucella abortus, Aspergillus niger, Saccharomyces cerevisiae, Candida utilis, Candida tropicalis, and Debaryomyces spp.

(Stock cultures, Department of Microbiology, Oregon State University, Corvallis).

H. Chromogenicity of Streptomyces Isolates

Before the chromatographic analysis of the antibiotics were carried out, it was necessary to test those isolates producing brown pigments in the medium used for production of antibiotic to see whether or not they were producing melanin. This test was essential to be sure that any new class of antibiotic was not due to the effect of melanin modifying the $R_f$ values to render the extracted antibiotics non-classifiable under the already established classes (6). Two methods were used, one for detection of both melanin and hydrogen sulfide (51), and the other specific for melanin alone (2).

The medium used for testing to see whether the brown pigment was melanin or hydrogen sulfide consisted of the following: bacto peptone 15 g, preteose peptone (Difco) 5 g, ferric ammonium citrate 0.5 g, dipotassium phosphate ($K_2HPO_4$) 1 g, sodium
thiosulfate 0.08 g, bacto agar 15 g, bacto yeast extract 1.0 g, distilled water 1.0 liter. The medium was adjusted to pH 7, liquified, autoclaved, cooled to 47°C, and poured into petri plates. Streptomyces for the test were grown at room temperature in yeast extract-malt extract broth for 7 days, centrifuged at 8000 x g, and the sediment used to streak the plates. One loopful of the sedimented mycelia was used to streak over the entire half of the plate, and the plates incubated at 27°C. The results were read on days 2, 4, 6, and 8. This was done for all isolates which elaborated brown pigment into the medium used to produce the antibiotic. Positive results meant either the brown pigment is melanin or hydrogen sulfide.

To further prove the identity of the brown pigment as being strictly melanin, a second test had to be done. The test medium used to grow the Streptomyces isolates to assay for melanin production included the following: glycerol 3 mls, monosodium glutamate 25 g, \( K_2HPO_4 \) 0.5 g, \( MgSO_4 \cdot 7H_2O \) 0.015 g, \( ZnSO_4 \cdot 7H_2O \) 0.13 g, \( CuSO_4 \cdot 5H_2O \) 0.005 g, distilled water 1 liter. Streptomyces isolates to be inoculated into this medium were prepared as for the first test, and one loopful was used to inoculate 100 ml of the test medium in 250 ml flasks. The inoculated flasks were shaken at 150 rpm. Assays were done in 24, 48, 72, 96, 120, 144, 168, and 192 hr. The assay system consisted of 2.0 ml of
culture medium, 2.0 ml of 0.1 M phosphate buffer pH 5.9, and 1.0 ml of 0.4% 3-(3,4-dihydroxy-phenyl)-L-alanine (L-dopa) as substrate. The reaction mixture was incubated at 37°C for 5 min, and the red coloration resulting from dopachrome formation was read at 480 nm. If no coloration appeared within the above time, the reaction mixture was further incubated for 30 min, and if no melanin formed, the sample was declared negative.

I. Development of M-7 into a Non-Melanin Producing Strain

1. Preparation of Cells for Ultra-Violet Irradiation

The stock culture of M-7 strain of Streptomyces from the yeast extract-malt extract agar slant was inoculated into 100 ml of yeast extract-malt extract broth in a 250 ml flask and grown shaken at room temperature for 8 days. The medium was centrifuged at 8000 x g to sediment the mycelia. The sedimented mycelial fragments were re-suspended in 10 ml of sterile 0.05% Tween 80 in distilled water and homogenized into a fine suspension in a blender at very low speed.

2. Ultra-Violet Irradiation of Cells to Obtain Mutant Strains

An ordinary germicidal lamp with a mercury vapor tube rated at 2537 Å wavelength was used for the induction of the mutation. One
ml of the fine suspension of the mycelial fragment was diluted in sterile 0.05% Tween 80 in distilled water and plated to determine an approximate cell count before treatment of the cells for mutation induction. The medium used for the cell count was yeast extract-malt extract agar, and 0.1 ml of the dilution was used for spread plating to determine cell counts. Typical dilutions onto the plates were $10^{-4}$ through $10^{-8}$, and each dilution was plated in duplicate. The remaining 9 ml were placed in a petri plate and subjected to mutation induction with the UV lamp. The distance from the UV lamp to the surface of the plate of the suspension was approximately 20.0 cm, and the plate was subjected to a range of exposures of 15, 30, 45, and 60 sec. The exposure times were controlled by opening and closing the glass petri plate cover. During the exposures, the suspension was agitated by gently rotating the plate. At time 0, the cover was removed, and after 15 sec it was replaced and a 1.0 ml volume was pipetted from the plate into a dilution blank containing 9.0 ml of sterile 0.05% Tween 80 in distilled water and labelled 15 sec ($10^{-1}$). The same procedure was repeated for an additional 15 sec, and 1.0 ml was diluted and labelled 30 sec ($10^{-1}$). The following 45 sec ($10^{-1}$) was obtained in a similar way but for 60 sec, 1 ml of the fine cell suspension was added to 9 ml of the blank and 1 ml spread plated to obtain the $10^{-2}$ plate. To obtain the $10^{-1}$ plate, 0.1 ml of cells was
plated directly. The procedure for obtaining $10^{-3}$ and $10^{-4}$ dilutions on the plates were as for others, and typical dilutions onto the plates were as follows:

<table>
<thead>
<tr>
<th>Time</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 sec</td>
<td>$10^{-4}$ through $10^{-8}$</td>
</tr>
<tr>
<td>15 sec</td>
<td>$10^{-4}$ through $10^{-8}$</td>
</tr>
<tr>
<td>30 sec</td>
<td>$10^{-3}$ through $10^{-6}$</td>
</tr>
<tr>
<td>45 sec</td>
<td>$10^{-3}$ through $10^{-6}$</td>
</tr>
<tr>
<td>60 sec</td>
<td>$10^{-1}$ through $10^{-4}$</td>
</tr>
</tbody>
</table>

Plates were then wrapped in aluminum foil to avoid photoreactivation and incubated at 27 C for 8 days. On the 8th day, plates were counted to obtain total number of cells per treatment time and the total number of mutants per treatment time. The mutants were located, picked, and re-streaked in yeast extract-malt extract agar as mutant stock for further melanin testing. The criteria for picking mutants was the lack of brown pigments surrounding the isolated colonies. The mutant isolates obtained by this treatment were subjected to the melanin test as described earlier. All those negative for melanin production were used for antimicrobial activity testing, and if still active, the antibiotic produced was characterized to see whether it had the same $R_f$ characteristics as the original strain.
J. Chromatography and Bioautographic Detection of Extracted Antibiotics from Overlay Plates

1. Comparison of the $R_f$ Values of Antibiotic of N-156 Isolate Extracted from the Overlay Plate to the Shake Flask Extract

The chromatographic papers were Whatman No. 1 filter papers cut to 35 cm x 1 cm strips, and the solvents were benzene saturated with water, ethyl acetate saturated with water, n-butanol saturated with water, and distilled water. Six chromatographic papers were used to spot each extract (six papers for the extract from overlays and six for the extracts from the shake flask) for each solvent used for development. The filtrate (0.02 ml) was spotted 3.0 cm from the lower end of the strip and developed using ascending chromatography. For the tank, a square chromatographic jar unit 12 in. square by 24 in. high made of pyrex glass was used. The cover was a pyrex glass 18 sq. in. The chromatographic papers were supported by clips on a stainless steel rack which carries angle brackets adjustable for height. There were normally six paper strips in each row. Before the assembly of the chromatographic paper on the rack for development, a drop of a solution of Sudan III in ether was spotted on the strip about 1 cm under the line where the front of the solvent system was to be stopped. When the front of the solvent reached the spot of Sudan III, its further movement was
shown by a red band. Development was stopped after the solvent front had moved 15 cm above the point where the sample was spotted. Chromatography was done at 20 C ± 4 C without preliminary saturation of the chromatograms with the vapors of the solvent. After drying the chromatograms in air at room temperature, the locations of the antibiotics on the chromatographic strips were detected bioautographically using Bacillus subtilis as the primary indicator organism. For the bioautographic detection, 2 qt. oblong baking dishes containing 200 ml of brain heart infusion agar were used. The agar dishes were seeded with an even lawn of the test organism when dry. Heavy duty aluminum foil was cut to fit the dish and absorbent paper towels were taped on the inside of the foil cover to absorb the moisture generated by autoclaving the dishes. The dishes were autoclaved at 121 C, 15 lbs, 20 min, and after the agar had solidified, were left to dry for 24 hr before use. The filter paper strips were laid on the agar (three per dish), and after 15, 30, and 90 min each strip was removed to assess whether the duration of the strip exposure on the agar would affect the sizes of the zones of inhibition. Extracts from both the overlay plates and the shake flask were compared as to their $R_f$ values to see whether the agar in the modified overlay plates would affect the $R_f$ in the four solvent systems.
2. **Characterization of the Antibiotics Produced by Streptomyces Isolates by Paper Chromatography**

Conditions for the characterization of the antibiotics produced by all the isolates consisted of paper chromatography of the antibiotics followed by bioautographic detection. For the bioautographic detection of the antibiotic, *Staphylococcus* spp and *Bacillus subtilis* were used as indicator organisms for those isolates showing activity against gram-positive organisms only. For those showing activity against gram-negative only, *Escherichia coli* and *Proteus vulgaris* were used. If the isolate showed activity against gram-positive and gram-negative, *Staphylococcus* spp. and *Proteus vulgaris* were used for detection. For those isolates which showed activity against gram-positive and gram-negative bacteria and fungi, *Staphylococcus* spp., *Proteus vulgaris*, and *Saccharomyces cerevisiae* were used for detection, and *Saccharomyces cerevisiae* was used for those active only against fungi and yeasts. These combinations were necessary to elucidate whether the concentrates contained one antibiotic with a broad spectrum of activity, or more antibiotics with different activities and with different Rf values in the solvent systems (3).

The medium for the bioautographic detection using the bacteria was always brain heart infusion, and for the yeast culture, potato dextrose agar. The chromatograms were always left in contact with
the agar for 30 min before being peeled off. For each antibiotic produced by each isolate, and for each solvent system, the chromatography was repeated three times to determine the reproducibility of the $R_f$ values.
IV. RESULTS AND DISCUSSION

The soil pH values were determined prior to sampling and the pH of the medium adjusted to that of the individual soil horizons. Streptomyces isolates were identified tentatively as described before. Colonies which were cartilaginous in consistency with either smooth surface of substrate mycelium or with a loose powdery aerial mycelium were presumptively called Streptomyces spp. and subjected to carbon utilization tests. All isolates tested utilized D-glucose, D-mannose, cellobiose, starch, dextrin, and glycerol; none of the isolates utilized phenol, o-cresol, p-cresol, sodium formate, or sodium tartrate. However, they were all identified as Streptomyces since the results agreed with Pridham and Gottlieb's tests (44), except those showing faint growth in sodium tartrate. It was felt that these did not actually represent a confirmed positive utilization as compared to other results which demonstrated a positive carbon utilization. Microscopic examination of isolates grown on yeast extract-malt extract agar revealed narrow hyphae and straight chains of cylindrical spores (arthospores).

A. Modified Overlay Method

In developing the modified overlay method, it was important to demonstrate that whatever might have caused the inhibition was a diffusible product. Figure 3 shows the antagonist on the basal layer
Figure 3. The modified agar overlay method showing inhibition of *Aspergillus niger* (the black colonies on the surface) by *Streptomyces* isolate D-144. A zone of inhibition extends beyond the edge of the antagonist. No antimicrobial agent could be extracted from this plate and the zone of inhibition did not show a steady and progressive increase with time.
Figure 3
while the overlay layer contains the test organism spread over the entire plate. It is apparent that an inhibitory zone (preventing the growth of *A. niger*) exists in the overlay immediately above the *Streptomyces* isolate and extends some distance out past the edge of colony of the antagonist. The presence of the zone of inhibition does not mean that the problem of nutrient deprivation (20) or the effect of pH (22) in causing a similar zone of inhibition has been eliminated. In fact, no antimicrobial agent was demonstrated with this isolate. The production of an antibiotic can only be confirmed if the substance can be extracted from the plates. The reason for this is that the *Streptomyces* spp. could still act as a sink for nutrients from the overlay. However, three types of inhibition zones could be distinguished. There were some plates of isolates with zones of inhibition which did not extend past the edges of their own colonies, and no antimicrobial substances could be extracted from their agar overlays. Inhibition was just over the producing organism. These isolates represent those showing the first type of inhibition and may be caused by the effect of pH change of the overlay by the antagonist on the basal layer. It may also be caused by nutrient deprivation by the antagonist acting on the overlay layer, or there may be antibiotics restricted to the periphery of the mycelia (38). The second type were those plates which showed a zone of inhibition between the edges of the colonies of the *Streptomyces* isolates and the test organism as represented on Figure 33.
The zones of inhibition represented by this second type did not increase markedly with time, and no antimicrobial substance could be extracted from the medium. This second type of inhibition could be caused by nutrient deprivation or the altering of the pH of the overlay layer by the underlying antagonist in the basal layer. These two types of inhibition were very common in plates of *Streptomyces* spp. which showed inhibition against fungi. The third type of inhibition was the only reliable indicator of a possibility of a diffusible product being elaborated into the medium. In those plates showing inhibition of the third type, the zones of inhibition increased steadily and very markedly with the time of assay, as shown in Figure 4. With all the 150 isolates which showed this type of inhibition, a diffusible product was always demonstrated.

It would appear to be highly probable that the type of antimicrobial activity demonstrated by those isolates on plates of the modified overlay which demonstrated a steady and marked increase in the size of the zone of inhibition with time was due to the production of a diffusible substance rather than any artifact. This alone makes the modified agar overlay technique superior to all previously described agar overlay techniques in selecting isolates for further testing for antibiotic production. Since a thicker layer of overlay agar was used, incubation of the overlay plates for 24 hr before the test organism was added became necessary to
Figure 4. Zones of inhibition produced by N-156 isolate overlaid after 2 days (upper picture) and 8 days (lower picture) of incubation and *Bacillus subtilis* (test organism) spread over the entire plate on day 3 and 9, respectively. Notice the increase in the zone of inhibition with time.
allow diffusion of the antibiotic into this overlay layer. Previous experiments had shown that some of the plates which demonstrated antimicrobial activity after incubation of the overlay plates for 24 hr. did not show any inhibition if the test organisms were spread immediately after the overlay layer was added. During this additional incubation time, more antibiotic diffused into the overlay layer. This is a key advantage of this modified overlay technique compared to the already described ones, in that more isolates were positive for antibiotic production. This interval period is necessary to allow the antibiotic to diffuse into the overlay agar and avoid false negatives which occurred quite often without the additional diffusion period.

B. Preliminary Screening: Comparison of the Modified Overlay to the Original Overlay and the Shake Flask-Disc Assay

Six hundred Streptomyces cultures were isolated from the three soil series, and their ability to produce antibiotics were tested by the modified overlay technique, unmodified overlay technique, and a combination of shake flask-disc assay. Table 2 represents the results of the preliminary screening of the soils by comparing the three techniques. From the data, it can be seen that a greater proportion of antimicrobially active isolates was detected with the modified overlay technique. The antimicrobial
Table 2. Modified overlay agar technique vs. shake flask-disc assay method and unmodified overlay technique in comparing fresh isolates as percent positive scores for antibiotic production. \(^a\)

<table>
<thead>
<tr>
<th>Soil Series</th>
<th>Horizon</th>
<th>Soil pH(^b)</th>
<th>Modified Overlay(^c)</th>
<th>Unmodified Overlay(^d)</th>
<th>Shake Flask-Disc Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marty</td>
<td>All</td>
<td>4.5</td>
<td>18.0</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>3.8</td>
<td>23.0</td>
<td>13.0</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Ap</td>
<td>5.6</td>
<td>32.0</td>
<td>20.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Nekia</td>
<td>B2t</td>
<td>5.1</td>
<td>26.0</td>
<td>16.0</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>Al</td>
<td>6.1</td>
<td>25.0</td>
<td>15.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Dixonville</td>
<td>B2t</td>
<td>4.7</td>
<td>21.0</td>
<td>13.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

\[ \bar{x} 24.3 \quad \bar{x} 14.5 \quad \bar{x} 9.3 \]

\(^a\) Isolates were tested against six indicator organisms, and the percent positive scores indicate that a given isolate inhibited the growth of one or more of the test organisms. Six hundred isolates at 100 isolates per horizon per soil series were tested.

\(^b\) All isolates were tested for antimicrobial activity in the media with the same pH as that of the soil horizon from which they were isolated.

\(^c\) The only isolates included are those with zones of inhibition showing a marked and continuous increase with time and producing antibiotic substances which could be extracted from the growth medium in aqueous form and shown to cause inhibition.

\(^d\) Extraction of the antibiotics were also done and shown to cause inhibition in aqueous form.
agents in both the modified and unmodified agar overlay techniques included in the results (Table 2) were extracted from the medium in aqueous form and shown to cause inhibition, but the concentrations of the antibiotic in the unmodified agar overlay plates were relatively very small compared to the modified overlay extracts as measured by the diameter of inhibition zones.

However, no method exists without its own disadvantages. Incubating the plate for an additional 24 hr after overlay before adding the test organism exposed the investigator to the danger of altering the pH of the overlay layer and obtaining more isolates demonstrating the inhibition of types 1 and 2 as already described. This problem did not, however, hinder the ability to distinguish true inhibitions from false inhibitions as shown in the third type which always had an extractable antimicrobial agent associated with it. The second problem associated with this method was that flooding the plate with an overlay layer sometimes dislodged the powdery aerial mycelia associated with some isolates. The powdery mycelia were carried along with the agar, and incubation of the plates for 24 hr before the test organism was added promoted the growth of the mycelia on the overlay layer and thus contaminated it. Spreading the test organism on this type of overlay layer redistributed the contaminant, and even if there was inhibition of the test organism, the zone was not apparent due to
the growth of the antibiotic-producer. This problem was overcome by at first flooding with 10 ml of the overlay layer to wet the aerial mycelia, allowing the agar to solidify along with the wetted mycelia, and finally putting on a thin film of overlay of about 5 ml of the same agar. Since Streptomyces are aerobic, no growth occurred even if some of the mycelial powder were picked up (or disturbed) during the first flooding. Other problems included the inability to assay large populations of the antagonists in the same plate, and the problem of extraction of a class of antibiotic substances which were not soluble in the acetone-water mixture.

C. Secondary Screening: Kinetics of Antibiotic Production

Five isolates (D-75, N-139, N-141, N-145, N-156) were compared for their abilities to produce antibiotics using both the modified overlay technique and the shake flask-disc assay in the same medium at both the pH of isolation of the antagonist organisms and at neutral pH. This was necessary to determine whether a sudden change in pH of the growth of the antagonist organisms can considerably affect the ability of the isolates to excrete antimicrobial agents into their growth medium. The organisms were selected for their ability to inhibit only one type of the test organisms (gram-positive or gram-negative or fungi and yeasts). Figure 5 shows the results of the secondary screening to determine the
Figure 5. Comparison of the kinetics of antibiotic production in the modified overlay and the shake flask-disc assay methods. Both methods were performed at both pH 7.0 and at the pH of the soil horizon from which the streptomycetes were isolated (a: pH 6.1; b-e: pH 5.6). Assay incubation (days) means days antagonist was incubated before the overlay was added.

(Δ-Δ) pH 7.0; shake flask-disc assay

(▲-▲) pH 7.0; modified overlay assay

(0-0) pH of isolation; shake flask-disc assay

(0-0) pH of isolation; modified overlay assay
relative kinetics during which the antibiotics were being produced. For all strains except N-156, it can be seen that on day 2 both the modified overlay and the shake flask showed a measurable antimicrobial activity. Figure 5 also shows that there were insignificant differences in production of the antibiotics when isolates were grown at neutral pH or the pH of isolation using the modified overlay technique. This result is not very surprising since the study was restricted to the acidoduric isolates which could grow favorably at the acidic pH of isolation and at neutral pH. The ability to produce reasonable amounts of antibiotics by these acidoduric isolates can always be expressed whether the culture is started at the pH of isolation or at neutral pH. Results also show that for further testing of the isolates for antibiotic production, 8 days would be a reasonable length of time to grow the isolates before the overlay because (from Figure 5), all of the isolates tested by the modified overlay technique showed near maximum production of the antibiotics by day 8.

Several of the isolates tested by the modified overlay technique also showed some periods of constant level of production, after which the activity increased again when assayed at both the pH of isolation and at neutral pH. This may mean that the culture had produced a maximum concentration of the antibiotic which it could tolerate and then stopped; but due to the diffusibility of the antibiotic outside the antagonist's growth zone, and the adsorption by the agar, the antibiotic
concentration had fallen below this critical concentration, and further synthesis was stimulated. Isolates N-139 (Figure 5b) and N-141 (Figure 5c) demonstrated a unique feature in the shake flask assay. There was a rapid degradation of the antibiotic shortly after an early peak, a feature that was not observed with the modified agar overlay technique. This early peak might represent a residual synthesis carried over from the culture during the preparation of the inocula, and the later peak might represent new synthesis. Martin and McDaniel (33) have evidence in the pattern of the candidin production which shows that about two-thirds of the total candidin appeared as mycelium-associated product during the production period and was later released into the medium after the synthesis had ceased. This early peak from N-139 and N-141 could represent the release into the medium of the antibiotics synthesized in the previous medium. N-139 and N-141 are both isolates producing antifungal agents, and candidin is also an antifungal compound.

It can be seen in Figure 5c (isolate N-156) that activity can be detected very early using the overlay method as compared to the disc assay method. When compared to the original overlay technique, no activity could be detected on day 2.

The antimicrobial activity exhibited in the modified overlay plates did not, during a 14-day incubation, ever fall to undetectable
levels for any of the antibiotic-producing *Streptomyces* cultures examined (of which those in Figure 5 are examples), but the level did fall with the shake flask method. The activity increased in stages throughout the incubation period in the modified overlay assay, and the upper limit of the assay procedure (45 mm, at which the test organism was inhibited throughout the entire plate) was reached by most of the cultures.

When compared to the normal, unmodified overlay assay, antibiotic activity did not fall below detectable levels during the entire assay period once it was detected, but the concentration in the medium was so small that it could not be detected early in the growth of the organism as compared to either the modified overlay or the shake flask assay. For the unmodified overlay assay, even though enough quantity was produced by some isolates at a latter stage of growth to be extracted in aqueous form and shown to cause inhibition, the concentration was so small that it could not be used for chromatographic analysis. To try to extract enough for chromatography would mean using so many plates that it could not be easily accomplished. This feature also makes the modified overlay technique very superior to the unmodified overlay.

Martin *et al.* (32) have evidence that Ca-montmorillonite (fine clay particles) at 0.25% concentration and Ca-humate markedly accelerated and increased growth, glucose consumption, and CO₂
evolution by various *Streptomyces*, *Micromonospora*, and *Nocardia* species. They invoked the explanation of Waksman and Iyer (55) that humic acid acts by adsorbing inhibitory molecules to explain their observation. Similarly, it could be that the agar in the overlay method is acting to adsorb the inhibitory substances causing the *Streptomyces* isolates to grow more rapidly and produce more antibiotics. This process of agar serving to adsorb the inhibitory molecules from metabolic wastes and thus allowing the *Streptomyces* isolates to grow more rapidly and produce more antibiotics in addition to the ability of the antibiotic being able to diffuse out of the zone of the growth of the antagonist (thereby keeping the concentration of the antibiotics below inhibitory concentration to the producing organism) could be the explanations for the observations which make the modified overlay technique superior to the shake flask method. These explanations have been supported by several findings by other investigators.

Kominek (23) has evidence that cyclohexamide production by *Streptomyces griseus* was affected by added cyclohexamide. He found that in the presence of various levels of cyclohexamide added to a standard fermentation in shake flasks, the net synthetic rate was found to be inversely proportional to the concentration of cyclohexamide in the medium. He also found that glucose exhaustion corresponds to the cessation of antibiotic accumulation which is
followed by a rapid degradation of cyclohexamide. In another study, Kominek (24) found that the use of dialysis fermentation with the continuous extraction of the dialysate resulted in a two-fold increase in cyclohexamide titer due to the relief from product inhibition.

This is not surprising because an earlier investigation (56) pointed out that accumulation of a certain antibiotic above a critical level would inhibit or completely suppress vegetative growth of the producing organism. Extensive use of this finding was made in selection of strains with elevated activities. The explanation for the superiority of the modified overlay agar technique over the routinely used shake flask method is further strengthened by the work of Benedict et al. (4). They showed that spore germination in *Streptomyces griseocarneus* was inhibited by 5.0 µg of streptomycin, whereas mycelial growth occurred in the presence of 200 to 300 µg of the antibiotic. This means that vegetative growth of this organism is very sensitive to this critical concentration of the antibiotic. The modified overlay technique serves as a self-dialysing system, encouraging more growth of vegetative cells and consequently more mycelial volume and more antibiotic.

Chao-Min Liu et al. (10) have shown that the mycelial inoculum size, and the total amount of mycelial dry weight in the culture medium is directly proportional to the amount of candididin produced.
The total volume of mycelia in the modified overlay layer is many times greater than in the unmodified overlay at the end of the incubation period. The reason is that a larger area is used for the modified overlay inoculation as compared to the unmodified overlay, hence a greater quantity of nutrients is available to support a larger volume of mycelia. The larger area of inoculation in the modified overlay technique results in more mycelial volume and hence more antibiotic production (10). After overlay, plates are left for an additional 24 hr for the antibiotic to diffuse into the overlay layer. Consequently, it is not surprising that the modified overlay technique is more sensitive (in terms of the numbers of isolates positive for antibiotic production) and superior (in terms of the concentration of antibiotic being produced) to the unmodified overlay technique.

D. Extraction of the Antibiotic and Disc Assay of Antibiotics of Isolates with Positive Inhibition in Overlay Plates

The extraction of the antibiotic in the modified overlay plates was performed to demonstrate that the antibiotic could be extracted, concentrated by freeze-drying, and used for disc assay detection of activity without a deterioration in activity. N-156 isolate from the A horizon of the Nekia soil series was used for the extraction and disc assay of antimicrobial activity. Isolate N-156 was overlaid at the end of two days, and the antibiotic was extracted from the
medium on day 4. Figure 4 (day 2) shows the inhibitory zone of N-156 using the modified overlay technique at pH 5.6 (after 48 hr of incubation before the addition of overlay). In Figure 6, the inhibitory zone in the center shows the extract from this N-156 overlay plate. To the right is the disc for the shake flask method after 2 days of growth pH 5.6, and to the left is a disc for unmodified overlay. The assumption here is that Streptomyces are strictly aerobic, and overlay causes its aerobic metabolic activity to cease, and any antibiotic activity demonstrated was evidently there before the overlay. This simply means that even though the antibiotic demonstrated in Figure 6 was extracted on day 4, it reflects the activity of the antibiotic up to day 2 of the production.

It is apparent that a more concentrated antibiotic could be extracted from the modified overlay plate when compared to normal overlay or shake flask at the end of 2 days of growth.

For all of the isolates which were examined and found to exhibit antimicrobial activity in the modified overlay method (with the zones of inhibition increasing steadily and markedly with time), inhibitory substances could be extracted from the overlay agar and the inhibition demonstrated by the disc assay. It is now apparent that inhibition represented by the third type of plates described earlier is a good indicator of the production of a diffusible product.
Figure 6. Comparison of the antimicrobial activities of extracts from the modified overlay plate, unmodified overlay plate and shake flask after 2 days of growth. Extracts from the N-156 (Figure 4, 2 days) modified overlay plate at the end of 2 days (center disc). The extraction was made on day 4, and the test organism is *Bacillus subtilis*. To the right is a shake flask sample assayed on day 2 and to the left is the original overlay assayed also on day 2. All discs received .02 ml of the concentrated extract.
The modified overlay agar method combines the features of the unmodified overlay agar technique in that you can detect inhibition without concentration of the growth medium, and the shake flask in that the antimicrobial agent can be extracted in aqueous form in a sizeable quantity to be used for chromatographic characterization.

Because of the unmanageable size of the number of isolates showing positive inhibition of the third type with which extractable antimicrobial agents were normally associated, a decision was made to restrict further studies to A horizons of the three soil series. The total number of isolates in A-horizon showing positive inhibition was 75. The extracts from the overlay plates of these isolates were subjected to disc assay activity screening against a battery of gram-positive and gram-negative bacteria, fungi, and yeasts. Figure 7 shows the distribution of these isolates according to their antimicrobial activity against both bacteria and fungi. It appears from the results that the number of isolates showing inhibitory activity against both bacteria and fungi increases from pH 4.8 to pH 6.1. These antibiotics could be broad spectrum, with one antibiotic showing activity against gram-positive, gram-negative bacteria, fungi, and yeasts. Such antibiotics actually exist, and examples are endomycin, mycomycin, streptothricin, thiolutin, aurantiogliocladin, and clavacin all of which are synthesized by actinomycetes. Alternatively, these antibiotics may be many
Figure 7. Distribution of antimicrobially active isolates according to their antimicrobial spectra as % of total number of isolates per soil series. Antimicrobial agents were extracted from the modified agar overlay plates and tested against a battery of gram-positive, gram-negative bacteria, fungi, and yeasts. G+ means gram-positive organism; G- means gram-negative organism. Result represents A-horizon only.
antibiotics elaborated by the same organism which show activity against different groups of test organisms. The same trend was also seen for isolates showing activity against fungi. The total number of isolates showing antimicrobial activity was less for the Marty series at pH 4.8 when compared to either the Nekia or Dixonville isolates. This is not surprising because at very low pH there exists a less diverse microflora, and quite possibly, less competition. With less competition, the probability of acquiring this mechanism of self-defense (antibiotic production) is less. This may serve as an indirect evidence that antibiotics may be produced in soils. Other workers have demonstrated this possibility by inoculating soils with known producing strains and demonstrating the antibiotics in the soil (14, 15, 34).

E. Chromogenicity and Melanin Production by Streptomyces Isolates

Before characterization of the antibiotics could be completed, it was necessary to assay those isolates producing brown pigments in the medium used for the fermentation of the antibiotic to see whether or not the brown pigments were melanin compounds. Table 3 shows the results of these tests on the isolates from the A-horizons of the three soil series. It can be seen from the data that the brown pigments elaborated by these isolates were actually melanin.
Table 3. Chromogenicity and melanin production by *Streptomyces* sp. which produced brown pigments in yeast extract-malt extract broth and the antibiotic fermentation medium.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Chromogenicity PYIA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Melanin formation L-DOPA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-30</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-22</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-51</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-49</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-103</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-121</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-72</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-156</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-61</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-164</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-148</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peptone-yeast extract-iron agar.

<sup>b</sup> 3-(3,4-dihydroxy-phenyl)-L-alanine.
Of interest is the fact that all the isolates which produced the brown pigments in the medium also showed inhibition against gram-positive organisms. The *Streptomyces* isolates which displayed these brown pigments were presumed to be producing an enzyme called phenolase (tyrosinase) which can oxidize monohydric phenol or dihydrophenol to quinone, a very reactive substrate which can copolymerize with similar compounds or proteins and appear as a brown amorphous structure termed melanin with a different molecular weight. If the antibiotic was a phenolic structure, this type of reaction may theoretically influence its $R_f$. However, the influence of phenolase in modifying the $R_f$ values of antibiotics produced by *Streptomyces* during the characterization of the unknown antibiotics has not been reported in the literature.

F. Development of M-7 into a Non-Melanin Producing Strain

The activity spots of M-7 in the four solvent systems were normally associated with the pigment spots when chromatographed and bioautographed. It was necessary, then, to develop M-7 into a non-melanin producing strain and see whether it would still be antimicrobially active, and if so, whether the antibiotic will run with the same $R_f$ values in the four solvent systems used for the systematic analysis of the M-7 melanin producing strain.
Isolates were obtained in which the ability to produce melanin pigment had been lost, but in the majority of them, the ability to produce antibiotic had also been lost. In some of those that were still able to produce antibiotics, the ability to produce a sizeable amount of antibiotics had been drastically reduced. This may be interpreted to mean that the ability to carry out the secondary metabolism which results in antibiotic production is tightly linked with pigment production in the M-7 isolate. Scribner et al. (47) had shown that the pigment associated with sporogenesis in *Streptomyces venezuelae* induced sporulation on vegetative hyphae of the same organism several hours before the sporogenesis started without the addition of pigment. A similar possibility was demonstrated for the melanoid-like pigment and spore ribosomes of *Streptomyces granaticolor* (36). The complexing of the pigment with sporal ribosomes appeared to be sufficiently stable, and complexes were not dissociated by treatment with ammonium chloride solutions or other routine purification procedures. This study shows that the pigment was not actually the antibiotic, but very importantly, the pigment may play some regulatory role in metabolic changes which lead to the production of the antibiotic. Figure 8 (upper) shows the original M-7 isolate and the genetic variant subjected to chromogenicity testing in peptone-yeast extract-iron agar (PYIA) it is obvious that the genetic variant has totally lost the ability to produce
Figure 8. Chromogenicity and the melanin production test of M-7 and its genetic variant in PYIA medium (upper picture) and using L-DOPA as a substrate (lower picture). For the melanin testing, the reaction mixture consisted of 2 volumes of the culture medium, 2 volumes of 0.1 M phosphate buffer at pH 5.9, and 1 volume of 0.4% L-DOPA.
Figure 8
the brown pigment, while the original M-7 is elaborating melanin into the medium (dark-brown pigment). Figure 8 (lower picture) shows M-7 and its genetic variant being subjected to the specific melanin production test using L-DOPA as the substrate. It can also be seen that the original M-7 still expresses this characteristic, whereas the genetic variant had lost this ability.

The antibiotics produced by both organisms were extracted and subjected to chromatography in the four solvent systems. Figure 9 shows the results of this chromatography assay. It can be seen that the chromogenicity did not in any way influence the $R_f$ values, although the antimicrobial activity of the M-7 genetic variant was by far less than the original isolate.

**G. $R_f$ Values of Antibiotic of the N-156 Isolate Extracted from Overlay Plate Compared to the Shake Flask Extract**

The antibiotic extract of N-156 was subjected to paper chromatography and subsequent bioautography to determine a suitable time for exposure of the chromatograms to the test organism during bioautography. Figure 10 shows the results of the bioautography of the extract from the N-156 overlay plate in Figure 4 (2 days). The test organism is *Bacillus subtilis*. From left to right, the chromatography strips were removed from the agar surface at the end of 15, 30, and 90 min. There appears to be little difference between 30 and 90 min;
Figure 9. Chromatography of antibiotics extracted from the overlay plates of M-7 (melanin producing strain) and its genetic variant (non-melanin producing). Extraction was done with 50% aqueous acetone (75 ml.), lyophilized to dryness and resuspended in 50% aqueous acetone (5 ml.) and 0.02 ml was assayed. (Distilled water = 1, butanol saturated with water = 2, ethyl acetate saturated with water = 3, benzene saturated with water = 4).
SOLVENT SYSTEMS

$M-7$ Melanin producing

$M-7$ Genetic variant
Figure 10. Bioautography of the extract of N-156 overlaid on day 2 and extracted on day 4. The solvent was benzene, and the chromatographic development was ascending. Each chromatogram received 0.02 ml. of the extract. Beginning from left to right, the chromatograms were removed at the end of 15, 30, and 90 min.
hence 30 min was found to be the most suitable time for allowing the chromatograms to stay on the agar against the test organism before removal. The solvent used in Figure 10 is benzene.

The extract from the modified overlay plate and the shake flask were compared by paper chromatography and subsequent bioautography to establish its class identity. Figure 11 shows the results of both chromatography assays. It can be seen that the extract from the modified overlay technique is as good as that from the shake flask method, which is the procedure routinely used for the paper chromatographic characterization of the antimicrobial agents. Both samples demonstrated that the isolate produces an antibiotic substance belonging to class IIIa according to Betina (6). Consequently modified overlay method was used for the production of antibiotics for further characterizations using all the isolates.

H. Paper Chromatographic Characterization of Antibiotics Produced by Streptomyces Isolates

It was not difficult to demonstrate the antimicrobial activity of extracts from overlay plates which showed inhibition of type 3 in which zones of inhibition increased steadily and markedly with time of assay. It was, however, difficult to characterize all of them because of the larger concentration needed to do this. Of the 75 isolates from the A-horizon of the three soil series, antibiotics from only 38 could be characterized. This is not surprising, because during paper chromatography, as the compounds move along with solvents, they are normally dispersed. This in turn causes a
Figure 11. Comparison of chromatography of the modified overlay extract (a) and the shake flask extract (b) of the N-156 isolate in the four principal solvent systems. (Distilled water = 1, butanol saturated with water = 2, ethyl acetate saturated with water = 3, benzene saturated with water = 4).
dilution effect and if the agent is diluted beyond its critical concentration necessary to inhibit the test organism, the bioautography will show no recognizable inhibition zones. With few of the extracts which were difficult to detect originally by bioautography, incubating the plates at 37 C for 5 hr and removing the plates to incubate at 25 C for 24 hr helped reveal some of the spots. Of the 75 isolates which the antimicrobial activities of their extracts could be demonstrated by disc assay, antibiotics from 18 out of 22 isolates active against gram-positive organisms could be characterized. For the isolates active against gram-negative bacteria, it was 2 out of 4, 11 out of 27 for fungi, and 7 out of 22 for isolates active against bacteria and fungi.

Several other solvents were tried to see whether the concentration of the antibiotic in the extract could be increased so as to ease the problem of characterization, but these were not successful.

It is not surprising that most of the antibiotics that could not be extracted in large enough quantities to use for chromatographic analysis and bioautographic detection belonged to the antifungal group. There is evidence to show that, for the actinomycetes producing the water insoluble antibiotics of the polyene family (mostly antifungal antibiotics), submerged cultures of these actinomycetes accumulate antibiotics in the form of crystals located at the cell surface or in the medium (26, 58) or in granules within
the hyphae as in flavofungin-type of polyene (25, 42). These supramolecular structures will be very difficult to extract totally into aqueous phase from agar plates since they will tend to sediment with the agar during centrifugation. The fermentation medium devised for the production of polyenes almost invariable includes oils, fats, insoluble particles, or compounds likely to provide heterophaseic conditions inductive for the formation of supramolecular structures of a miscellar nature (18). The agar may provide just such a medium, and extraction of a sizeable amount of the antibiotic into the aqueous phase will require a solvent in which the antibiotic is very soluble. This may be a possible reason why most of the antifungal antibiotics were not extracted in sufficient quantities for chromatographic analysis; even though enough was always extracted to show inhibitory effects in aqueous form.

Figure 12 shows a typical chromatographic analysis of an antibiotic produced by D-140 (an isolate from the Dixonville series, A-horizon). This antibiotic belongs to class IIIb according to Betina (6). Fifty-one antibiotics were found in the crude concentrates prepared from the cultivation medium of the 38 isolates of Streptomyces. Paper chromatographic studies showed that 13 isolates produced two antibiotics, and 25 isolates produced one antibiotic. Classification of the antibiotics according to their classes
Figure 12. Bioautographic detection of antibiotics on paper chromatograms. The extract was from the modified overlay plate of D-140. It was extracted with 100% acetone (75 ml) and, after lyophilization, it was redissolved in 80% aqueous acetone (5 ml) and 0.02 ml assayed. The lines at the bottom indicate where the extracts were spotted and the lines at the top represent where the solvent front was stopped (a = distilled water, b = n-butanol saturated with water, c = ethyl acetate saturated with water and d = benzene saturated with water).
in the three soil series is given in Table 4. It can be seen that no new classes or subclasses were found, and the subclasses were evenly distributed in the three soil series.

The classification of the antibiotics according to their activity and chromatographic behavior is given in Table 5. It is apparent from the data that most of the antibacterial antibiotics belong to class I through III in all the three soil series, while the antifungal agents belong to class IV and V. This means that the antibacterial antibiotics are more hydrophilic than the antifungal antibiotics. This is not surprising when we consider the mode of action of the polyene antibiotics, for example, nystatin, amphotericin B, and candididin. They display selective toxicity towards organisms whose membranes contain sterols. Lampden (28) has suggested that this group of antibiotics destroys the selective permeability properties of a wide variety of fungi and other eukaryotic organisms (membranes that contain sterols). Hammond and Kliger (19) demonstrated that polyene antibiotic candididin produces a rapid efflux of $K^+$ ions from a suspension of Candida albicans. For a polyene antibiotic to interact effectively with the sterols of the fungal and yeast cell walls, the bulk of the molecule must be hydrophobic.

All of the 7 (out of the 22) isolates which produced characterizable antifungal and antibacterial antibiotics simultaneously, produced different antibiotics with different spectra of activity. One
Table 4. Classification of 51 diffusible antibiotics produced by 38 *Streptomyces* isolates from the A-horizon of three acidic soil series.

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Table 5. Classification of 51 diffusible antibiotics produced by 38 *Streptomyces* isolates according to their activities and systematic chromatographic analysis with respect to three acidic soil series: Marty, Nekia, and Dixonville.

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TOTAL
type was specific solely for gram-negatives and gram-positives, and the other produced by the same organism was specific solely for fungi and yeasts. None were characterizable which possessed activity against bacteria and fungi simultaneously.
V. SUMMARY

The development of a method which successfully reduces the problems of discriminating between true inhibition by an antibiotic substance and inhibition due to other artifacts such as nutrient deprivation and the effect of pH alterations has been reported. This new method simultaneously increases the sensitivity of detecting antimicrobial activities without preliminary concentration of the antibiotic in the growth medium. The method was used in an investigation of acidoduric populations of *Streptomyces* inhabiting the A-horizons of three different soil series located in western Oregon.

The modified overlay method developed in these studies allowed the detection of antibiotic production within 3 days for all the active isolates examined.

Antibiotics with antibacterial and antifungal activities in crude concentrations from the cultivation medium of 38 isolates of soil *Streptomyces* were classified by the systematic paper chromatographic analysis. The data on the chromatographic classification and on antimicrobial activity of 51 antibiotics found in the concentrates were presented.

The modified overlay technique combines the features of the older, unmodified overlay technique and the shake flask method in that one can detect antimicrobial activity without preliminary concentration of the antibiotic in the growth medium which is similar
to the unmodified overlay, and one can extract enough quantity from the agar medium to use for chromatographic analysis as done in the shake flask method.
BIBLIOGRAPHY


