EXTRACELLULAR COMPOUNDS HAVING ANTIBACTERIAL PROPERTIES PRODUCED BY THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA*

By

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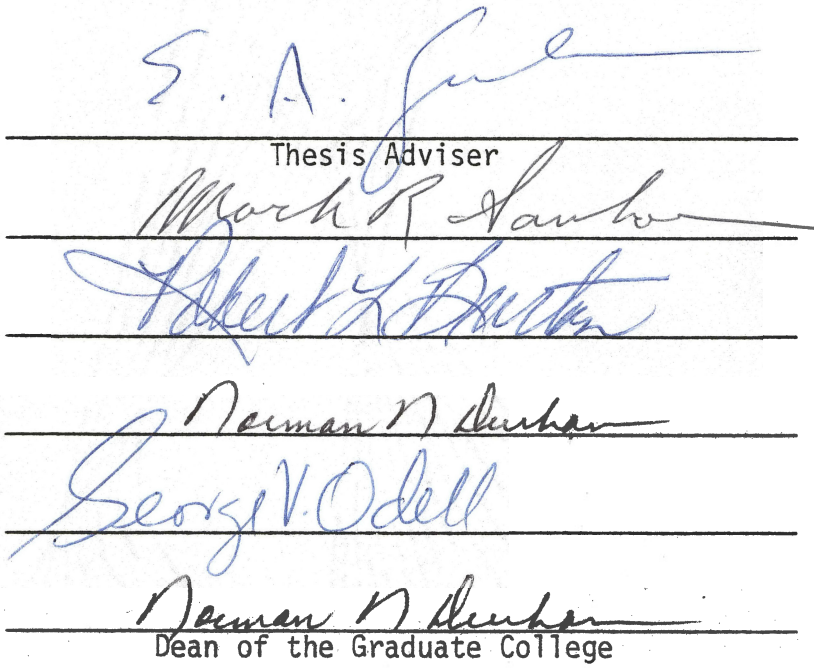

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CHAPTER I

INTRODUCTION

Work in this laboratory has included a study of the biochemical events involved in the mechanism of entomopathogenicity of the white muscardine fungus *Beauveria bassiana*, a member of the class Deuteromycetes. Various ultraviolet-induced mutants differing in their abilities to produce extracellular proteolytic enzymes have been selected and utilized in the investigation (Grula et al., 1978). It was observed early in the course of the study that the growth of bacterial colonies would often be inhibited in areas contiguous with colonies of *B. bassiana* on agar plates. This initial observation prompted an ancillary investigation into the production and mechanism of the extracellular antibiosis of *B. bassiana*.

A search through the existing literature pertaining to *B. bassiana* revealed that the ability of the fungus to inhibit the growth of other micro-organisms has been realized for many years. It has been reported that over 2000 years ago the Chinese used mummified muscardine silkworms as drugs and practiced a custom wherein precious stones bearing the figures of silkworms were placed in the mouths of the dead with hopes of preventing putrification and initiating revival (Kobayasi, 1977).

Several references to the antibiotic-producing potential of *B. bassiana* have been made in recent years. The observations that mycotic

silkworms *(Bombyx mori)* infected by *B. bassiana* do not become soft and black upon death (Kodaira, 1961) and that they are mummified and remain unputrified (Kobayasi, 1977) suggest that the fungus does produce one or more antibiotic factors which inhibit the secondary proliferation of digestive tract-inhabiting bacteria. A similar conclusion was reached by Walstad et al. (1970) after finding pure fungal growth on moribund pales weevil larvae *(Hylobius pales)* in the field whereas weevils dead from other diseases were usually found to be infected with a variety of secondary fungi. They went on to show that *B. bassiana* inhibits saprophytic fungi *in vitro* by the excretion of toxic material on agar plates.

In addition to the reports of antibiotic activity exhibited by *B. bassiana,* a number of investigators have demonstrated that the organism also produces several insecticidal toxins. Dresner (1950) was the first to propose that *in vivo* toxin production might be involved in entomopathogenesis. He found that representatives from three different insect genera are paralyzed upon exposure to germinating spores in much less time than it takes for hyphal penetration into the body cavity as determined using histological techniques. Furthermore, the fungus produced the same paralytic effect even after germinating spores had been treated with a copper sulfate solution or autoclaved. Kodaira (1961) reported that culture filtrates of *B. bassiana* grown in an artificial medium consisting of beef extract and peptone are toxic to silkworm larvae upon direct injection. The production of the toxic factor is dependent upon the presence of an organic nitrogen source in the medium and independent of the carbon source. West and Briggs (1968) later showed that *in vitro* toxin production by various *B. bassiana* isolates could be assayed by the injection of culture filtrates into

the haemocoels of greater wax moth larvae *(Galleria mellonella)*. Isolates from infected insects exhibited higher toxin titers than did isolates which had been repeatedly subcultured on artificial media. The toxin first appears in the medium at the time of conidia formation and there is a gradual decrease in toxin production with age subsequent to the peak titer. This decrease was correlated with mycelial degeneration leading the authors to conclude that if the fungus produces the same toxins within the insect as in the artificial medium, it could be expected to elaborate them from intact hyphae during the early stages of infection.

The first *B. bassiana*-produced toxins to be characterized were determined to be extracellular proteases which are insecticidal upon injection into *G. mellonella* larvae (Kucera and Samsinakova, 1968). Two toxic fractions were obtained by ammonium sulfate precipitation of culture fluids followed by Sephadex gel filtration chromatography. The first toxic fraction contained a high molecular weight protein having an acidic pH optimum while the second protein was of a lower molecular weight and optimally active at an alkaline pH. The authors hypothesized that both fractions exhibited toxic effects by either damaging certain principal functions of the haemolymph or by producing toxic by-products within the insect.

More recently, a methanol-extractable toxin has been isolated from the mycelia of *B. bassiana* and designated bassianolide (Suzuki et al., 1977). The compound was determined to be a cyclic octadepsipeptide ionophore consisting of four residues each of L-N-methylleucine and D-α-hydroxyisovaleric acid. Murakoshi et al. (1978) found that the purified bassianolide is responsible for the atonicity (muscular relaxation)

and death observed when silkworm pupae *(B. mori)* are fed an artificial diet containing a powder prepared from moribund silkworms. Kanaoka et al. (1978) reported that the oral administration of artificial diet containing bassianolide at a concentration of 4 ppm would induce the atonic symptom in fourth instar silkworm larvae while dosages of 8 ppm or greater are lethal. The direct intrahaemocoelic injection of 2 µg per larva will also induce atony with injections of 5 µg per larva being lethal. The same authors also studied the related compound beauvericin (discussed below) and found it to be nontoxic to silkworm larvae when administered orally at concentrations of up to 1000 ppm and when up to 100 µg per larva were injected.

Work in our own laboratory has provided evidence for the fact that bassianolide is significantly less toxic to the corn earworm *(Heliothis zea)*. Intrahaemocoelic injections of up to 6 µg per fourth instar larva produced an atonic condition (similar to that reported in the silkworm) from which recovery appears to be complete within 48 to 72 hours (Champlin and Grula, 1979).

Although both the *in vivo* and *in vitro* antibiotic activities of *B. bassiana* have been alluded to in the literature for many years, only two compounds exhibiting antibiotic activity have been isolated and fully characterized to date.

The first antibiotic to be isolated was a deep red pigment which was found to be present in submerged culture filtrates under certain growth conditions by Vining et al. (1962). They were able to obtain the compound in a pure form by lowering the pH of the filtrates and extracting with ethyl acetate. The extract was then crystallized from methanol yielding a copper-red substance which was analyzed in a paper

chromatography system and shown to contain a single red pigment. Ultraviolet, visible and infrared absorption spectra and melting point data were used to identify the pigment as oosporein, a dibenzoquinone pigment known to be produced by several different fungi. In addition, they utilized a nutrient agar disc assay procedure to demonstrate an antibiotic activity for oosporein. Low concentrations of the compound caused the inhibition of growth by *Staphylococcus aureus, Bacillus subtilis* and *Proteus vulgaris*; significantly higher concentrations were necessary to inhibit the growth of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

A paper dealing with the biosynthesis of oosporein reported it to be the product of the dimerization of a benzoid compound which is derived from the condensation of acetic and malonic acids (Basyouni and Vining, 1966). A later study showed the production of oosporein to not be a universal characteristic of all *B. bassiana* strains nor is it necessarily constant within one strain (Basyouni et al., 1968). Only 5 of 16 strains tested synthesized oosporein and only then in certain media containing peptone. All but one of the producing strains lost the ability when repeatedly subcultured on artificial media. When 13 monospore cultures were transfered from the one parent strain which retained the ability to synthesize the compound, only two continued to produce oosporein and one of these again lost the ability upon repeated transfer.

Beauvericin, the second antibiotic known to be produced by *B. bassiana,* has been more extensively studied. Hamill et al. (1969) first isolated beauvericin from mycelia using a methanol extraction procedure and reported it to be a cyclic hexadepsipeptide ionophore consisting of three residues each of D-α-hydroxyisovaleric acid and N-methyl-L-

phenylalanine in alternating sequence. This structure is quite similar to the enniatins, a family of neutral ionophoric antibiotics differing only in the nature of the N-methylamino acids (Ovchinnikov et al., 1971). Beauvericin has been synthesized by two independent laboratories (Ovchinnikov et al., 1971 and Roeske et al., 1974) and has been shown to form lipophilic complexes with a broad spectrum of both mono- and divalent cations (Roeske et al., 1974). This is in contrast with the enniatins, which bind only monovalent cations (Hamilton et al., 1975), and valinomycin, also a neutral depsipeptide ionophore, which is able to bind only potassium, rubidium and cesium (Pressman, 1976). The ability of beauvericin to first form complexes with various cations, then to dissolve into hydrophobic domains allows the molecule to render biological membranes cation permeable. Cation translocation by beauvericin has been studied by several investigators using black lipid films, mitochondrial membranes, phospholipid liposomes and bacterial chromatophores (Estrada-O et al., 1972; Prince et al., 1974; and Yafuso et al., 1974). Dorschner and Lardy (1969) demonstrated that the ability to transport alkali metal cations into mammalian mitochondria enables beauvericin to uncouple oxidative phosphorylation albeit less potent than valinomycin. In addition, beauvericin has been reported to alter nuclear morphology and inhibit migration of insect cardiac cells in tissue culture (Vey et al., 1973) and to induce the aggregation of human blood platelets with the concomitant release of serotonin and nucleotides while stimulating the uptake of calcium (Massini and Naf, 1980).

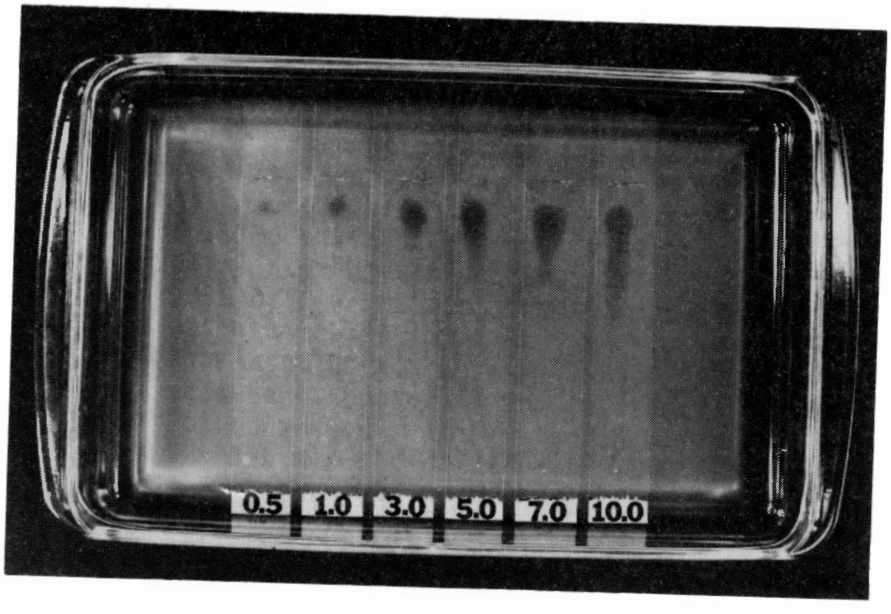
We found that beauvericin completely inhibited the growth of seven different Gram-positive bacteria when small squares of Whatman number 1 chromatography paper, each containing a 50 µg deposition of the compound,

were applied to seeded agar plates (Champlin and Grula, unpublished data). Five Gram-negative bacteria examined showed no sensitivity while *Escherichia coli* was only slightly inhibited. These results agree with observations by Hamill et al. (1969), who reported beauvericin to be moderately toxic to Gram-positive bacteria, and Ovchinnikov et al. (1971), who reported *Staphylococcus aureus* to be about five times more sensitive to the antibiotic than *E. coli*.

Although beauvericin has been shown to be toxic to mosquito larvae (Hamill et al., 1969), the literature contains no evidence either implicating or ruling out its involvement in the entomopathogenic process of *B. bassiana* in members of the insect order Lepidoptera. To study this question, we exploited the antibiotic nature of the molecule by developing a bioautographic assay for the detection of beauvericin (Figure 1) in order to assess its possible involvement in the muscardine infection of *H. zea* (Champlin and Grula, 1979). The absence of detectable amounts of beauvericin in both culture filtrates and haemolymph from moribund larvae, coupled with the finding that the direct intrahaemocoelic injection of the synthetic compound into fourth instar larvae exerts no deleterious effect, led us to conclude that synthesis of the antibiotic is not directly involved in the pathogenic process.

A third antibiotic activity has been attributed to *B. bassiana* when grown in a medium containing an organic nitrogen source by Kodaira (1961). He describes a partial purification scheme utilizing charcoal adsorption and aluminum oxide adsorption chromatography to extract the antibiotic from culture filtrates. A crude powder was obtained which is soluble in water but insoluble in organic solvents. The powder inhibited the growth of *Staphylococcus aureus* even after a 320,000-fold dilution yet there

Figure 1. Bioautographic Assay for Beauvericin. Zones of bacterial growth inhibition can be observed at R_f 0.90 due to the presence of various concentrations of synthetic beauvericin after development in a paper chromatography system. Assay bacterium is a member of the genus *Bacillus* which was isolated from soil and determined to be extremely sensitive to the antibiotic. Values are given in µg of beauvericin.


0.5
1.0
3.0
5.0
7.0
10.0

has been no apparent effort made to further purify and characterize the antibiotic factor.

With the use of paper chromatography and bioautography, we have observed two different antibiotic factors in culture filtrates of several *B. bassiana* strains. Neither can be attributed to either beauvericin, since it is retained in the fungal membrane (Hamill et al., 1969), or oosporein, which is synthesized by only a few strains when grown in special media and is visible as a deep red pigment in culture fluids (Vining et al., 1962). None of the strains studied in our laboratory exhibit such extracellular pigment formation. Since we are unable to extract any antibiotic activity from our culture filtrates using the purification scheme reported by Kodaira (1961), we are presumably observing antibiotics other than that produced by Kodaira's strain.

In this study, we have sought an understanding of the molecular basis for the extracellular antibiotic activity exhibited by the *B. bassiana* mutants utilized in our laboratory to study entomopathogenesis. A 3-fold investigation was undertaken to accomplish this goal. Initially, experiments were devised to define the cultural requirements of the fungus with respect to antibiotic production in order to establish the conditions under which the antibiotics could be maximally produced. Secondly, we attempted to purify the antibiotics so that their molecular composition could be elucidated. Lastly, we investigated the mechanisms of their inhibitory effects upon the growth of sensitive bacteria.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The *B. bassiana* strain utilized to produce the antibiotic factors under study was selected from a group of 15 mutants on the basis of its culture filtrates (Sabouraud dextrose broth; Difco Laboratories) having the greatest amount of antibiotic activity as determined by disc antibiotic assay. The mutants were obtained originally by the ultraviolet irradiation of wild type conidiospore suspensions followed by the selection of colonies exhibiting varying degrees of extracellular proteolytic enzyme activity when plated on Sabouraud dextrose agar (SDA; Difco Laboratories) containing casein (Grula et al., 1978). The high antibiotic-producing mutant had been assigned the designation strain number 7 (S-7).

Stock cultures were grown and maintained on SDA slants supplemented with 0.3% yeast extract (Difco Laboratories). The fungus was transferred every 2 weeks and incubated at 25^{0}C until complete sporulation had occurred (about 14 days) after which it was stored at 2 to 4^{0}C. Conidiospores from 14 to 21 day old cultures were used as inocula for various experiments.

Assay Organism

The bacterium utilized in both the disc antibiotic and bioautographic

assays for antibiotic activity, as well as in studies of the mechanisms of action of the antibiotics on growing and resting cells, was an aerobic, Gram-positive, spore-forming rod belonging to the genus *Bacillus*. The organism had been isolated previously by Dr. E.A. Grula and was selected for further research from a large group of soil bacteria on the basis of its being judged most sensitive when cross-inoculated against *B. bassiana* on SDA plates. The morphological and biochemical characteristics of the bacterium (Table I) precluded its being assigned a species classification based upon the descriptions given in either the seventh or eighth editions of Bergey's Manual of Determinative Bacteriology (Smith and Gordon, 1957 and Gibson and Gordon, 1974). In addition to the extracellular antibiotics produced by *B. bassiana* S-7, Champlin and Grula (1979) showed the *Bacillus* sp. to also be sensitive to beauvericin, but not to bassianolide.

Cultures of the *Bacillus* sp. were grown on tryptic soy agar (TSA; Difco Laboratories) slants at 25^{0}C and maintained on the same medium at 2 to 4^{0}C. Cells from 12 to 18 hour old cultures were used to inoculate the defined bacterial growth medium used in the mechanistic studies and to seed SDA plates for use in both the disc antibiotic and bioautographic assays.

Growth Conditions and Media for Antibiotic Production

Several different growth media were used at different times in the course of this study for the production of extracellular antibiotics by *B. bassiana* S-7 in submerged cultures. Cultures in all cases were grown in 250 ml Erlenmeyer flasks containing 100 ml of the indicated

TABLE I

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE *Bacillus* sp. USED IN THE BIOAUTOGRAPHIC ASSAY

Parameter[a]	Results
Gram stain	Positive, stains unevenly
Motility	+
Spore formation[b]	Terminal, oval, cell distended
Acid production with glucose	-
Nitrate reduction to nitrite	-
Starch hydrolysis	+
Tributyrin hydrolysis	+
Gelatin hydrolysis	+
Catalase production	+
Urease production	-
Growth with citrate	+
Growth with allantoin	-
Indole	-
Methyl red	-
Voges-Proskauer	-
Litmus milk[c]	-
Chromogenesis	Pale yellow (water insoluble)
Growth in Sabouraud dextrose broth	+
Lipid vacuoles	Numerous (Sudan black B)
Volutin granules	Numerous (Lindegren's stain)

[a]Biochemical tests were read after incubation for 24 hrs. at 25°C with the exception of litmus milk.
[b]Spore formation occurred by 36 hrs. on tryptic soy agar (Difco) at 25°C.
[c]There was no detectable change in pH or curd formation in litmus milk within 72 hrs.

growth medium and all inocula consisted on one loopful of 14 to 21 day old conidiospores from refrigerated stock cultures. The flasks were incubated for various times at 25^{0}C on a gyrotory shaking apparatus (New Brunswick Scientific Company, Model V) having a rotational speed of 180 rpm.

Sabouraud fructose broth (SFB), a complex medium containing fructose in place of dextrose found in commercially prepared Sabouraud dextrose broth, was utilized initially for the production of the antibiotic factors because greater amounts were produced on fructose than on dextrose. The medium contained D-fructose (4 g) and Difco Neopeptone (1 g) per 100 ml of distilled water and was sterilized by autoclaving for 15 minutes at 250^{0}F after which the pH was adjusted to 7.5 with filter-sterilized 1 N NaOH.

Antibiotics were also produced using Neopeptone-citrate broth (NCB), a growth medium which afforded a buffering capacity sufficient to partially preserve the initial pH conditions in the presence of the large amount of acidic products produced by the fungus when grown with Neopeptone as the source of carbon, nitrogen and energy. The medium contained the following components per 100 ml total volume: Neopeptone (1 g), trisodium citrate (5.16 g to yield a final concentration of 0.2 M; Sigma Chemical Company), 20 ml of trace minerals solution (Grula, 1960) and 80 ml of glass-distilled water. The pH of the medium was adjusted to 8.5 with 1 N NaOH prior to autoclaving for 15 minutes at 250^{0}F.

Acid-hydrolyzed casein broth (CASE) contained the following components per 100 ml total volume: acid-hydrolyzed casein (0.5 g; Sigma Chemical Company), trisodium citrate (2.58 g to yield a final

concentration of 0.1 M; Sigma Chemical Company), $MgSO_4 \cdot 7H_2O$ (3 mg in 3 ml of glass-distilled water), K_2HPO_4 and KH_2PO_4 (174 and 136 mg respectively in 20 ml of glass-distilled water), 20 ml of trace minerals solution (Grula, 1960) and 57 ml of glass-distilled water. The pH of the medium was approximately 6.95 after autoclaving for 15 minutes at 250^0F.

A similar growth medium containing glucose, NH_4Cl, trisodium citrate and only three amino acids (GAC defined medium) was utilized to produce antibiotics under chemically-defined conditions in order to facilitate biosynthetic, purification and chemical characterization studies. This is possible since a reduced number of amino acids are present in the medium to begin with. The GAC medium contained the following components per 100 ml total volume: D-glucose (1 g in 10 ml of glass-distilled water), NH_4Cl (0.1 g), L-alanine (100 mg; Sigma Chemical Company), L-phenylalanine (100 mg; Sigma Chemical Company), L-valine (100 mg; Sigma Chemical Company), trisodium citrate (2.58 g to yield a final concentration of 0.1 M; Sigma Chemical Company), $MgSO_4 \cdot 7H_2O$ (3 mg in 3 ml of glass-distilled water), K_2HPO_4 and KH_2PO_4 (174 and 136 mg respectively in 20 ml of glass-distilled water), 20 ml of trace minerals solution (Grula, 1960) and 47 ml of glass-distilled water. The pH of the medium was approximately 6.85 after autoclaving the 1 g of glucose in 10 ml of water separately from the remainder of the medium (15 minutes at 250^0F) followed by the combining of the two solutions after cooling to give the total 100 ml volume. Variations of this medium having certain of the carbon, nitrogen and energy sources omitted were utilized to selectively control the synthesis of the individual antibiotics.

Studies involving the growth of and antibiotic production by *B. bassiana* S-7 with various organic substrates provided as the sole sources of carbon and energy were undertaken by adding 0.5 g of each compound singly or in combination to the NH_4Cl and basal salts and minerals of the GAC defined medium. In the cases of carboxylic acid intermediates of glycolysis and the tricarboxylic acid cycle, the compounds were first filter-sterilized in solutions having concentrations such that the desired final concentration (0.5 g/100 ml) was obtained upon addition to the autoclaved basal medium after cooling.

Preparation of Antibiotic-Containing Culture Filtrates

B. bassiana S-7 submerged cultures were cleared after the desired periods of incubation by centrifugation (Sorvall RC2-B) at 5000 rpm for 15 minutes followed by passage of the supernatants through 0.45 µm Millipore filters. The culture filtrates were then either used unconcentrated or divided into 10 ml aliquots, frozen overnight and lyophilized to dryness (Virtis Automatic Freeze-Dryer). The lyophilizates were then stored at minus 20^0C until needed at which time they were solubilized with 1 ml of glass-distilled water thereby concentrating the antibiotics by a factor of 10.

Paper Chromatography

Paper chromatography followed by bioautography revealed two different antibiotic factors to be present in culture filtrates of *B. bassiana* S-7. Sample depositions were applied to Whatman number 1 chromatography paper (8 by 8 inches square and 0.16 mm thick) and

air-dried under a blowing hair dryer at room temperature. Chromatograms were developed in a solvent system consisting of n-butanol, water and isopropanol (12.5:65:22.5) after allowing the solvent mixture to equilibrate for at least 30 minutes. Running time for the system was about 5.5 hours at 25^{0}C.

Samples were analyzed for the presence of free amino acids either one or two dimensionally on Whatman number 1 chromatography paper using the solvent systems of Heathcote and Jones (1965).

Developed chromatograms were air-dried for at least one hour and then either tested for the presence of antibiotics using bioautography or sprayed with reagents specific for the detection of certain types of compounds. Compounds containing unsubstituted amino groups, such as amino acids and peptides, were detected by spraying paper chromatograms with 0.4% ninhydrin in acetone followed by incubation for 3 minutes at 100^{0}C. Compounds capable of chelating iron, such as phosphate esters and citrate, were detected using the $FeCl_3$-sulfosalicylic acid spraying procedure of Wade and Morgan (1953). Reducing sugars were detected using the periodate-potassium permanganate spraying procedure of Lemieux and Bauer (1954).

Bioautographic Assay

Bioautography was utilized as the means for analyzing culture filtrates and various fractions obtained in the process of antibiotic purification for the presence of the individual extracellular antibiotics. It also permitted direct comparisons of Rf values exhibited by the *B. bassiana*-produced antibiotics with those of known commercially-prepared antibiotics.

Assay plates were prepared by pouring 190 ml of melted SDA (56.3 g SDA per liter rather than the 65.0 g suggested by Difco, thereby lowering the agar concentration from 1.5 to 1.3% to better facilitate antibiotic diffusion) seeded with 6 ml of the *Bacillus* sp. in water suspension (OD_{540} of 0.80 after sonicating with one short burst) into a sterile Pyrex plate (22 by 34 cm) covered with aluminum foil. Cells were dispersed by tilting the plate back and forth at least 20 times. After solidification of the agar, paper chromatograms, air-dried for 1 hour and cut into strips 3 cm wide, were laid onto the seeded agar surface and permitted to remain during the incubation of the plate at 25^0C. Zones of bacterial growth inhibition underneath specific areas of the paper chromatograms could be observed after incubation for 18 to 23 hours.

The relative migrations of both *B. bassiana*-produced antibiotics and commercially-prepared standards on developed chromatograms were determined by calculating R_f values.

Disc Antibiotic Assay

Relative amounts of total antibiotic activity present in culture filtrates and various fractions obtained in the process of antibiotic purification were determined using a disc antibiotic assay. Assay plates were prepared by pouring 12 ml of melted SDA (56.3 g SDA per liter) seeded with 0.35 ml of the *Bacillus* sp. in water suspension (OD_{540} of 0.50 after sonicating with one short burst) into a sterile pertri plate. After swirling the agar to disperse the cells and allowing the agar to solidify, sterile paper discs (13 mm in diameter; Whatman number 3 MM chromatography paper) impregnated with the various

samples were laid onto the seeded agar surface and permitted to remain during the incubation of the plates initially at 4^0C for one hour to allow antibiotic diffusion to precede bacterial growth. Zones of bacterial growth inhibition could then be observed surrounding those discs containing antibiotic after 12 to 18 hours of incubation at 25^0C. Since the diameter (mm) of the clear area surrounding any given disc is proportional to the degree of antibiotic diffusion from that disc, this method yields a quantitative measure of the antibiotic concentration initially present in the saturated disc assuming that all of the discs in an experiment absorb equal volumes and the antibiotic molecule is water-soluble and freely diffusible through the agar.

Methanol Fractionation of Antibiotics from Spent SFB

The two antibiotics present in SFB culture filtrates (final pH of 2.8 after 72 hours growth) were separated from each other using a differential methanol fractionation procedure. A 10 ml culture filtrate lyophilizate was extracted by suspending the dried material in 1 ml of absolute methanol and agitating on a Vortex mixer for 1 minute. The insoluble material was then removed by clinical centrifugation and the supernatant decanted yielding two different samples, each containing a single antibiotic factor. One ml of glass-distilled water was added to each sample thereby dissolving the methanol-insoluble pellet and diluting the methanol supernatant. To each sample was then added 1 ml of chloroform followed by vortex agitation for 1 minute. After the two phases had been allowed to separate (about 10 minutes), the two antibiotics could then be detected in their respective water layers by either

disc antibiotic assay or paper chromatography followed by bioautography.

Acetone Precipitation of Antibiotics from Spent NCB

A partial purification of both antibiotics directly from spent NCB was achieved by precipitation with acetone. Two volumes of reagent grade acetone were added to one volume of culture filtrate (final pH of 5.5 after 96 hours growth) at room temperature with constant magnetic stirring bar agitation. The resulting heavy white precipitate was collected by centrifugation (RC2-B for 15 minutes at 5000 rpm) and washed one time with ½ volume of absolute methanol. The precipitate was again pelleted and resuspended in ½ volume of glass-distilled water with constant magnetic stirring bar agitation for 30 minutes. The remaining insoluble material was removed by centrifugation leaving the water supernatant containing the extracted antibiotics. Chromatographic alumina (Sigma Chemical Company) was added to the water extract at a 2% concentration and adsorption was allowed to proceed for 15 minutes at room temperature with constant magnetic stirring bar agitation. The alumina was then removed by centrifugation leaving the water extract which was divided into 10 ml aliquots, frozen overnight and lyophilized to dryness (Virtis Automatic Freeze-Dryer). The lyophilizate consisted of a white powder which was stored at minus 20^0C until needed, at which time each 10 ml lyophilizate was solubilized with 1 ml of glass-distilled water thereby further concentrating the antibiotics by a factor of 10.

n-Butanol Extraction of Migrating Antibiotic from Spent Glucose-NH_4Cl-Citrate Defined Medium Culture Filtrates

The migrating antibiotic was extracted from spent glucose-NH_4Cl-citrate defined medium culture filtrate lyophilizates using a water-n-butanol biphasic partitioning procedure. A 50 ml lyophilizate was dissolved in 5 ml of glass-distilled water (thereby concentrating by a factor of 10). The pH was adjusted from 6.4 to about 2.5 with 3 N HCl in a 50 ml conical glass-stoppered tube to which 25 ml of n-butanol were added. The mixture was agitated by repeatedly inverting the tube for one minute at room temperature. After allowing the immiscible phases to separate completely, the n-butanol layer was removed and concentrated to 5 ml by evaporation under either flowing nitrogen gas or air. Five ml of glass-distilled water (pH 6.5) were added to the n-butanol extract and the mixture was again agitated by repeatedly inverting the tube for one minute at room temperature. After allowing the phases to separate, the 5 ml water layer was removed, frozen and lyophilized to dryness. The migrating antibiotic-containing lyophilizate was stored at minus 20^{0}C until needed.

Gel Filtration Chromatography

Sephadex gel filtration column chromatography of water-extracted spent glucose-NH_4Cl-citrate or amino acids defined media culture filtrate lyophilizates was used to obtain the migrating and origin antibiotics separately in desalted preparations free of all extracellular proteins. Sephadex G-25-150 gel filtration beads (particle size, 50 to 150 μm; bed volume, 4 to 6 ml per g dry gel; exclusion limit, 5000

daltons; Sigma Chemical Company) were prepared by suspending 160 g in 1600 ml of glass-distilled water. After allowing the beads to settle, the supernatant was decanted and the beads resuspended in an additional 1600 ml of water prior to heating in an Arnold steam sterilizer for one hour. The beads were allowed to cool overnight at room temperature and then resuspended forming a slurry which was poured into a one liter Econo-Column glass reservoir (Bio-Rad Laboratories) attached to a 4 by 60 cm Pyrex chromatographic tube (Corning Glass Works). After allowing the gel to pack at a flow rate of about 1.5 ml per minute, a column having a void volume of 338 ml and a total bed volume of 653 ml was obtained. The column was loaded with samples of from 5 to 15 ml and developed by eluting with glass-distilled water at a constant flow rate of 1.2 ml per minute. An automatic fraction collector (Instrumentation Specialties Company, Model 328) was used to collect 5 ml fractions, all of which were assayed for antibiotic activity using the disc antibiotic assay. Those fractions exhibiting antibiotic activity were pooled, frozen and lyophilized to dryness after it had been determined with bioautography that only one of the antibiotics was present.

Treatment of Growing and Resting Cells with Antibiotic Preparations

Growing and resting *Bacillus* sp. cells were treated either with crude culture filtrate extracts (containing a mixture of both antibiotics) or one of the two defined media culture filtrate extracts after gel filtration chromatography (containing only one of the antibiotics) in order to determine the mechanism(s) by which the molecules inhibit bacterial growth. Cells were grown in 100 ml batch cultures in 250 ml

Erlenmeyer side-arm flasks which were incubated at 25°C on a gyrotory water bath shaker (New Brunswick Scientific Company, Model G76) having a rotational speed of 180 rpm. The growth medium utilized was a glucose-aspartic acid minimal medium (Grula, 1960) which contained the following components per 100 ml total volume: D-glucose, 150 mg; L-aspartic acid, 280 mg; K_2HPO_4, 174 mg; KH_2PO_4, 136 mg; $MgSO_4 \cdot 7H_2O$, 3 mg and a trace minerals solution. The inoculum was prepared by suspending a 12 to 18 hour TSA slant culture of the bacterium in 3 ml of a sterile physiological saline solution (PSS). The cells were then pelleted in a clinical centrifuge, washed one time in 10 ml of PSS and resuspended in 3 ml of PSS. This suspension was then aseptically added in a drop-wise manner to 5 ml of sterile PSS until an OD_{540} of 0.30 was reached. Each flask was inoculated with 0.1 ml of the final washed cell suspension.

Once middle log phase growth had been obtained, 5 ml culture aliquots were transferred to sterile 18 by 150 mm Kimax culture tubes. The cultures were treated with appropriate antibiotic preps and incubation was resumed. The subsequent effects upon cell growth and morphology were monitored spectrophotometrically (Bausch and Lomb Spectronic 20) and visually (Nikon phase-contrast light microscope) at 30 minute intervals.

To obtain resting state cultures, middle log phase cells were harvested by centrifugation for 10 minutes at 5000 rpm (Sorvall RC2-B) and washed twice in 100 ml of the basal salts and minerals of the growth medium. The initial cell density was then restored by diluting the cells with the basal salts and minerals and 5 ml aliquots were transferred to 18 by 150 mm tubes, appropriate treatments were added and incubation was resumed. This procedure causes the endogenous carbon, nitrogen and

energy reserves to become depleted and a state of nongrowth results. The resting cultures were then monitored spectrophotometrically and visually at 30 minute intervals.

Phospholipid Analysis

Lipids were extracted from *Bacillus* sp. whole cells using the method of Folch et al. (1957) in order to determine qualitatively the major phospholipid components of the cell membrane.

Thin-layer chromatography of phospholipid-containing extracts in chloroform-methanol (2:1) solution was performed using glass plates (20 x 20 cm square) layered with Silica Gel H (Sigma Chemical Company) which were activated at 100^{0}C for one hour and prewashed with acetone-petroleum ether (1:3) immediately prior to use. Sample depositions were made in microliter amounts and chromatograms were developed in a solvent system consisting of chloroform, methanol, acetic acid and water (80:13:8:0.3) after allowing the solvent mixture to equilibrate for at least 30 minutes. Phospholipids were detected on developed plates with a phosphate ester specific molybdate spray reagent (Dittmer and Lester, 1964). Phospholipids containing unsubstituted amino groups were detected by spraying with 0.4% ninhydrin in acetone followed by incubation of the plates for 3 minutes at 100^{0}C.

The tentative identification of the extracted phospholipids was achieved by the comparison of R_f values with those of known standards including phosphatidic acid, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin and phosphatidylcholine (all obtained from Sigma Chemical Company).

CHAPTER III

RESULTS

Production of Antibiotics

Selection of a High Antibiotic-Producing Strain of *Beauveria bassiana*

The disc antibiotic assay method using Sabouraud dextrose agar (SDA) plates seeded with the *Bacillus* sp. was chosen over the cylinder plate method for the purpose of analyzing culture filtrates of the various *B. bassiana* mutants for extracellular antibiotic activity. This agar diffusion technique affords a means by which the total amounts of antibiotic activity present in spent growth media can be determined at any given time following inoculation and comparisons of the antibiotic-producing potentials of the strains can be made. The disc method is best suited for this study because it allows for the analysis of a large number of samples to be made in a relatively short period of time. Both methods were shown to be of equal efficiency in an early study of polymyxin, a highly water-soluble antibiotic (Stansly and Schlosser, 1947).

A difficulty exists in that the assay *Bacillus* tends to come off of tryptic soy agar (TSA) slants in the form of visible sheets and clumps when water suspensions are made. This causes growth to occur unevenly in the seeded agar resulting in a situation whereby the assay

exhibits nonuniform sensitivity over the surface of any one plate. This problem is alleviated by treating the cell suspension with one short burst of sonic oscillation which breaks up the clumps and a homogenous cell suspension is thus obtained.

As can be seen in Figure 2, the diameters of the zones of bacterial growth inhibition vary with the concentration of antibiotic absorbed when assay discs are impregnated with various dilutions of a given culture filtrate sample. It has been reported that a linear relationship usually exists between the diameters of zones of inhibition and the logarithms of the concentrations of the molecule responsible for the zones for most antibiotics (Collins and Lyne, 1976). This linearity made it possible to construct a standard curve by plotting the dilution factors logarithmically against the corresponding zone of of inhibition diameters surrounding each disc (Figure 3). The expected linear relationship is observed to exist between antibiotic concentration and inhibition implying that the molecule (or molecules) present in the *B. bassiana* S-7 culture filtrate which is responsible for the inhibition is both water-soluble and freely diffusible through the agar medium. A means is then provided for the quantitative estimation of antibiotic concentrations in culture filtrates as well as for the initial detection of the antibiotic factor. The amounts of antibiotic being excreted by the various fungal strains at any given time can be compared in order to determine which strain possesses the capability for the greatest antibiotic production. It should be noted at this point that the standardization of the disc antibiotic assay does not allow for direct comparisons of the potencies of different antibiotic agents in this system because certain antibiotics, such as vancomycin

Figure 2. Standard Disc Antibiotic Assay Plate. A 10 ml lyophilizate of a spent glucose-amino acids-citrate (GAC) broth culture filtrate (after growth for 108 hours) was dissolved in 1 ml of water (thereby concentrating by a factor of 10) and a 2-fold serial dilution performed. Each disc was impregnated with a different dilution and applied to a SDA plate seeded with the assay bacterium (*Bacillus* sp.). Dilutions are indicated on the discs.

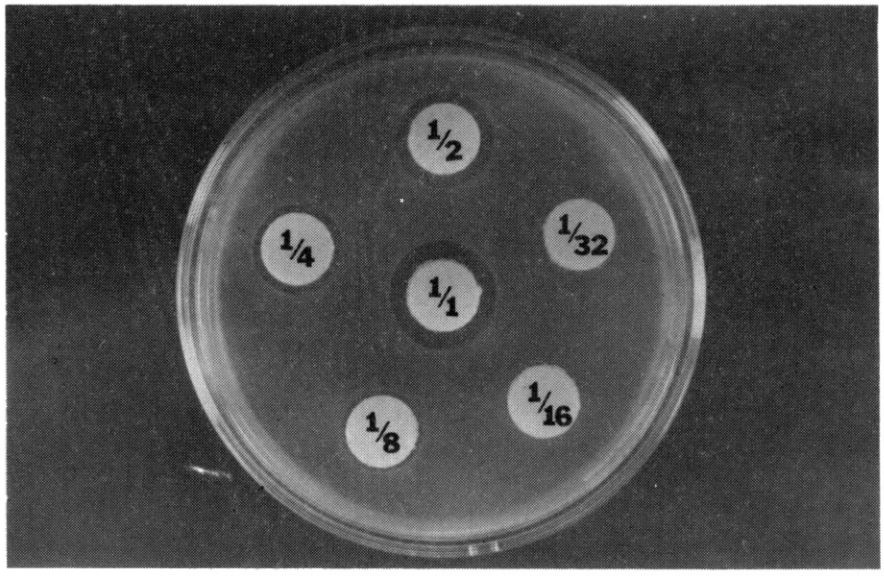
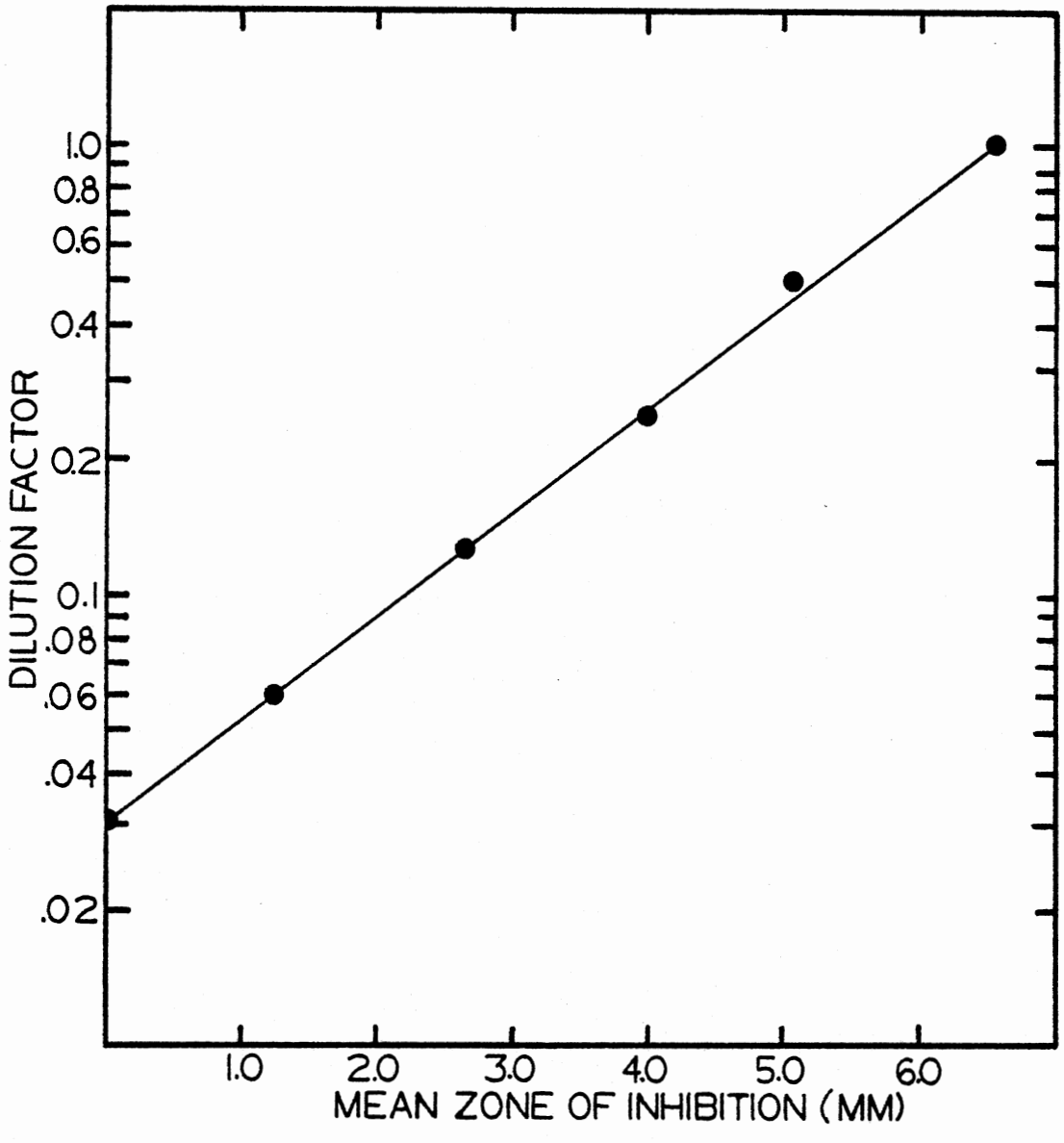

1/2
1/4
1/32
1/1
1/16
1/8

Figure 3. Disc Antibiotic Assay Standard Curve. Dilution factors are plotted logarithmically as functions of the diameters of the zones of bacterial growth inhibition minus 13 mm (see Figure 2). Each point represents the mean value in mm for the zones of inhibition observed for that dilution on three replicate plates.


DILUTION FACTOR
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
.04
.02
1.0
2.0
3.0
4.0
5.0
6.0
MEAN ZONE OF INHIBITION (MM)

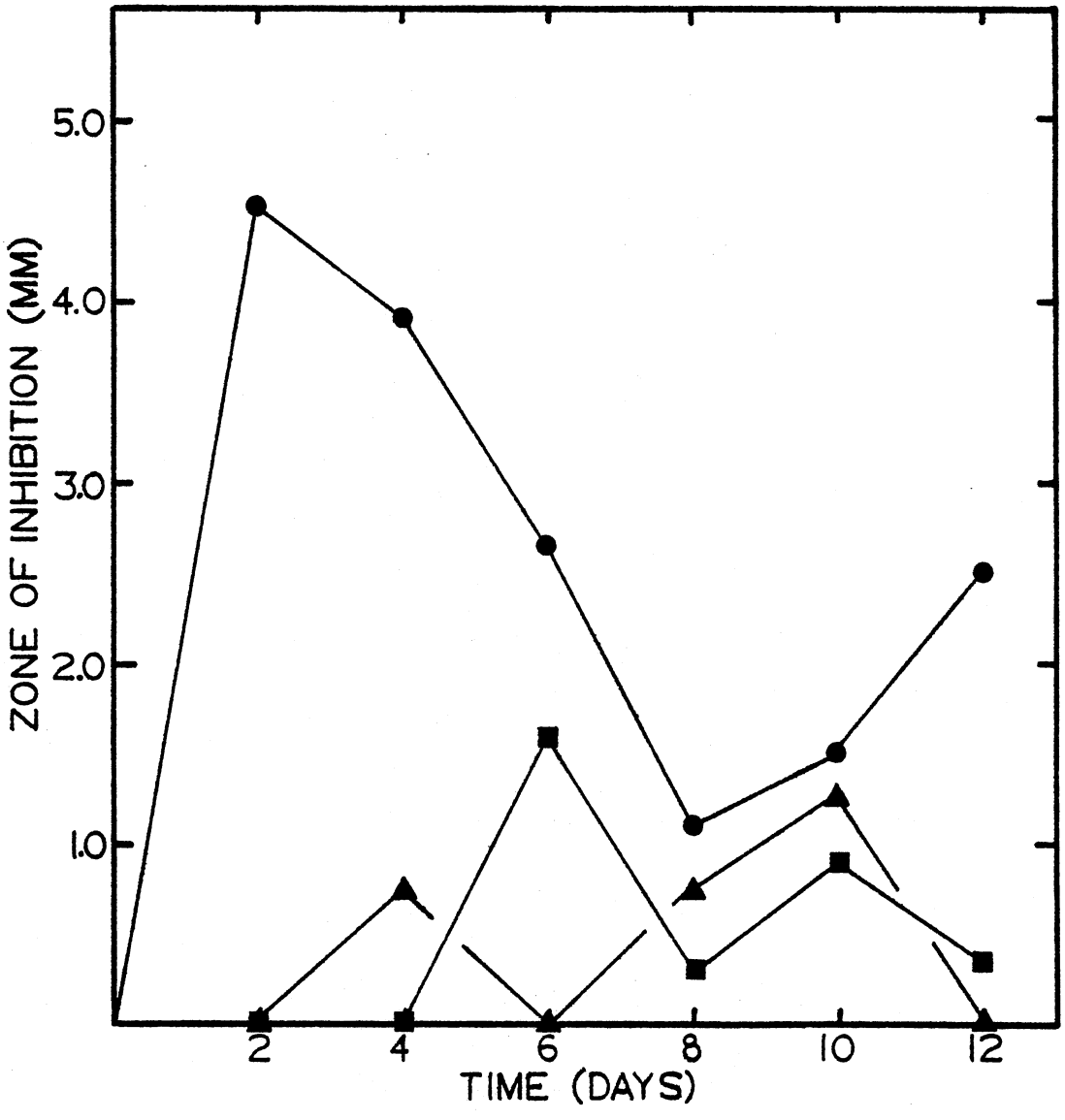
and polymyxin-B, diffuse less freely than others in any given medium (Bauer et al., 1966).

Spent Sabouraud dextrose broth (SDB; used prior to the finding that fructose could be substituted for glucose at the same concentration yielding an increase in the amount of extracellular antibiotic activity) culture filtrates of all 15 *B. bassiana* mutant strains were screened for the presence of antibiotic activity after growth for 96 hours using the disc antibiotic assay. It was determined that those strains designated as 6, 7 and 12 contain more of the antibiotic factor in their culture filtrates by 96 hours than any of the others. When these high antibiotic-producing strains are grown on SDB and the culture filtrates tested at two day intervals, it is clear that strain 7 produces the most antibiotic at the earliest time and maintains the synthesis at a higher level for a greater period of time than either strain 6 or 12 (Figure 4). For this reason, *B. bassiana* S-7 was selected as the mutant for utilization in the production of antibiotic in all further research.

Paper Chromatography and Bioautography to Study Nutritional Requirements of *B. bassiana* S-7 with Regard to Antibiotic Production

Due to the lack of previous information in the literature concerning production of extracellular antibiotics other than oosporein and the Kodaira antibiotic, several media were used to grow *B. bassiana* S-7 for the production of the antibiotic factor and then replaced by better formulations as they were found. Since important information pertaining to extracellular antibiosis was obtained with each system,

Figure 4. Antibiotic Production by the Three Best *B. bassiana* Strains. Zones of bacterial growth inhibition obtained with the disc antibiotic assay of SDB culture filtrates are expressed as the diameter of each zone of inhibition minus 13 mm plotted (in mm) against time (in days). ●, strain 7; ▲, strain 12; ■, strain 6.


5.0
4.0
3.0
2.0
1.0
ZONE OF INHIBITION (MM)
2
4
6
8
10
12
TIME (DAYS)

they are all included in this discourse in the order in which they were utilized.

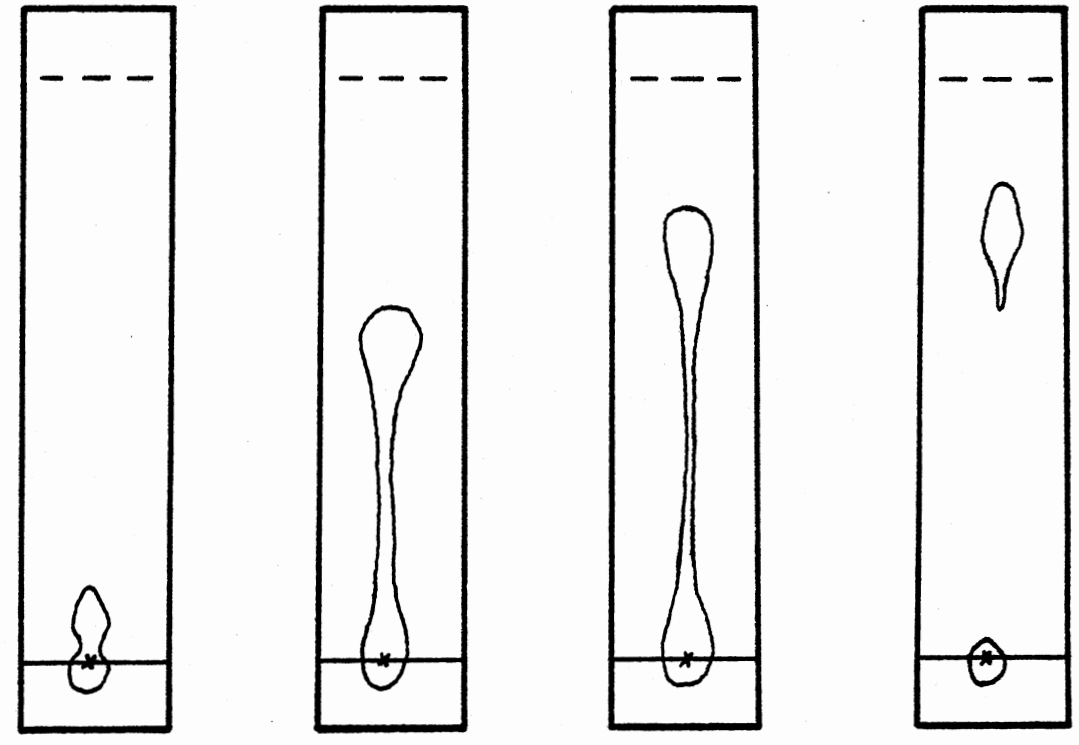
Sabouraud fructose (SFB), containing 4% D-fructose and 1% Difco Neopeptone and having an initial pH of 7.5, was used initially as the growth medium of choice for antibiotic production. It had been found that antibiotic production could be enhanced by substituting D-fructose for the D-glucose in SDB. Since a maximal amount of antibiotic was found to be present in the SDB at 48 to 96 hours of growth (see Figure 4), culture filtrates were obtained after 72 hours of growth. The pH of the medium at this time is quite low (2.80) due to the production of acid products in an unbuffered system.

In order to determine whether the observed antibiosis in the culture filtrates was due to one or more separate molecules, as well as to obtain a characteristic R_f value for the responsible compound(s), numerous paper chromatography systems were screened. Ten ml culture filtrate lyophilizates were dissolved in 1 ml of glass-distilled water each, thereby concentrating by a factor of 10, and depositions were made on Whatman number 1 chromatography paper. Chromatograms were then developed in the various solvent systems which were chosen initially on a trial-and-error basis. Detection was accomplished using the same bioautographical system employed in the study of beauvericin (Champlin and Grula, 1979).

A solvent system composed of n-butanol, water and isopropanol (35:20:45) revealed the antibiotic factor found in culture filtrates of *B. bassiana* S-7 to be composed of two chromatographically separable antibiotic molecules as indicated by two overlapping zones of inhibition at and just ahead of the origin (Figure 5). By increasing

Figure 5. Bioautography of Water Extracts of Spent SFB Culture Filtrate Lyophilizates Developed in the Antibiotic Solvent System. Solvent system components are given in volume-to-volume ratios (ml) and sample deposition volumes are 10 µl for each chromatogram.

N-BUTANOL, H_2O, ISOPROPANOL



35:20:45 25:40:35 20:50:30 15:60:25

the polarity of the solvent mixture (i.e. increasing the amount of water while decreasing the amounts of n-butanol and isopropanol proportionately), it is possible to further separate the two compounds. One remains at the origin (designated origin antibiotic) while the other migrates a gradually increasing distance from the origin as the polarity is increased (designated migrating antibiotic). A complete separation is obtained as the water concentration approaches 60%.

When the polarity is increased further by mixing n-butanol, water and isopropanol (12.5:65:22.5), the tailing of the migrating antibiotic is alleviated and a compact oval zone is obtained. This system was chosen for all subsequent research and designated as the antibiotic solvent system.

In order to obtain the two antibiotics in separate samples, a fractionation scheme was devised. When SFB culture filtrate lyophilizates are extracted with methanol, the migrating antibiotic is solubilized and the origin antibiotic can then be recovered by dissolving the pelleted methanol-insoluble material in water. The differential solubilities of the two antibiotics in methanol at the low pH (2.8) of the spent SFB is then the basis for the methanol fractionation procedure depicted in flow chart form in Figure 6 and described in detail in Chapter II.

Subsequent paper chromatography of the methanol-soluble and methanol-insoluble fractions in the antibiotic solvent system followed by bioautography reveals that the separation is complete and that the relative migrations remain unchanged (Figure 7). As can be seen in the photograph of a bioautographic assay plate of paper chromatograms

Figure 6. Methanol Fractionation to Separate the Two Antibiotics Present in Spent SFB Culture Filtrate Lyophilizates.

METHANOL FRACTIONATION OF ANTIBIOTICS

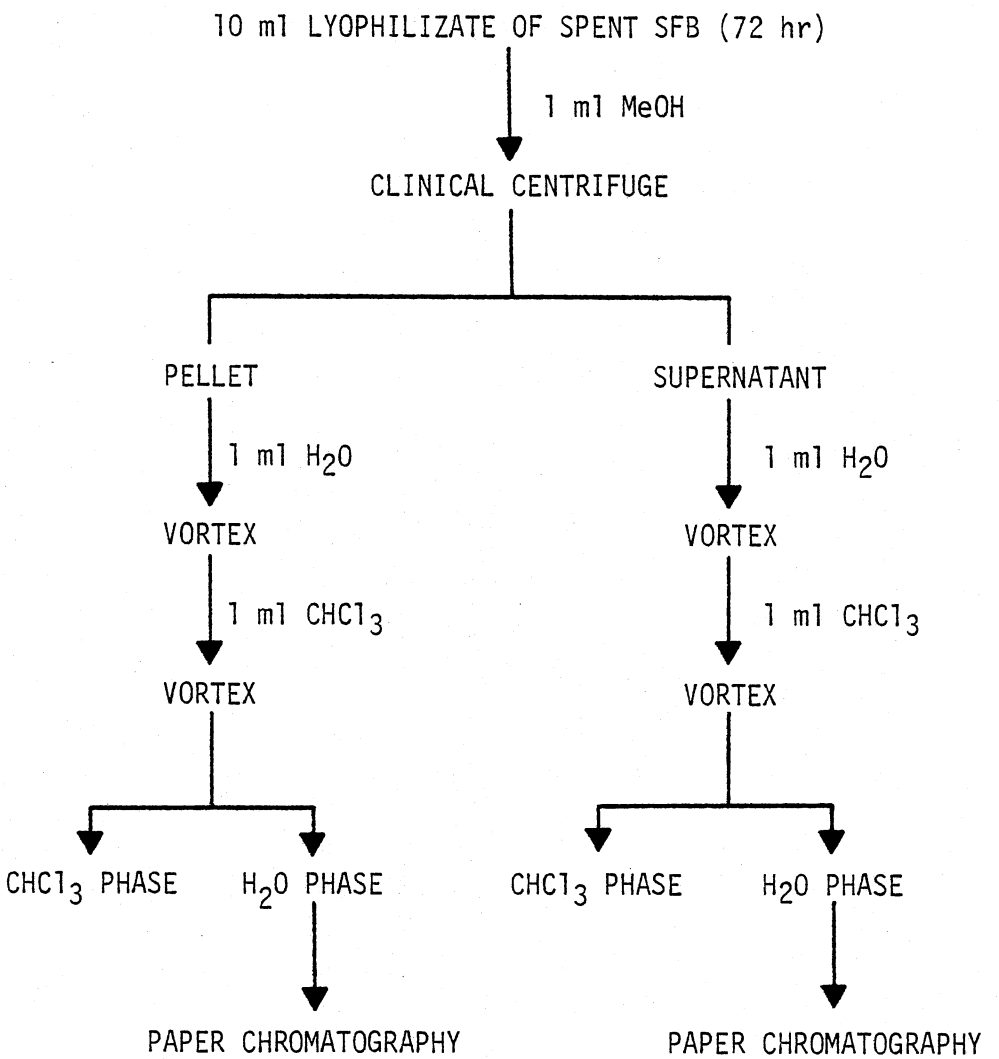
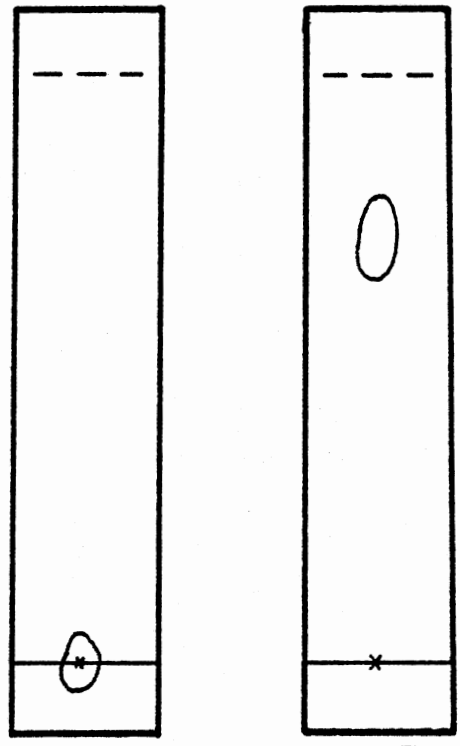


Figure 7. Bioautography of Samples Obtained by the Methanol Fractionation of Spent SFB Culture Filtrate Lyophilizates. Solvent system components are given in a volume-to-volume ratio (ml) and sample deposition volumes are 10 µl for both chromatograms.

N-BUTANOL, H_2O, ISOPROPANOL
12.5:65:22.5

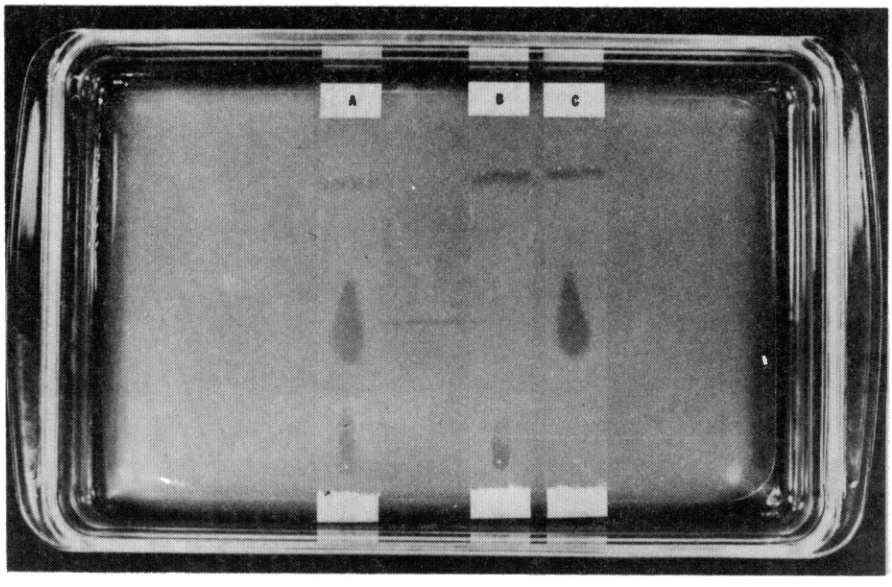


containing the antibiotics (both before and after methanol fractionation) in which the sample deposition volumes were increased 3-fold over those in Figures 5 and 7 (Figure 8), the subsequent increase in the sizes of the zones is accompanied by the slight heading of the origin antibiotic and a decrease in the R_f value of the migrating antibiotic.

In order to be sure that the zones of inhibition observed on bioautographic plates are indeed due to antibiotic factors being produced by the fungus rather than components of the medium which are themselves toxic to the assay organism, control samples consisting of 1 ml water extracts of 10 ml uninoculated SFB lyophilizates were tested and found to contain no toxic factors. However, when the pH of the sterile medium was artificially lowered to 2.8 (the pH of spent SFB after growth of the fungus for 72 hours) with 1 N HCl and incubated under the same conditions as inoculated cultures, a slight zone of inhibition could be observed under paper chromatograms with bioautography. The R_f value for this zone (0.53) corresponded with that of the much larger, more complete zone of inhibition obtained with the spent medium (0.50). Since the zone obtained with spent medium samples is so much greater than that obtained with the apparent acid catalysis in the absence of fungal growth, it was concluded that the latter was due to the low pH conversion of a component of the medium to a bacterial toxin which coincidentally exhibits an R_f value similar to that of the migrating antibiotic in the antibiotic solvent system.

When *B. bassiana* S-7 is grown on 1% Neopeptone (i.e. SFB having the fructose omitted) for 72 hours, a degree of growth comparable to that observed in the presence of the sugar is obtained. The final pH

Figure 8. Photograph of a Bioautographic Assay Plate.
(A) Ten ml lyophilizate of spent SFB culture filtrate dissolved in 1 ml of water.
(B) Methanol-insoluble fraction of a 10 ml lyophilizate of spent SFB.
(C) Methanol-soluble fraction of a 10 ml lyophilizate of spent SFB.
Sample deposition volumes are 30 µl for each chromatogram.


A
B
C

of the culture filtrate is again drastically lower (approximately 3.0) than the initial pH of the medium (7.5) and both zones of inhibition are observed with chromatograms of the water extracts of culture filtrate lyophilizates. As is the case with SFB, a slight zone of inhibition exhibiting an R_f value similar to that of the migrating antibiotic can be observed when the pH of uninoculated 1% Neopeptone is artificially lowered to 3.0 with 1 N HCl. These data indicate that Neopeptone is all that is necessary in the growth medium for the production of the antibiotics, the drastic lowering of the pH and is the component which contains the acid-activated toxin (possibly as an impurity in the Difco product).

Further evidence for the fact that the migrating antibiotic is indeed an antibiotic resulting from the metabolic activity of the growing fungus, rather than a result of the action of low pH on a component of the Neopeptone in the growth medium, is provided by the fact that both antibiotics are produced in very large amounts in SFB containing 0.2 M K_2HPO_4. This medium possesses a buffering capacity sufficient to maintain the original pH of 7.5. It is interesting to note that the production of both antibiotics is actually enhanced in the buffered situation.

Since *B. bassiana* S-7 will grow and produce both antibiotics with a concomitant lowering of the culture filtrate pH utilizing Neopeptone as the only component in the growth medium, three different peptones (enzymatic hydrolyzates of various proteins) were examined with regard to growth and antibiotic production (Table II). Both antibiotics are present in large amounts in Neopeptone and Proteose Peptone No. 3 culture filtrates but only the origin antibiotic is present in a detectable

TABLE II

GROWTH[a] AND ANTIBIOTIC PRODUCTION IN THE PRESENCE OF THREE PEPTONE CARBON, NITROGEN AND ENERGY SOURCES

Peptone[b]	Initial pH	Final pH	Sugar Present[c]	Antibiotic Production[c]	
				Origin	Migrating
Neopeptone	7.20	3.09	+++	+++	+++
Bacto-peptone	6.84	8.27	+	+++	0
Proteose Peptone No. 3	7.21	8.29	++++	++++	++++

[a] *B. bassiana* S-7 was grown in each case for 72 hrs. in a 1% solution of the specified peptone at 25°C on a gyrotory shaking apparatus (New Brunswick Scientific Company, Model V).

[b] All peptones were obtained from Difco Laboratories.

[c] Relative amounts of sugar present in the peptone solutions (as determined using thin-layer chromatographic analysis) and antibiotics present in culture filtrates (as determined with paper chromatography in the antibiotic solvent system followed by bio-autography) are indicated as follows: 0, none detected; ++++, greatest amount detected.

amount in the Bacto-Peptone culture filtrates. The final pH values of the spent Bacto-Peptone and Proteose Peptone No. 3 media are significantly higher than the initial values. This is an expected result if it is assumed that the fungus is growing at the expense of peptides and amino acids (the major components of peptones) which would be expected to undergo deamination in the process of their metabolism and thereby cause an increase in the pH. The unique lowering of the pH observed with growth in the 1% Neopeptone medium is then unexplained.

To investigate this anomaly, 15 mg/ml solutions of all three peptones were analyzed for the presence of carbohydrate impurities so as to determine if the acid production in Neopeptone could be accounted for by the fermentative utilization of a sugar. Thin-layer chromatography plates were prepared using Silica Gel-G powder (EM Laboratories) suspended in 0.1 M K_2HPO_4 and 10 µl depositions of each peptone solution were made. Following development in an acetone, n-butanol and water (5:4:1) solvent system, the plates were sprayed with the periodate-potassium permanganate sugar detection reagent of Lemieux and Bauer (1954). As can be seen in Table II, all three peptones contain varying amounts of a sugar impurity which was tentatively identified as maltose by virtue of its exhibiting an R_f value comparable to that of a maltose standard in this system. Since all three of the peptone media examined contain the same sugar impurity, yet the decrease in pH is observed only with growth in Neopeptone, it can be concluded that the acid production cannot be due simply to the utilization of a sugar present only in Neopeptone.

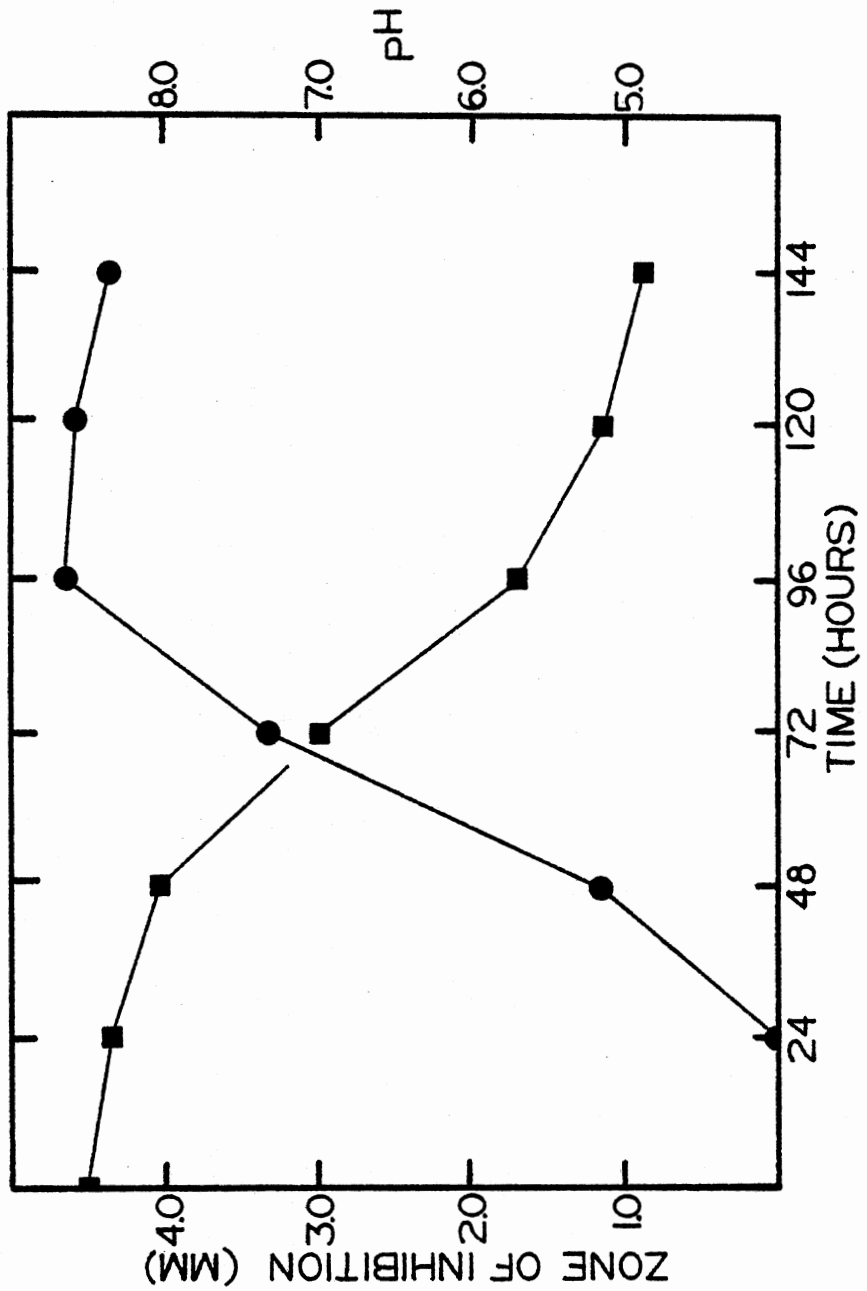
Various buffers were incorporated into the SFB growth medium in order to maintain a higher pH as growth and antibiotic production progressed in an attempt to circumvent the coincidental production of the

bacterial toxin under acidic conditions. Tris, K_2HPO_4, Na_2HPO_4 and trisodium citrate were all found to possess buffering capacities capable of maintaining a final pH after 72 hours of growth greater than 5.5 when present at concentration of 0.1 M or greater. HEPES and Bicine were found to be ineffective buffers under these growth conditions. The largest amounts of both antibiotics were obtained in the presence of the citrate salt. Greater amounts of both antibiotics were produced in the presence of trisodium citrate than with SFB alone or with SFB plus any of the other buffers tested indicating that the fungus may be able to transport and metabolize citrate.

Since it had been previously determined that fructose could be omitted from the medium with no decrease in the amounts of growth or antibiotic obtained, a new medium consisting of 1% Neopeptone, 0.2 M trisodium citrate and a trace minerals solution was formulated. This high concentration of buffer in the Neopeptone-citrate broth (NCB) prohibited the pH from dropping from the initial 8.5 to levels which would be toxic to bacteria due to acidity alone.

It was determined using the disc antibiotic assay that the maximum amount of antibiotic activity is present in the NCB culture filtrates after 96 hours of growth and that the pH is lowered gradually when both antibiotic production and pH are monitored as functions of time (Figure 9). Detectable amounts of antibiotic activity first appear some time between 36 and 48 hours post-inoculation. It is also at this time that blastospores, which are conidiospores produced by *B. bassiana* in submerged culture following a period of germination and extensive hyphal elongation (Samsinokova and Hrabetova, 1969), are first observed to be growing from the hyphae when viewed under the light microscope. This

Figure 9. Antibiotic Production and Change in pH as Functions of Time in NCB. Amounts of total antibiotic activity present in culture filtrates at various time intervals (in hours) were determined with the disc antibiotic assay. Each point represents the mean zone of inhibition (expressed as the diameter in mm of each zone minus 13 mm) or pH value obtained from three replicate cultures. ● , zone of inhibition; ■ , pH.


ZONE OF INHIBITION (MM)
1.0
2.0
3.0
4.0
pH
5.0
6.0
7.0
8.0
24
48
72
96
120
144
TIME (HOURS)

observation is in line with the hypothesis that the antibiotics are secondary metabolites which are synthesized in concert with sporulation. However, this cannot be stated conclusively since hyphal growth continues after the initiation of blastospore formation. During the period of from 48 to 96 hours, the blastospores continue to increase in number and are released from the hyphae concomitant with the further increase in antibiotic production. After 96 hours, the amount of antibiotic activity is seen to level off and then decrease slightly with time. Microscopic observation of the culture at this time reveals that both hyphae and free blastospores are present in the medium. Subsequent to this time, the onset of hyphal lysis occurs and gradually increases leaving the free blastospores to predominate at 120 to 144 hours post-inoculation. Uninoculated medium controls, having had their pH values lowered artificially with varying amounts of 1 N HCl, do not cause any inhibition of bacterial growth in the disc assay until the pH is significantly less than 5. Therefore, the antibiotic activity observed as a consequence of the growth of *B. bassiana* S-7 in NCB is not due to the lowering of the culture pH by the fungus since the pH is about 5.5 at 96 hours, the time at which the maximal amount of antibiotic activity is observed in the culture filtrate.

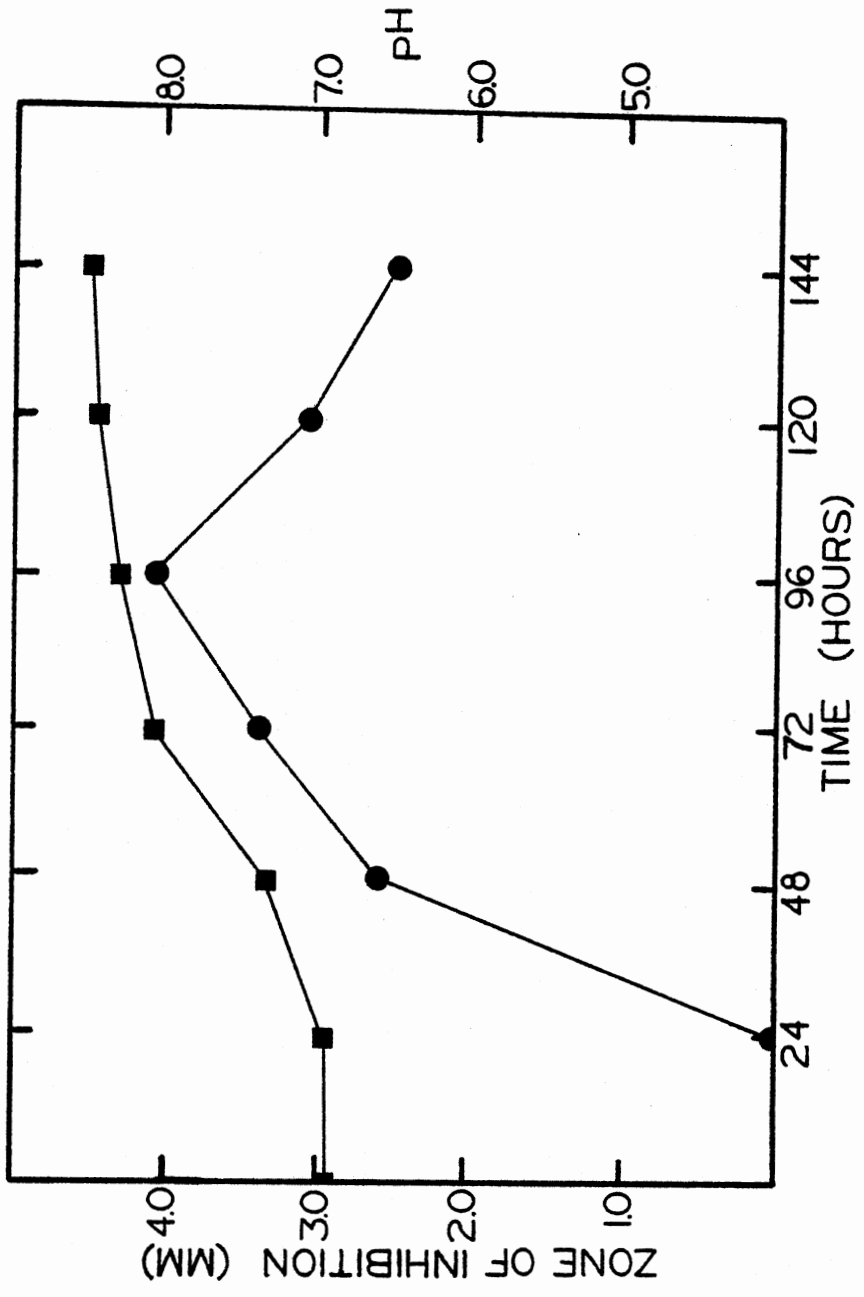
Paper chromatograms of spent 10 ml NCB culture filtrate lyophilizates extracted with 1 ml each of glass-distilled water and developed in the antibiotic solvent system reveal that the migrating antibiotic co-migrates with the Neopeptone peptides (a very large zone can be observed by spraying with ninhydrin) and citrate (as determined by spraying with $FeCl_3$-sulfosalicylic acid). For this reason, as well as to better facilitate the separation of the antibiotics from the medium

components, it was decided to substitute 0.5% acid-hydrolyzed casein for the 1% Neopeptone in NCB. This provides the fungus with the same organic carbon, nitrogen and energy sources (ultimately amino acids) present in Neopeptone while alleviating the potential purification difficulties due to the large peptides and other impurities. In addition, the citrate concentration was lowered from 0.2 M to 0.1 M since low pH no longer presented a problem in the absence of Neopeptone.

An acid-hydrolyzed casein (CASE) broth was formulated consisting of 0.5% acid-hydrolyzed casein, 0.1 M trisodium citrate, 3 mg of $MgSO_4 \cdot 7H_2O$, 174 mg of K_2HPO_4, 136 mg of KH_2PO_4, trace minerals solution and glass-distilled water to give a final volume of 100 ml. Both antibiotics are produced in the medium in amounts greater than those obtained in NCB (as determined by paper chromatography in the antibiotic solvent system followed by bioautography). By growing *B. bassiana* S-7 in various combinations of these components, it was found that the citrate enhanced production of the migrating antibiotic while the addition of the phosphate salts enhanced production of both antibiotics as well as the growth rate of the fungus. As was the case with NCB, the maximum amount of antibiotic activity in the culture filtrate is obtained at 96 hours, after which there is a gradual decrease (Figure 10). However, in contrast with growth in the NCB, the culture pH can be seen to rise from an initial value of 6.95 to 8.30 after growth for 96 hours. This is expected since amino acid deamination precedes subsequent metabolism. Additionally, Neopeptone is absent thereby removing the precursor for the synthesis of acidic product in NCB.

Further nutritional studies were carried out in an attempt to develop a medium which would support the growth and antibiotic production

Figure 10. Antibiotic Production and Change in pH as Functions of Time in CASE Broth. Amounts of total antibiotic activity present in culture filtrates at various time intervals (in hours) were determined with the disc antibiotic assay. Each point represents the mean zone of inhibition (expressed as the diameter in mm of each zone minus 13 mm) or pH value obtained from three replicate cultures. ● , zone of inhibition; ■ , pH.


ZONE OF INHIBITION (MM)
1.0
2.0
3.0
4.0
pH
5.0
6.0
7.0
8.0
24
48
72
96
120
144
TIME (HOURS)

of *B. bassiana* S-7 under chemically-defined conditions in order to facilitate studies on the biosynthesis, purification and chemical characterization of the antibiotic compounds. This was accomplished by removing the complex acid-hydrolyzed casein component from CASE broth and incorporating a mixture of 1 mg/ml L-alanine, 1 mg/ml L-phenylalanine and 1 mg/ml L-valine with the trisodium citrate and basal salts and minerals. These three amino acids had been previously shown to meet the minimal requirements of *B. bassiana* for good germination and growth when only various amino acid mixtures are provided as the sole sources of carbon, nitrogen and energy (Smith and Grula, 1981). D-Glucose and NH_4Cl were incorporated into the formulation initially to enhance the growth rate of the fungus by providing a readily usable alternate carbon, nitrogen and energy source. The glucose-amino acids-citrate (GAC) growth medium thus formulated can be considered chemically-defined yet both antibiotics are produced in significant amounts by 72 hours post-inoculation. However, in contrast to the results obtained with the NCB and CASE growth media, the maximum amount of antibiotic activity is not obtained in the culture filtrate until after 120 hours of growth and is significantly less than that observed with the two more complex media (Figure 11). This is most likely due to the fact that *B. bassiana* S-7 grows at a much slower rate in the simpler defined medium. The culture pH can be seen to decrease only slightly (from an initial value of 6.84 to 6.30) over the course of 144 hours of growth.

It is possible to selectively produce either of the two antibiotics by omitting certain components of the GAC medium and incubating for various times (Table III). The amino acids defined medium (GAC medium lacking D-glucose, NH_4Cl and trisodium citrate; 6.85 initial pH)

Figure 11. Antibiotic Production and Change in pH as Functions of Time in GAC Defined Medium. Amounts of total antibiotic activity present in culture filtrates at various time intervals (in hours) were determined with the disc antibiotic assay. Each point represents the mean zone of inhibition (expressed as the diameter in mm of each zone minus 13 mm) or pH value obtained from three replicate cultures. ● , zone of inhibition; ■ , pH.

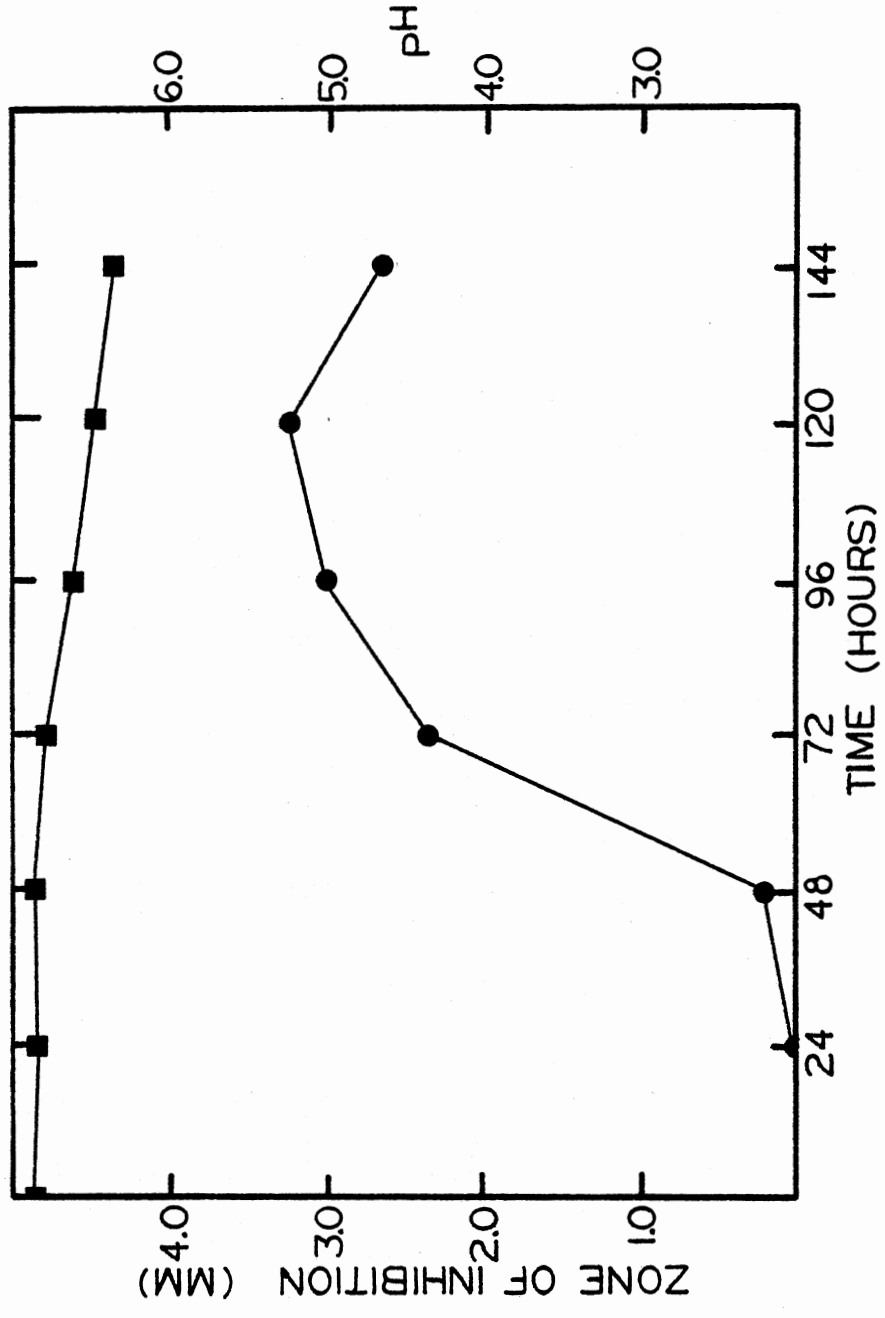

ZONE OF INHIBITION (MM)
4.0
3.0
2.0
1.0
TIME (HOURS)
24
48
72
96
120
144
pH
6.0
5.0
4.0
3.0

TABLE III

RELATIONSHIP BETWEEN ANTIBIOTIC PRODUCTION[a], GROWTH SUBSTRATES AND TIME OF INCUBATION IN THE CHEMICALLY-DEFINED MEDIA

Time of Incubation (hrs.)	Amino Acids Defined Medium[b]		Glucose-NH_4Cl-Citrate Defined Medium[b]	
	Origin	Migrating	Origin	Migrating
24	0	0	0	0
48	+	0	0	+
72	++	0	0	++
96	+++	+	+	+++
120	++++	++	++	++++
144	++++	++	+++	++++

[a]Relative amounts of each antibiotic in culture filtrates at the specified times (as determined with paper chromatography in the antibiotic solvent system followed by bioautography) are indicated as follows: 0, none detected; ++++, greatest amount detected.

[b]Both defined media consist of the GAC medium having either the glucose, NH_4Cl and citrate omitted (amino acids defined medium) or the three amino acids omitted (glucose-NH_4Cl-citrate defined medium).

provides the three amino acids mixture as the sole source of carbon, nitrogen and energy. Paper chromatography followed by bioautography of spent culture filtrate lyophilizates of this medium reveals that only the origin antibiotic is present in a detectable amount up to 96 hours of growth. After 96 hours, a small amount of the migrating antibiotic is also produced.

The glucose-NH_4Cl-citrate defined medium (GAC medium lacking the three amino acids; 6.90 initial pH) provides glucose as the source of carbon and energy with NH_4Cl as an inorganic nitrogen source. As can also be seen in Table III, a significant amount of the migrating antibiotic, but very little of the origin antibiotic, is present in the culture filtrate up to 96 hours of growth in this medium. After 96 hours, both antibiotics begin to be excreted in increasingly greater amounts.

Growth in the amino acids defined medium results in an increase in the pH of the culture filtrate and growth in the glucose-NH_4Cl-citrate defined medium results in a decrease in the pH of the culture filtrate. However, in neither case is the overall change greater than 0.6 of a pH unit within 144 hours post-inoculation. Therefore, the synthesis or activation of the antibiotic molecules is most likely not dependent upon a net change in the pH of the culture medium due to fungal growth.

Hence, in addition to finding a chemically-defined growth medium for the production of the antibiotics, the means by which the individual antibiotics can be selectively produced by simply altering the time of incubation and manipulating the various carbon, nitrogen and energy sources added to the basal salts and minerals of the CASE medium was discovered. This makes it possible for culture filtrates to be obtained

which contain only one of the two extracellular antibiotics. It is necessary to incubate the fungus for longer periods of time (120 to 144 hours) in these simpler media than in the complex media used previously in order to obtain the maximum amounts of antibiotics from the culture filtrates owing to the slower growth rates obtained.

In order to determine more fully the nutritional requirements of *B. bassiana* S-7 with regard to the selective production of the individual antibiotics, the fungus was cultured on all combinations of the carbon, nitrogen and energy sources (D-glucose, the three amino acids mixture, trisodium citrate and NH_4Cl) included in the composite GAC defined medium. The GAC basal salts and minerals ($MgSO_4 \cdot 7H_2O$, K_2HPO_4, KH_2PO_4 and trace minerals solution) were provided in all cases. Several important conclusions can be drawn on the basis of observations made by the spectrophotometric determination of the relative degrees of growth obtained in each culture as well as pH, disc antibiotic assay and bioautographic analyses of culture filtrates obtained at 24 hour intervals for up to 144 hours post-inoculation.

Maximum amounts of growth were obtained in media containing glucose plus all combinations of the amino acids mixture, citrate and NH_4Cl with the exception of glucose plus NH_4Cl which yielded only an average amount of growth. Significantly less growth was obtained with the amino acids mixture plus combinations of citrate and/or NH_4Cl in the absence of glucose. No growth occurred in the citrate plus NH_4Cl medium indicating the inability of *B. bassiana* S-7 to utilize citrate as the sole carbon and energy source. Citrate does however function to supplement the buffering capacity of the K_2HPO_4 and KH_2PO_4 since the final pH values of

glucose-containing culture filtrates were lowered only slightly in its presence while pH decreases as large as 2 units were observed in its absence.

Overall antibiotic activity, which was determined using the disc antibiotic assay and is therefore a nonspecific measure of the inhibition affected by both antibiotics, reached maximum levels in all media at 96 to 120 hours post-inoculation. Higher levels were obtained in media containing citrate than in media lacking citrate.

Data obtained with the paper chromatography and bioautography of culture filtrates, which allows for the relative amounts of the individual antibiotics to be determined, revealed that the elevated levels of overall antibiotic activity in the presence of citrate were due to the increased production of the migrating antibiotic with no increase in the amount of origin antibiotic. The incorporation of citrate into all of the media studied resulted in an approximately 3-fold enhancement of the amount of migrating antibiotic synthesized over that observed in the same media lacking citrate for up to 96 hours of growth. In contrast, the production of the origin antibiotic was stimulated by the presence of the amino acids mixture. All media combinations containing the three amino acids exhibited an approximately 2-fold enhancement in the amount of origin antibiotic present in culture filtrates when compared to media lacking amino acids after 96 hours of growth. These observations appear to implicate citrate as a necessary precursor for the biosynthesis of the migrating antibiotic, possibly as an intermediate involved in secondary metabolism since it cannot be used as the sole source of carbon and energy for growth, and one or more of the

three amino acids as necessary precursors for the biosynthesis of the origin antibiotic.

Lastly, data obtained from these bioautographs support the results presented in Table III relative to the times the antibiotics first appear in detectable amounts in culture filtrates and the amounts produced at various times in both the amino acids and glucose-NH_4Cl-citrate defined media. Additionally, this series of experiments led to the finding that these two media (see Table III) represent the correct combinations of medium components necessary for providing *B. bassiana* S-7 with optimal growth conditions under which the two antibiotics can be most selectively produced. That is to say, one antibiotic is produced at an earlier time and in greater amounts relative to the other antibiotic during growth in a given medium.

Although at least one of the two extracellular antibiotics is produced in relatively large quantity in all of the growth media mentioned which support the growth of *B. bassiana* S-7, it is interesting to note that this is not the case with all of the substrates which have been studied. For example, in a medium consisting of 1% chitin (Sigma Chemical Company) plus the basal salts and minerals of the GAC broth, only a small amount of the origin antibiotic and none of the migrating antibiotic were detected in the culture filtrate after 144 hours of growth.

Growth and Antibiotic Production in the Presence of Various Glycolytic and Tricarboxylic Acid Cycle Precursors and Intermediates

Since the addition of 0.1 M trisodium citrate enhances the pro-

duction of the migrating antibiotic over that produced with glucose alone, yet the fungus is unable to grow when citrate is provided as the sole source of carbon and energy, it was decided to study the relationship between this tricarboxylic acid (TCA) cycle intermediate and antibiotic production in greater detail.

Four flasks, each containing the glucose-NH_4Cl-citrate defined medium with varying concentrations of citrate (100, 10, 1 and 0.1 mM) were inoculated with *B. bassiana* S-7 and incubated at 25^0C on the gyrotory shaker. A fifth flask contained no citrate. Culture filtrate samples were obtained after 72 and 120 hours of growth and concentrated by a factor of 10 by lyophilization and reconstitution in 0.1 volume of glass-distilled water. Paper chromatograms of the concentrated samples were then developed in the antibiotic solvent system and the relative amounts of both antibiotics present were judged with bioautography. The relative degrees of growth obtained in each flask and culture filtrate pH values were also measured at both times.

As can be seen in Table IV, the buffering capacity of the medium increases with the citrate concentration when greater than 1 mM while there is no significant change below this value. In addition, 100 mM citrate appears to inhibit the total amount of growth obtained when compared with glucose alone yet growth is stimulated by citrate in concentrations of from 1 to 10 mM. The growth inhibitory effect had been observed previously with high citrate concentrations and could possibly be due to chelation of certain cations in the medium which are necessary for optimal growth to occur.

With regard to antibiotic production, the amount of origin antibiotic present in the culture filtrates for up to 120 hours of growth is

TABLE IV

CULTURE GROWTH AND ANTIBIOTIC PRODUCTION WITH VARIOUS CONCENTRATIONS OF CITRATE IN THE GLUCOSE-NH_4Cl-CITRATE DEFINED MEDIUM

Citrate Conc. (mM)	Initial pH	72 Hours Post-Inoculation				120 Hours Post-Inoculation			
		pH	OD_{540}[a]	Antibiotic Production[b]		pH	OD_{540}[a]	Antibiotic Production[b]	
				Origin	Migrating			Origin	Migrating
0.0	6.44	6.37	.054	0	½+	5.97	.212	++	++
0.1	6.45	6.37	.079	0	½+	5.88	.211	++	++
1.0	6.47	6.41	.075	0	½+	5.86	.272	++	++
10	6.55	6.46	.082	0	+	6.07	.340	++	+++
100	6.77	6.70	.040	0	++	6.54	.199	++	++++

[a]Degrees of growth obtained at times indicated were determined by diluting 1 ml of each culture in 9 ml of water and measuring optical density at 540 nm.

[b]Relative amounts of each antibiotic in culture filtrates at the specified times (as determined with paper chromatography in the antibiotic solvent system followed by bioautography) are indicated as follows: 0, none detected; ++++, greatest amount detected.

clearly independent of the citrate concentration. However, the addition of 10 and 100 mM amounts of citrate stimulated migrating antibiotic production over that obtained with glucose alone while concentrations of 1 mM or less did not affect production to any detectable degree. These data confirm previous observations that a relatively large amount of citrate will serve to maximize the buffering capacity of the medium and provide optimal conditions for production of the migrating antibiotic. The inability of *B. bassiana* S-7 to grow when provided only citrate as the sole carbon and energy source then allows for the possibility that the compound may be providing the fungus with a carbon chain which is more efficiently utilized in migrating antibiotic biosynthesis yet cannot be used as the sole source of carbon and energy for growth.

Several other TCA cycle intermediates were added to the basal medium at a concentration of 5 mg/ml both singly and in combination with glucose in order to determine if they can be utilized by the fungus as sole carbon and energy sources for growth and if they too act to increase the amount of migrating antibiotic produced (Table V). Citrate and citrate plus glucose were included for control situations and again, the fungus is seen to be unable to grow with citrate alone yet, when citrate is added to glucose, more migrating antibiotic is present in the culture filtrate at 72 and 120 hours than is present with glucose alone.

Precisely the same situation exists with α-ketoglutarate, succinate and malate as with citrate. None of the three is capable of serving as the sole carbon and energy source yet they all act to increase the amount of migrating antibiotic produced in 72 hours when in combination with glucose over that amount produced with glucose alone. The

TABLE V

CULTURE GROWTH AND ANTIBIOTIC PRODUCTION
WITH VARIOUS TCA CYCLE INTERMEDIATES[a]

Compound Added[b]	Initial pH	72 Hours Post-Inoculation				120 Hours Post-Inoculation			
		pH	OD_{540}[c]	Antibiotic Production[d]		pH	OD_{540}[c]	Antibiotic Production[d]	
				Origin	Migrating			Origin	Migrating
glc	6.50	6.48	.034	0	+	6.10	.135	+++	+++
cit	6.79	---	NG	--	--	---	NG	--	--
glc + cit	6.80	6.82	.007	0	++	6.62	.128	+++	++++
α-kg	6.58	---	NG	--	--	---	NG	--	--
glc + α-kg	6.60	6.53	.056	0	++	6.11	.400	+++	+++
succ	6.61	---	NG	--	--	---	NG	--	--
glc + succ	6.61	6.61	.018	0	++	6.46	.218	+++	+++
mal	6.62	---	NG	--	--	---	NG	--	--
glc + mal	6.58	6.58	.042	0	++	6.20	.268	+++	+++

[a]Abbreviations are as follows: NG, no growth; glc, D-glucose; cit, trisodium citrate; α-kg, potassium α-ketoglutarate; succ, potassium succinate; mal, potassium malate.
[b]All compounds were filter-sterilized and added to the autoclaved NH_4Cl and basal salts and minerals of the glucose-NH_4Cl-citrate defined medium to give a final concentration of 5 mg/ml except glucose which was autoclaved separately and added to give a final concentration of 10 mg/ml.
[c]See Table IV, legend a.
[d]See Table IV, legend b.

enhancement appears to be by the same amount as with citrate at 72 hours; however, only citrate still exhibits an enhanced effect by 120 hours.

It is also interesting to note that, although less growth is obtained with glucose plus citrate than with glucose alone, all three of the other TCA cycle intermediates act to stimulate the growth of the fungus when present in combination with glucose as judged by the degree of growth at 120 hours post-inoculation. This enhancement of growth is most notable with α-ketoglutarate, a direct precursor of glutamic acid.

With these observations in mind, the only means by which any TCA cycle intermediate could support the growth of *B. bassiana* S-7 would be for the fungus to convert oxaloacetate to pyruvate which would then be routed through the gluconeogenic pathway. As can be seen in Table VI, pyruvate itself cannot be utilized as the sole source of carbon and energy. As a result, none of the TCA cycle intermediates should be expected to be able to provide carbon and energy for growth. The addition of a combination of pyruvate and citrate to the basal medium also failed to support growth. Pyruvate does, however, stimulate the production of migrating antibiotic in combination with glucose over that seen with glucose alone by precisely the same amount as does citrate at both 72 and 120 hours of growth without affecting the amount of origin antibiotic being produced.

Glycerol, which enters the glycolytic pathway at the level of dihydroxyacetone phosphate, does support growth when provided as the sole source of carbon and energy or in combination with glucose. In both cases, the amount of growth obtained is significantly greater than that on glucose alone yet there is no proportional increase in migrating

TABLE VI

CULTURE GROWTH AND ANTIBIOTIC PRODUCTION WITH GLYCEROL, PHOSPHOENOLPYRUVATE AND PYRUVATE[a]

Compound Added[b]	Initial pH	72 Hours Post-Inoculation				120 Hours Post-Inoculation			
		pH	OD_{540}[c]	Antibiotic Production[d]		pH	OD_{540}[c]	Antibiotic Production[d]	
				Origin	Migrating			Origin	Migrating
glc	6.50	6.48	.034	0	+	6.10	.135	+++	+++
gly	6.63	6.43	.023	0	0	5.75	.229	+++	+++
glc + gly	6.59	6.26	.098	0	++	3.89	.405	+++	+++
K^+-pep	6.79	---	NG	--	--	---	NG	--	--
glc + K^+-pep	6.82	5.85	.010	0	¼+	3.80	.080	0	++
NH_4^+-pep	6.78	---	NG	--	--	---	NG	--	--
glc + NH_4^+-pep	6.75	5.50	.005	--	¼+	3.70	.050	--	½+
pyr	6.50	---	NG	--	--	---	NG	--	--
glc + pyr	6.50	6.40	.030	0	++	5.92	.289	+++	++++
pyr + cit	6.76	---	NG	--	--	---	NG	--	--

[a]Abbreviations are as follows: NG, no growth; glc, D-glucose; gly, glycerol; K^+-pep, potassium phosphoenolpyruvate; NH_4^+-pep, monocyclohexylammonium phosphoenolpyruvate; pyr, sodium pyruvate.

[b]All compounds were filter-sterilized and added to the autoclaved NH_4Cl and basal salts and minerals of the glucose-NH_4Cl-citrate defined medium to give a final concentration of 5 mg/ml except glucose which was autoclaved separately and added to give a final concentration of 10 mg/ml and glycerol which was autoclaved separately and added to give a final concentration of 0.5% (v/v).

[c]See Table IV, legend a.

[d]See Table IV, legend b.

antibiotic production. These observations suggest that glycerol can be utilized in gluconeogenic reactions; however, it does not provide the same carbon chain as pyruvate and citrate which are more efficiently utilized in migrating antibiotic biosynthesis.

The fungus was also unable to grow on either potassium phosphoenolpyruvate or monocyclohexylammonium phosphoenolpyruvate when provided as the sole source of carbon and energy in an attempt to determine if the inability to grow on pyruvate alone might be due to the inability to reverse the pyruvate kinase reaction of glycolysis. This question remains unanswered because the failure of either of the two salt forms to support growth could be due to the inability of the fungus to transport phosphorylated organic acids such as phosphoenolpyruvate rather than the inability to utilize it as the sole source of carbon and energy. Much less of the migrating antibiotic is produced when the phosphoenolpyruvates are added to glucose than that obtained with glucose alone. This is most likely due to the fact that the fungus grows so poorly on these combinations of substrates.

Physical and Chemical Characteristics of Antibiotics Present in Culture Filtrates

Effects of pH Extremes on Antibiotic Activity

Paper chromatograms having 30 µl depositions of the methanol-insoluble and methanol-soluble fractions (containing the origin and migrating antibiotics respectively) of a spent SFB culture filtrate lyophilizate were developed in chambers containing the antibiotic solvent system at different pH values in an attempt to determine if the

relative migrations of the antibiotics are pH-dependent. Bioautography of the chromatograms revealed that the position of the origin antibiotic is unchanged until the pH is raised to about 11.8 at which point a slight degree of migration occurs. The migrating antibiotic exhibited essentially the same R_f value under all pH conditions (Table VII). These results suggest that the migrating antibiotic contains no titratable goups while the origin molecule may have one or more such groups having an extremely high pK.

In spite of the fact that changes in solvent pH exert no detectable effect on the movement of the migrating antibiotic in the antibiotic solvent system, exposure of culture filtrate samples to low pH conditions either prior to or during paper chromatography was seen to affect both the degree of inhibition observed in bioautography and the solubility characteristics of the molecule. Culture filtrate samples from both SFB and NCB were prepared for chromatography after approximately the same degrees of growth had been reached (72 and 96 hours of incubation respectively) yet the migrating antibiotic from SFB (unbuffered and having a final pH of 2.8) yielded a much larger and clearer zone of inhibition than did that from NCB (buffered and having a final pH of 5.5). This difference in antibiotic activity could have been due to the fact that the fungus was grown on different media but it should be noted that both contained 1% Neopeptone.

In addition, treatment of the water extract of the acetone precipitate of NCB culture filtrates (a white powder containing both antibiotics) with dilute HCl yielded enhanced migrating antibiotic activity over that seen with white powder extracted with the same volume of water in the absence of acid treatment. The acid also rendered the migrating

TABLE VII

EFFECT OF VARIOUS SOLVENT pH VALUES
ON ANTIBIOTIC MIGRATION

Solvent pH[a]	Antibiotic R_f Values[b]	
	Origin Antibiotic	Migrating Antibiotic
2.45	origin	0.55
3.20	origin	0.57
5.80	origin	0.55
7.25	origin	0.54
10.00	origin	0.56
11.80	0.03	0.53

[a]The pH of the antibiotic solvent system in each chamber was adjusted to the pH indicated with either 1 N NaOH or 1 N HCl.

[b]Depositions of 30 µl each were made on Whatman no. 1 chromatography paper and developed in solvents having various pH values. Antibiotic migration was determined bioautographically.

antibiotic somewhat methanol-soluble. This observation is in keeping with the fact that the migrating antibiotic is methanol-extractable from lyophilized spent SFB (2.8 final pH) but not from the NCB white powder (6.5 pH prior to lyophilization to obtain the powder).

Further evidence for the acid modification of the migrating antibiotic molecule is provided by the fact that it can be partially extracted with n-butanol from a water extract of the NCB acetone precipitate which has had the pH lowered from the normal 6.5 to 2.0 whereas none of the antibiotic can be extracted with n-butanol when the pH remains unadjusted. Bioautography of the n-butanol-partitioned antibiotic reveals a much larger and clearer zone of bacterial growth inhibition after development of paper chromatograms in the Heathcote-Jones I solvent system (Heathcote and Jones, 1965), than is seen after development in the antibiotic solvent system. This enhancement of the antibiotic activity is presumed to be due to the 4% formic acid present in the Heathcote-Jones I solvent system.

Effect of Heat on Antibiotic Activity

In order to determined the effect of heat on the antibiotic activity present in an NCB culture filtrate after 96 hours of growth, six 5 ml aliquots were obtained and each incubated at a different temperature ranging from 20 to 100^{0}C for 10 minutes. Total antibiotic activity remaining after the heat treatment was judged using the disc antibiotic assay against *Bacillus* sp. As can be seen in Table VIII, there is no detectable decrease in antibiotic activity after heating for 10 minutes at temperatures of up to 55^{0}C while total inactivation is affected by heating at 82 and 100^{0}C for the same period of time. These data would

TABLE VIII

DISC ANTIBIOTIC ASSAY OF NCB CULTURE FILTRATE SAMPLES AFTER HEATING[a]

Temperature (^{0}C)	Zone of Inhibition (mm)[b]
25	3
33	3
37	3
55	3
82	0
100	0

[a]Each of six 5 ml culture filtrate samples was heated for 10 minutes at one of the temperatures indicated after which total antibiotic activity remaining was determined with the disc antibiotic assay.

[b]Diameter of the discs (13 mm) was subtracted from the total zone of clearing measured.

seem to indicate that the antibiotics are relatively complex molecules consisting of a thermal-sensitive moiety.

Effects of Various Cations on Antibiotic Activity

Certain cations have been reported in the literature to exert an antagonistic effect upon the activities of several known antibiotics (Newton, 1954; Brown and Melling, 1969; and Davis and Ianetta, 1972). It was therefore decided to determine how the incorporation of various cations into the bioautographic assay medium might affect the sensitivity of the assay *Bacillus* sp. to the extracellular antibiotics produced by *B. bassiana* S-7.

A 10 ml SFB culture filtrate lyophilizate (obtained after 72 hours of growth) was dissolved in 1 ml of glass-distilled water and replicate 30 µl depositions were made on Whatman no. 1 chromatography paper. The chromatograms were developed in the antibiotic solvent system and assayed for antibiotic activity on SDA bioautographic plates containing 0.25 mg/ml of the chloride salts of the various cations. The relative activities of the two antibiotics in the presence of the different cations were judged by comparing the sizes and degrees of clarity of the zones of inhibition obtained in the presence of the various cations with those obtained in the normal SDA assay medium lacking additional cations.

The data given in Table IX show that the incorporation of the monovalent cations Li^+, K^+, Na^+, Cs^+ or Rb^+ into the SDA assay medium yields either no or very little change in zone size or clarity for either antibiotic when compared to zones on the control assay medium. In contrast, the divalent cations Sr^{2+}, Ca^{2+} and Ba^{2+} appear to decrease the growth

TABLE IX

EFFECTS OF VARIOUS CATIONS ON ANTIBIOTIC ACTIVITY[a]

Cation[b]	Effect on Antibiotic Activity[c]	
	Origin Antibiotic	Migrating Antibiotic
Control	+++	+++
Li^+	++++	++++
K^+	++++	++
Na^+	++++	+++
Cs^+	+++	++++
Rb^+	+++	+++
Sr^{2+}	0	+++
Ca^{2+}	0	+
Mg^{2+}	+++	+++
Zn^{2+}	NG	NG
Cd^{2+}	NG	NG
Ba^{2+}	+	+++

[a]Abbreviations are as follows: control, no cation added to the medium; NG, no growth due to toxicity of the cation for the assay *Bacillus* sp.

[b]Chloride salts of the various cations were included individually in the SDA medium of the bioautographic plates at a concentration of 0.25 mg/ml.

[c]Relative antibiotic activities, as judged by the increase or decrease in size and degree of clarity of the zones of inhibition when compared to zones of inhibition in the control situation (i.e. SDA assay medium without the addition of a cation), are indicated as follows: 0, no antibiotic activity detected; ++++, greatest antibiotic activity detected.

inhibitory activity of the origin antibiotic markedly while only Ca^{2+} decreases activity of the migrating antibiotic. The presence of Mg^{2+} exerted no effect on bacterial inhibition by either antibiotic while both Zn^{2+} and Cd^{2+} were themselves toxic to the assay bacterium.

It is not clear whether the antagonistic relationship observed between certain divalent cations (Sr^{2+}, Ca^{2+}, Ba^{2+}) and the activities of the antibiotics against the assay *Bacillus* sp. is due to a modification of the antibiotic molecules which makes them less active or to a modification of the bacterial cells which renders them less sensitive.

Previous reports in the literature regarding the effects of Ca^{2+} and Mg^{2+} on the activities of other antibiotics toward *Pseudomonas aeruginosa* lend support to the results reported herein for the relationship between these two cations and the *B. bassiana*-produced antibiotics. Brown and Melling (1969) reported that *P. aeruginosa* loses sensitivity to polymyxin B when grown under conditions of Mg^{2+} depletion and Davis and Iannetta (1972) found that physiological concentrations of Ca^{2+} in serum antagonize the activities of gentamycin, colistin and polymyxin B against *P. aeruginosa*.

Solubilities of Antibiotics from SFB Culture Filtrates in Various Solvents

In order to determine the solubility characteristics of the two antibiotics in various organic solvents, spent SFB culture filtrates (pH 2.8) were obtained after 72 hours of growth and divided into 10 ml aliquots which were then frozen and lyophilized to dryness. Each lyophilizate was then extracted with 1 ml of a given solvent by agitation (Vortex Jr. Mixer, Scientific Industries Inc.) for one minute followed

by the removal of all insoluble material by centrifugation. The presence of either antibiotic in the extracts was detected by paper chromatography of the supernatants (20 µl depositions) followed by bioautography.

As should be expected for compounds which are excreted into the extracellular medium, both antibiotics are soluble in water (Table X). However, the origin antibiotic cannot be extracted with any of the organic solvents tested while the migrating antibiotic is soluble in methanol and only slightly soluble in ethanol. These solubility characteristics closely resemble those of the surfactant antibiotics which are generally soluble only in water and lower molecular weight alcohols and insoluble in relatively nonpolar organic solvents (Coleman, 1969).

Isolation and Purification of Antibiotics

Charcoal Adsorption of Antibiotics from Spent SFB Culture Filtrates

The only previous investigation into the extracellular antibiosis exhibited by *B. bassiana* strains which do not produce the red pigment oosporein is one detailed in a monograph on the biochemistry of the muscardine infection of silkworms by Kodaira (1961). In this paper he outlined the extraction and partial purification of an antibiotic factor which was found to be produced both *in vivo* during the infection process and *in vitro* in artificial growth media containing organic nitrogen sources. The best growth medium found for antibiotic production is quite similar to the SFB utilized initially in this study in that it is composed of 3% glucose and 0.5% peptone (as opposed

TABLE X

SOLUBILITIES OF ANTIBIOTICS
IN VARIOUS SOLVENTS[a]

Solvent	Antibiotic Solubility	
	Origin	Migrating
Water	sol.	sol.
Methanol	insol.	sl. sol.
Ethanol	insol.	insol.
n-Propanol	insol.	insol.
Isopropanol	insol.	insol.
Isobutanol	insol.	insol.
2-Chloroethanol	insol.	insol.
2-Ethoxyethanol	insol.	insol.
Ethylacetate	insol.	insol.
Methylethylketone	insol.	insol.
Pyridine	insol.	insol.
Hexane	insol.	insol.
Acetone	insol.	insol.
Chloroform	insol.	insol.
Benzene	insol.	insol.

[a]Abbreviations are as follows: sol., soluble; sl. sol., slightly soluble; insol., insoluble.

to the 4% fructose and 1% Neopeptone in SFB). Differences between the two growth media include the incorporation by Kodaira of KH_2PO_4, $NaNO_3$ and trace minerals at an initial pH of 6.0 versus the SFB initial pH of 7.5. Also, Kodaira's spent medium samples were obtained after 12 days of growth in contrast with the three days that *B. bassiana* S-7 was incubated in SFB.

Kodaira was able to achieve the partial purification of the antibiotic factor using a relatively involved procedure which utilized, as a first step, the batch adsorption of certain culture filtrate components to 0.5% charcoal at pH 2. This was followed by the adsorption of the antibiotic to 2% charcoal after the pH of the filtrate had been adjusted up to 8. The antibiotic was then selectively eluted from the charcoal with acidic methanol (pH 2) after washing with water.

In keeping with the observation by Kodaira, neither of the antibiotics produced by *B. bassiana* S-7 in SFB are adsorbed by 0.5% charcoal at pH 2 as determined after removal of the charcoal with Millipore filtration and paper chromatography of the filtrate in the antibiotic solvent system followed by bioautography. When the pH of the filtrate is adjusted up to 8, adsorption to 2% charcoal is slightly less than complete; in addition, that portion which is adsorbed cannot be eluted with acidic methanol. These differences, plus the fact that only one antibiotic was eluted from an aluminum oxide adsorption column in a later step in the Kodaira procedure, lead to the conclusion that at least one, and probably both, of the antibiotic factors produced by *B. bassiana* S-7 are compounds other than that reported in 1961. Further evidence lending support to this conclusion is provided in that while the Kodaira antibiotic was produced only with growth media

containing an organic nitrogen source, both antibiotics studied here are produced during growth in media containing either an organic nitrogen source (Neopeptone or amino acids) or NH_4Cl as the sole source of nitrogen.

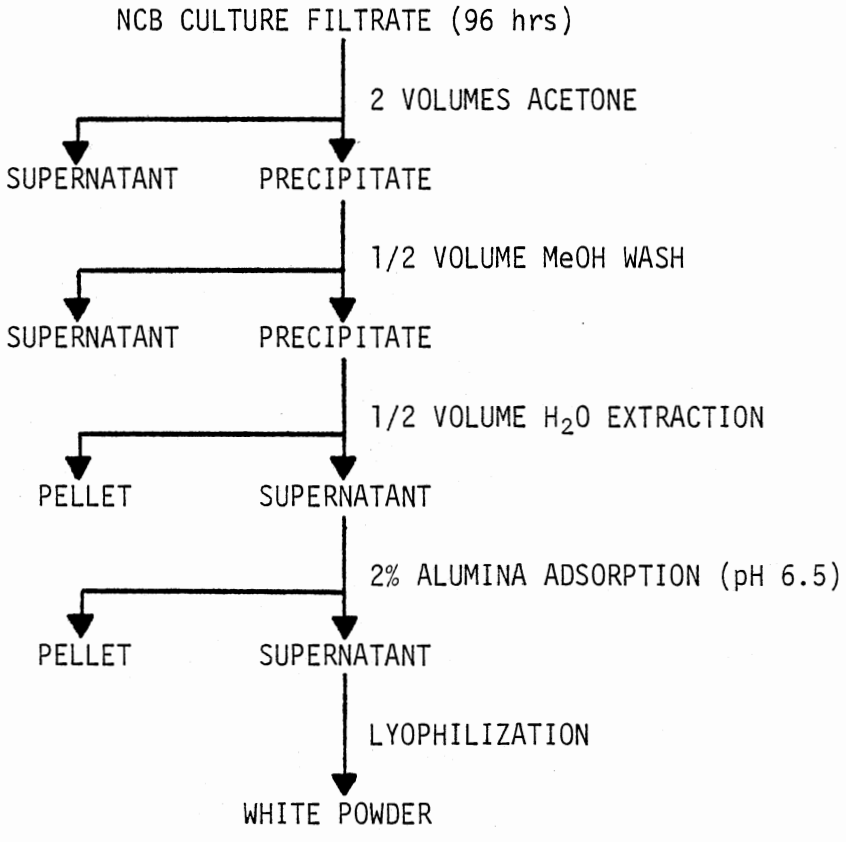
Acetone Precipitation of Antibiotics from Spent NCB Culture Filtrates

The antibiotic activity present in spent NCB culture filtrates after growth for 96 hours (about pH 5.5) can be precipitated out of the medium by the addition of acetone. Disc antibiotic assays of culture filtrate aliquots after titration with varying volumes of acetone reveal that a minimum of one volume is required while volume ratios of from 1.5 to 2.0 affect the precipitation of all antibiotic activity present initially. The heavy white precipitate obtained in this manner can be washed with methanol without the extraction of the antibiotics and the evaporation of the methanol supernatant leaves a yellow oily residue indicating that some relatively nonpolar impurities are being removed by the methanol. In contrast, washing the acetone precipitated material with water (0.5 of original volume of the filtrate) results in the extraction of the antibiotics without dissolving the entire precipitate. It had been determined previously that the antibiotics adsorb to 2% chromatographic alumina at high and low pH extremes but not at intermediate values. These findings allowed for the development of a procedure by which the antibiotics can be obtained in a purer form (Figure 12).

Lyophilization of 10 ml aliquots of the alumina supernatant yields a fine white powder that can be shown to contain both antibiotics when

Figure 12. Acetone Precipitation of Both Antibiotics from Spent NCB Culture Filtrates.

ACETONE PRECIPITATION OF ANTIBIOTICS



extracted with 1 ml of water and chromatographed in the antibiotic solvent system. However, paper chromatograms sprayed with ninhydrin or $FeCl_3$-sulfosalicylic acid reveal that significant amounts of both peptides and trisodium citrate (components of the NCB growth medium) are also extracted from the white powder indicating that the antibiotics are, at best, only partially purified using this procedure.

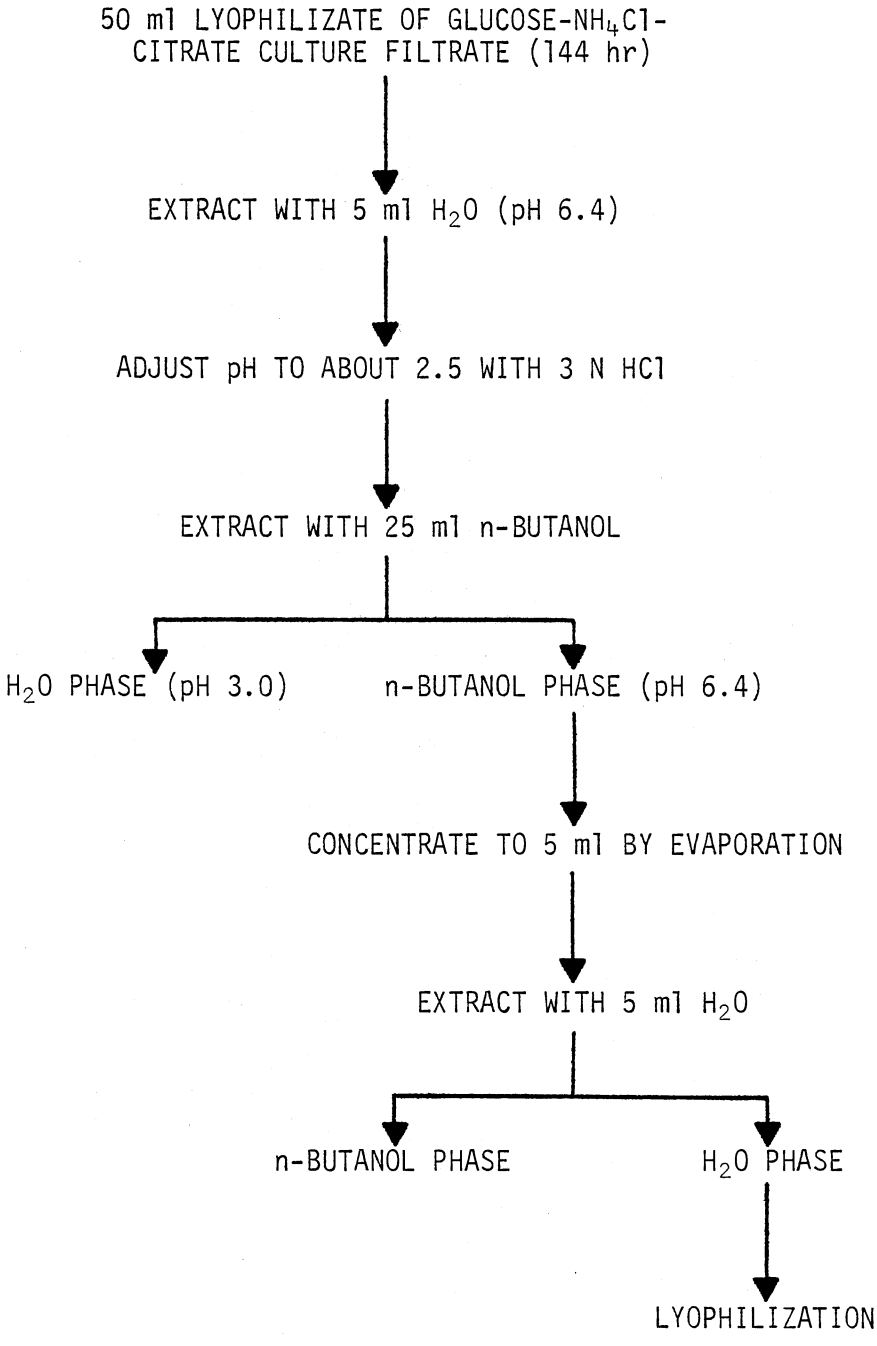
n-Butanol Extraction of Migrating Antibiotic from Spent Glucose-NH_4Cl-Citrate Defined Medium Culture Filtrates

Previous experimentation had revealed that neither antibiotic could be extracted directly from the acetone precipitate of a 96 hour NCB culture filtrate with any of several organic solvents tested. The migrating antibiotic was found to be extractable with 2% aqueous acetic acid but so were the water-soluble Neopeptone peptides present in water extracts of the same material. This problem was lessened when it was found that a portion of the total amount of migrating antibiotic present in the acetone precipitate would partition into n-butanol from a water extract after lowering the pH from the initial 6.5 to about 2.5 with 3 N HCl.

The formulation of the glucose-NH_4Cl-citrate defined medium made it possible to circumvent the problem of coextracting ninhydrin-positive material with the migrating antibiotic altogether since the medium contains no peptides or amino acids. This allowed for the development of a procedure by which the migrating antibiotic can be isolated from a crude culture filtrate on the basis of its partial solubility in n-butanol at low pH (Figure 13). Once the migrating antibiotic has

Figure 13. Low pH Extraction of Migrating Antibiotic from Spent Glucose-NH_4Cl-Citrate Defined Medium Culture Filtrates with n-Butanol.

n-BUTANOL EXTRACTION OF MIGRATING ANTIBIOTIC



partitioned into the n-butanol (about 20% of the total amount initially present in the water extract), it can be concentrated by evaporation and extracted back into water at pH 6.4. The water extract is then lyophilized to dryness and stored at minus 20^{0}C.

Bioautography of the final water phase of the extraction procedure on paper chromatograms developed in the antibiotic solvent system reveals an R_f value of about 0.80 for the migrating antibiotic. Analysis of paper chromatograms using various reagents (ninhydrin, $FeCl_3$-sulfosalicylic acid and periodate-potassium permanganate) indicates the absence of any detectable ninhydrin-positive compounds or citrate and the presence of a small amount of a reducing sugar exhibiting an R_f value similar to that of the antibiotic. This is evidence for the fact that either the antibiotic contains a carbohydrate moiety or that there is some glucose remaining in the sample as an impurity from the original growth medium.

Antibacterial Spectrum

Origin Antibiotic

Preparations containing only the origin antibiotic were obtained by eluting concentrated water extracts of lyophilized amino acids defined medium culture filtrates through a Sephadex G-25 gel filtration column. Fractions containing only origin antibiotic were pooled and lyophilized yielding a white, flaky powder. A water solution of this powder (50 mg/ml) was tested using the disc antibiotic assay against a number of different bacterial species. Previous testing using lower

centrations had revealed that the growth of sensitive bacteria was inhibited only directly under the discs with no diffusion out from the discs.

In view of the water-solubility of the molecule, the possibility that the antibiotic might be binding to the paper was considered likely. Several lines of evidence lend support to this idea. The antibiotic never migrated during paper chromatography even though numerous solvent mixtures of various hydrophilic and hydrophobic compositions were tested. However, heading was seen to occur with overloaded solute depositions, conceivably due to saturation of the paper at the point of deposition. Zones of inhibition under the origin area in bioautography were never significantly larger than the size of the deposition itself implying that the antibiotic diffused into the agar only from the area of highest concentration. Finally, it was not possible to elute the origin antibiotic from paper chromatograms after development in the antibiotic solvent system with either water or water-NH_4OH mixutres (pH values ranging up to 10.5).

Disc antibiotic assays of the 50 mg/ml solution indicate that, of the bacteria tested, only *Bacillus* sp. and *Bacillus cereus* are appreciably sensitive to the origin antibiotic (Table XI). None of the other strains tested showed any less growth under the antibiotic-impregnated discs than was present under sterile water-impregnated control discs.

Migrating Antibiotic

Preparations containing only the migrating antibiotic were obtained by eluting concentrated water extracts of lyophilized glucose-

TABLE XI

DISC ANTIBIOTIC ASSAY[a] SENSITIVITIES OF SELECTED BACTERIA TO EXTRACELLULAR ANTIBIOTICS

Bacterium	Incubation Temperature (°C)	Zone of Inhibition (mm)[b]		
		Origin	Migrating	
			Clear	Hazy
Bacillus sp.	25	2.0	4.0	18.0
Bacillus thuringiensis	25	0.0	0.0	10.0
Bacillus subtilis	37	0.0	0.0	5.0
Bacillus cereus	37	2.0	3.0	0.0
Staphylococcus aureus	30	0.0	0.0	15.0
Streptococcus lactis	30	0.0	0.0	22.0[c]
Streptococcus faecalis	37	0.0	0.0	22.0[c]
Micrococcus lysodeikticus	25	0.0	3.0	0.0
Corynebacterium equi	37	0.0	0.0	12.0
Escherichia coli	37	0.0	0.0	0.0
Enterobacter aerogenes	37	0.0	0.0	0.0
Pseudomonas aeruginosa	37	0.0	0.0	8.0
Proteus vulgaris	37	0.0	0.0	0.0
Serratia marcescens	25	0.0	0.0	3.0
Erwinia carotovora	25	0.0	0.0	20.0[c]
Salmonella typhimurium	37	0.0	0.0	0.0

[a]Disc antibiotic assay was performed for each organism as previously described for *Bacillus* sp. using nutrient agar in place of SDA with test plates incubated for 12 hours at appropriate temperatures in all cases. All cultures were obtained from Oklahoma State University Department of Microbiology research labs or departmental stock culture collection.

[b]Diameter of the discs (13 mm) was subtracted from the total zone of growth inhibition being reported.

[c]Zones of growth inhibition surrounded wide bands of growth stimulation contiguous with the discs.

NH_4Cl-citrate defined medium culture filtrates through a Sephadex G-25 gel filtration column. Fractions containing only migrating antibiotic were pooled and lyophilized yielding a white granular powder. A water solution of this powder (200 mg/ml) was tested using the disc antibiotic assay against a number of different bacterial species.

The zones of inhibition obtained with certain of the organisms after incubation for 12 hours (Table XI) reveal the possible presence of a heretofore undetected third antibacterial factor. Several of the bacteria tested exhibit quite large zones of only partial growth inhibition (i.e. hazy growth) at this time. In some cases, the hazy zone surrounds a smaller clear zone of complete growth inhibition. If it is assumed that the migrating antibiotic exists in only one form in the absence of another growth inhibitor in these preparations, it would appear that the large areas of incomplete inhibition represent only a decreased growth rate in the presence of relatively low concentrations of the antibiotic. This explanation would be acceptable but for the fact that, in cases where both clear and hazy zones exist with the same organism, the margin between the two is distinct rather than gradual and, also, the hazy zone has an equal amount of clearing from the innermost to the outermost boundary. It is for these reasons that the data obtained with the migrating antibiotic-containing sample are tabulated in Table XI as being due to the presence of either two different forms of the migrating antibiotic or two altogether different antibiotics. The hazy zone is not usually detected after incubation for longer periods of time implying only a lessening of the growth rate rather than complete growth cessation after a certain cell density has been reached.

Clear, complete zones of inhibition were obtained with *Bacillus* sp., *Bacillus cereus* and *Micrococcus lysodeikticus*. The hazy zone of incomplete inhibition surrounding the disc with *Bacillus thuringiensis* must be considered also due to sensitivity to the migrating antibiotic since this had been previously determined using bioautography (albeit less sensitive than the assay *Bacillus*). *Erwinia caratovora* can also be considered significantly sensitive to the migrating antibiotic since bioautographic analysis of paper chromatograms using this organism also yielded a large zone of inhibition due to the migrating antibiotic, the center of which contained an area of growth. This is analogous to the large band of growth observed in the disc assay which is contiguous with the disc and extends out to a band of slight inhibition.

Situations similar to that of *E. carotovora* were observed for both *Streptococcus lactis* and *Streptococcus faecalis*. The streptococci exhibited bands of slight inhibition (22 mm diameter) surrounding large areas of growth stimulation contiguous with the discs. The stimulation of growth is presumed to be due to the glucose which is present as an impurity in the antibiotic preparations. Growth on the nutrient agar assay plates was minimal but was greatly enhanced when the medium was supplemented with 1% glucose. It is interesting to note that no inhibition was observed on the glucose-supplemented plates indicating an antagonistic effect upon antibiotic activity. It is not clear whether the overcoming of the antibiotic effect is due to the increased growth rate or the increased acid present in the medium, both seen in the presence of glucose. The pH of the nutrient agar-glucose plates was about 4.0 after growth as opposed to approximately

7.0 after growth on nutrient agar alone.

Mechanistic Studies

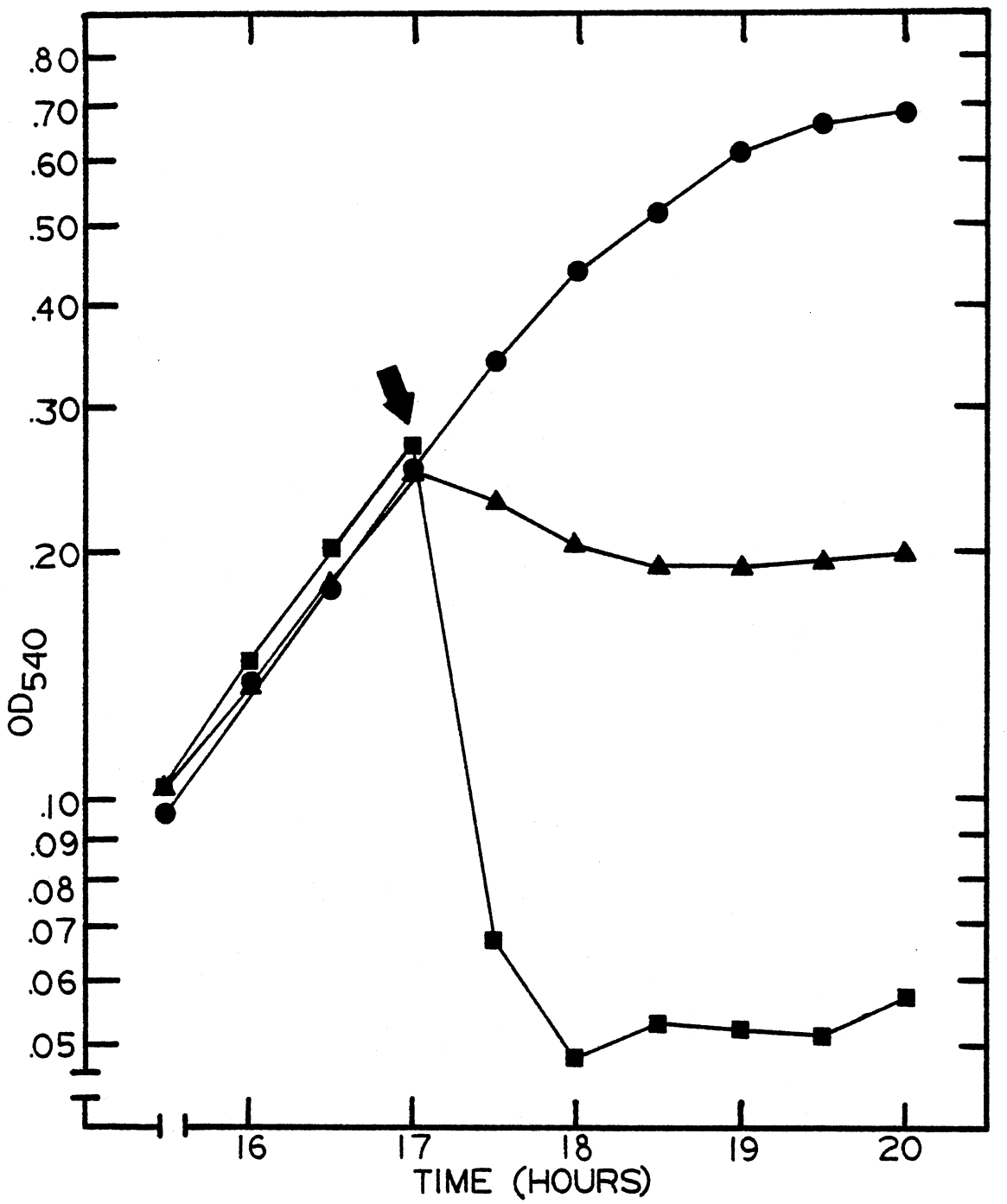
Treatment of Cells with Crude Culture Filtrate Extracts

Experiments designed to study the effects of the antibiotics on growing and nongrowing *Bacillus* sp. cells were carried out initially by adding water extracts of lyophilized spent culture filtrates (either SFB or NCB concentrated by a factor of 10) to 100 ml glucose-aspartic acid minimal medium (Grula, 1960) cultures. Both SFB and NCB extracts prepared in this manner contained a mixture of both extracellular antibiotics and exerted the same effects on *Bacillus* sp. cultures.

The addition of both 0.1 and 0.5 ml volumes of an antibiotic mixture to growing *Bacillus* sp. cells resulted in immediate decreases in culture turbidity indicating a rapid loss of cell density and eventual lysis (Figure 14). The precipitous decrease seen with the higher antibiotic concentration suggests that the onset of cell lysis occurs very shortly after treatment and is complete within 60 minutes. The rapidity of the initial decrease in optical density was confirmed by measuring turbidity at five minute intervals.

The bactericidal activity of the antibiotics in combination involves morphological changes in the cells which occur concomitantly with the decline in optical density and can be visualized readily using phase-contrast microscopy (Figure 15). Cells are seen to undergo a detectable decrease in phase density beginning within the first

Figure 14. Treatment of Log Phase *Bacillus* sp. Cells with a Water Extract of a Spent SFB Culture Filtrate Lyophilizate. A 10 ml culture filtrate lyophilizate was extracted with 1 ml of water and the volumes indicated were added after 17 hours of growth (arrow). ● , control cells (addition of 0.5 ml of a 1 ml water extract of a 10 ml uninoculated SFB lyophilizate having had the pH lowered to 2.8 with 1 N HCl to approximate the acidic conditions of the spent medium); ▲ , 0.1 ml; ■ , 0.5 ml.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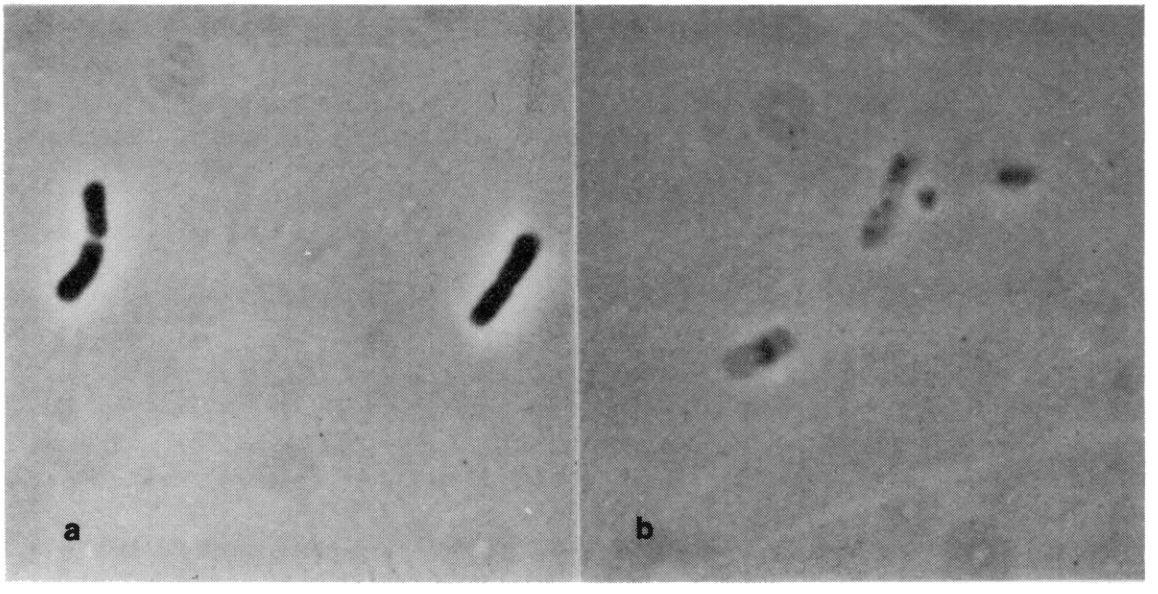
TIME (HOURS)

Figure 15. Phase-Contrast Photomicrographs of Log Phase *Bacillus* sp. Cells After Treatment with Antibiotics (0.5 ml of antibiotic-containing extract; 3,800 X).
(a) Control cells after 25 minutes.
(b) Treated cells after 25 minutes.
(c) Control cells after 50 minutes.
(d) Treated cells after 50 minutes.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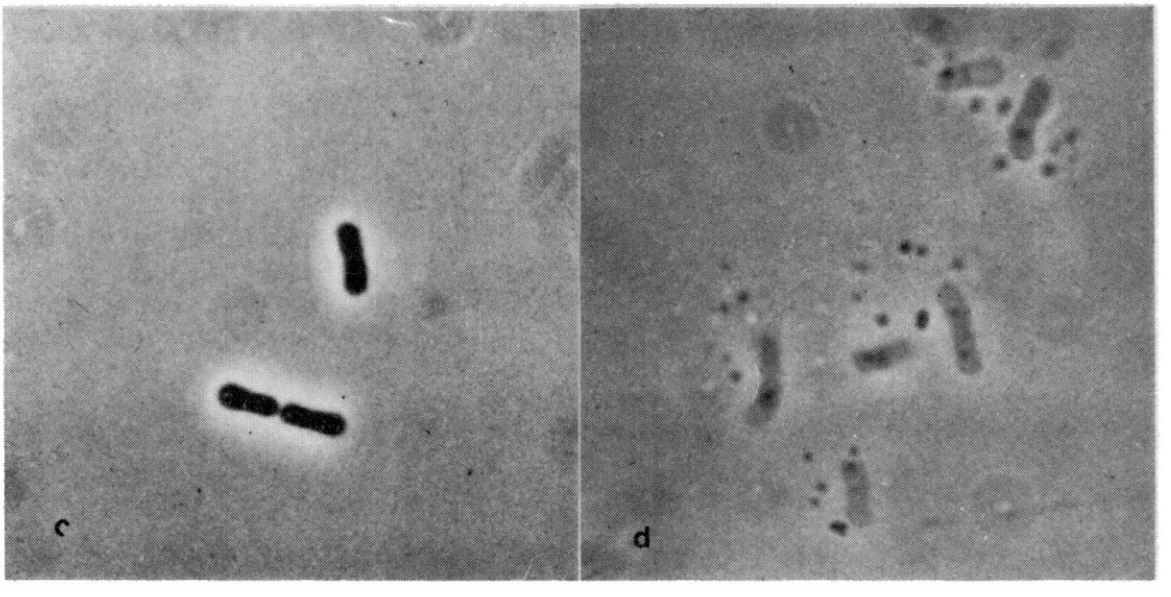
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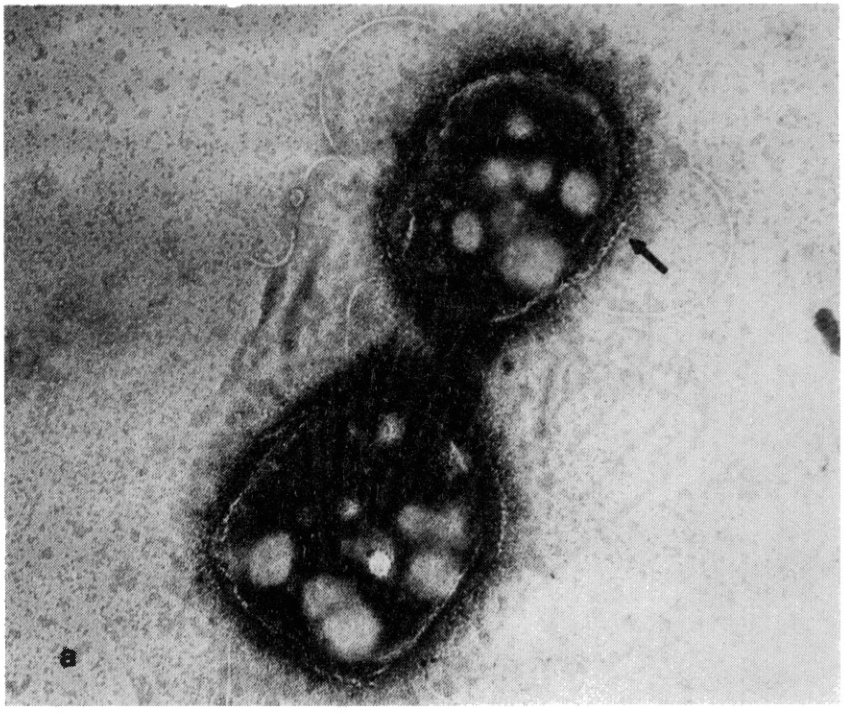
5 to 10 minutes while the intracellular granules (volutin; Grula and Hartsell, 1954) gradually become more distinct. Vacuolization and marked lateral swelling occur prior to cell lysis at which time the granules are released. No cell wall "ghosts" can be observed in either phase-contrast wet mounts or crystal violet-stained smears.

Electron microscopic observations of treated cells confirm the degradation of the cell wall as evidenced by the apparent peeling off of mucopeptide from grossly distorted rods (Figure 16). The exaggeration of lateral swelling seen in Figure 16 a is presumed to be due to distortion caused by the vacuum of the electron microscope acting upon an already weakened cell wall. Figure 16 b clearly shows the release of some flagella and electron transparent vacuoles upon cell dissolution.

Rapid clearing of liquid cultures and increased cell permeability (loss of phase-density and increased vacuolization) with the retention of large intracellular granules are all observations which are consistent with the bactericidal activity which would be expected for a membrane-directed surfactant antibiotic. It is then possible to conclude that either one or both of the *B. bassiana*-produced antibiotics exerts its deleterious effect upon bacterial growth at the level of the osmotic barrier of the cell. In order to verify this, nongrowing *Bacillus* sp. cultures were treated in the same manner as were log phase cultures and the resulting effects were monitored both spectrophotometrically and microscopically. As can be seen in Figure 17, the resting suspensions exhibited decreases in optical density commencing immediately following antibiotic treatment. The more dramatic decrease occurred again with the 5-fold higher concentration. The absence of a requirement for cell growth in order for the inhibitory activity to be

Figure 16. Electron Micrographs of *Bacillus* sp. Cells After Treatment with Antibiotics. Cells were placed on Formvar-coated grids, negatively stained for 45 seconds with 2% uranyl acetate and observed with an RCA EMU3G transmission electron microscope.
(a) 25 minutes after treatment (28,000 X). Note the increased vacuolization, peeling of the cell wall (arrow) and continued presence of flagella.
(b) 50 minutes after treatment (7,600 X). Note the complete loss of structural integrity, loss of vacuoles, general dissolution of the cell and free as well as attached flagella.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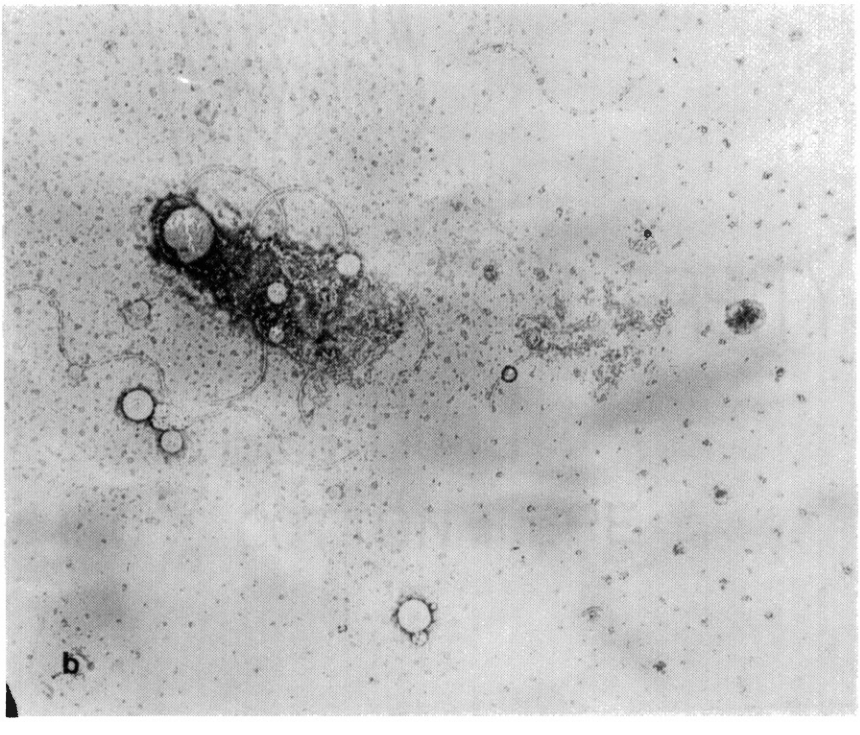
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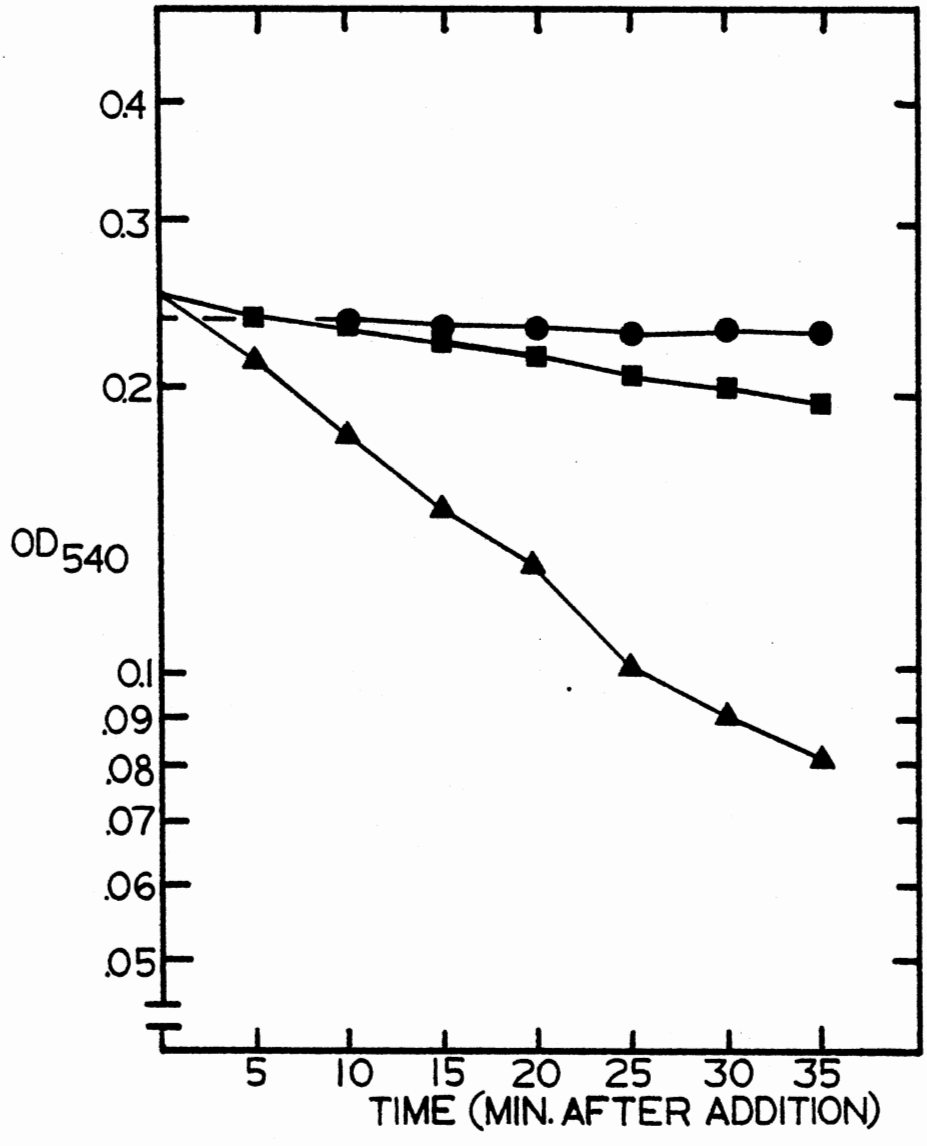
Figure 17. Treatment of Nongrowing *Bacillus* sp. Cells with a Water Extract of a Spent NCB Culture Filtrate Lyophilizate. A 10 ml culture filtrate lyophilizate was extracted with 1 ml of water and the volumes indicated were added to resting cells at time zero. ● , control cells (addition of 0.5 ml of a 1 ml water extract of a 10 ml uninoculated NCB lyophilizate); ■ , 0.1 ml; ▲ , 0.5 ml.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TIME (MIN. AFTER ADDITION)

exerted lends further support for the involvement of a membrane-directed activity.

As was the case with growing cells, no intact cell wall "ghosts" could be observed with the nongrowing cells. This leaves open the possibility that one of the antibiotics acts at the level of the cell wall, a rationalization which must be considered unlikely in view of the time frame involved (less than one generation time) and the absence of a requirement for growth. A more attractive explanation would assign cell wall degradation the role of a secondary effect of cell membrane disorganization due to the activation of one or more constitutive autolytic enzymes. In a proposed sequence of events occurring during the action of certain surface-active agents on bacterial cells, Salton (1968) has previously reported that some bacteria do eventually undergo complete lysis which is ultimately dependent upon intrinsic autolytic enzyme systems.

Treatment of Cells with Isolated Antibiotics

In order to determine the effects of the antibiotics acting individually upon growing and nongrowing *Bacillus* sp. cells, concentrated water extracts of lyophilized amino acids and glucose-NH_4Cl-citrate defined media culture filtrates (containing relatively high concentrations of the origin and migrating antibiotics respectively) were eluted through a Sephadex G-25 gel filtration column. Fractions containing antibiotic activity were pooled, lyophilized and concentrated by reconstitution in glass-distilled water. Samples obtained in this manner contained only one of the antibiotics, as determined using paper chromatography followed by bioautography, and were free of extracellular

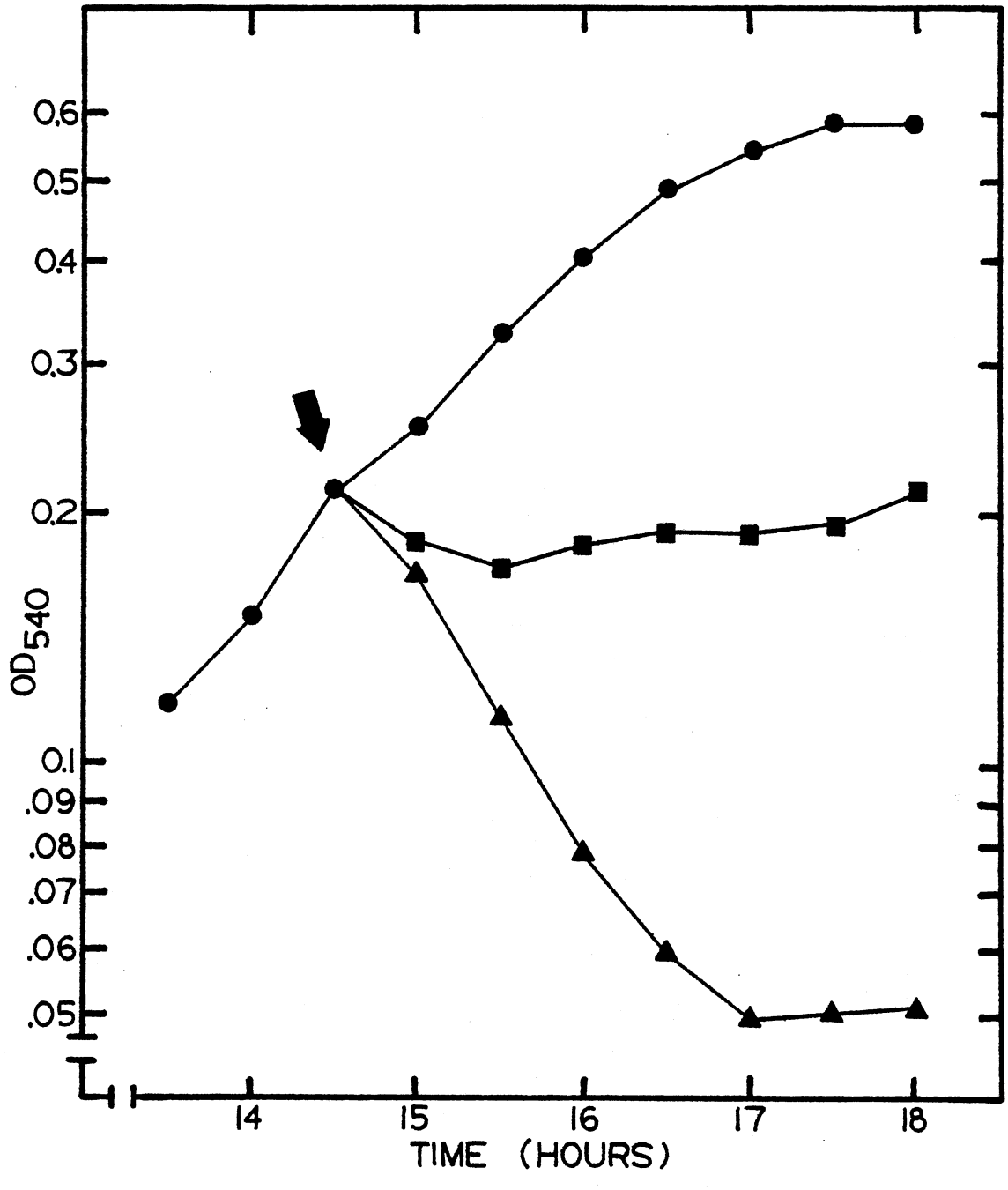
proteins and salts. These preparations were then added to growing and nongrowing cultures of *Bacillus* sp.

The treatment of log phase cells with the migrating antibiotic resulted in a precipitous decline in cultural turbidity which was fully complete within 2.5 hours (Figure 18). Phase-contrast microscopy revealed a gradual decrease in cell phase density commencing within minutes while the intracellular granules concomitantly became more distinct. Subsequent vacuolization and lateral swelling became increasingly noticable as cytoplasmic leakage progressed and granules were released upon total cell lysis. These observations are consistent with those seen with the addition of crude culture filtrate extracts in the previous section suggesting that all of the bactericidal effects can be accounted for by the activity of the migrating antibiotic alone.

The treatment of log phase cells with the origin antibiotic resulted in the cessation of growth with only a slight lowering of the optical density (Figure 18). However, when viewed microscopically, the cells were seen to undergo a lesser degree of the same type of damage seen with the migrating antibiotic. Total clearing of the culture was never obtained with the origin antibiotic even though both antibiotics exhibited comparable zones of inhibition at these concentrations using bioautography.

Since both antibiotics cause, to varying degrees, the same morphological changes in *Bacillus* sp., it is possible that both act to inhibit growth via a similar mechanism which ultimately results in disorganization of the cell membrane. The more drastic response illicited by the migrating antibiotic could then be due to either a greater

Figure 18. Treatment of Log Phase *Bacillus* sp. Cells with Isolated Antibiotics. Preparations containing individual antibiotics were added to 5 ml aliquots of cells growing in glucose-aspartic acid medium (arrow). ●, control cells; ■, 2.0 mg/ml origin antibiotic; ▲, 8 mg/ml migrating antibiotic.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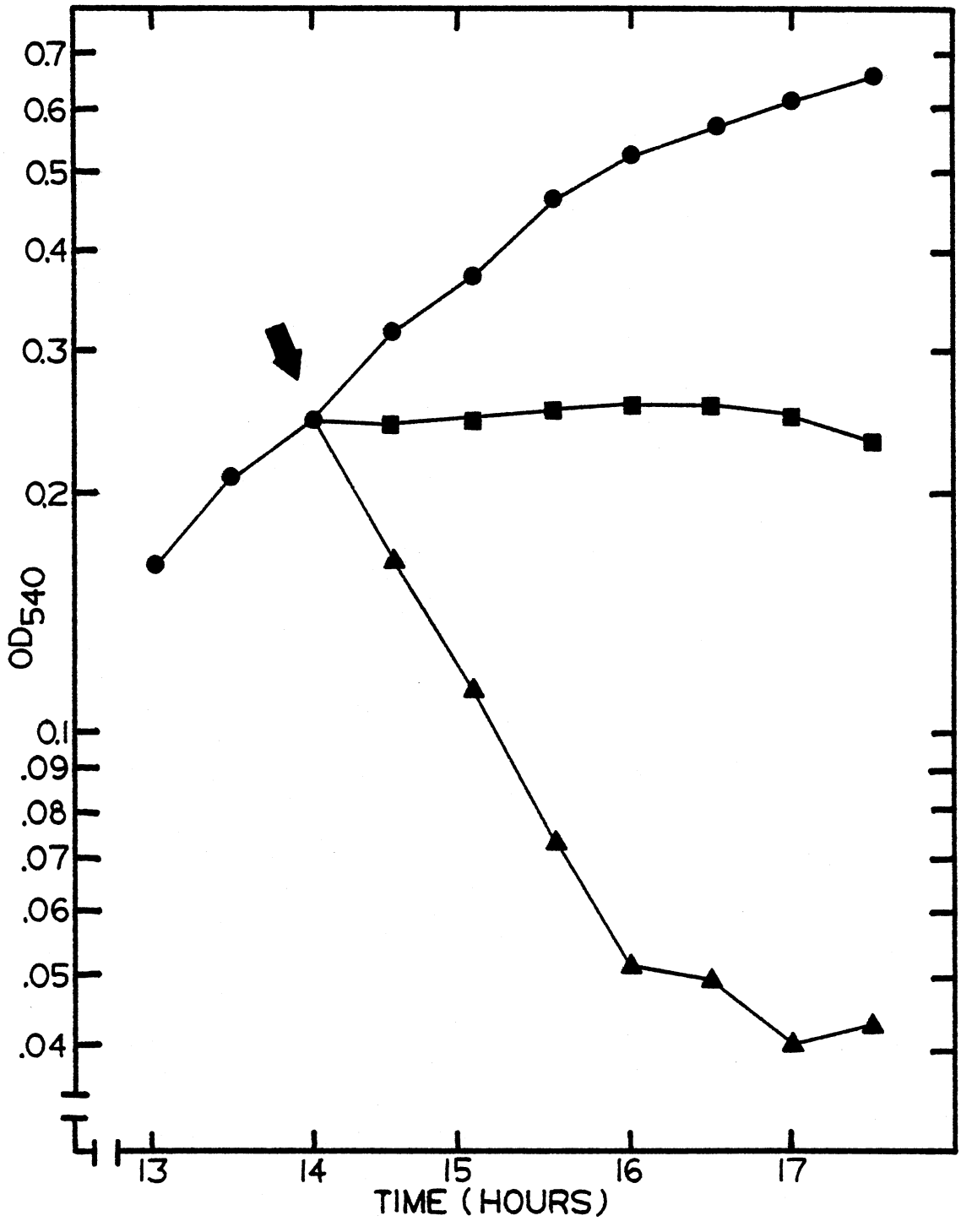
TIME (HOURS)

affinity for certain binding sites on the surface of the organism or an effectively higher concentration of migrating antibiotic, a disparity which could not be determined using bioautography.

As was the case with the addition of crude culture filtrate extracts containing both antibiotics to *Bacillus* sp. cultures, intact cell wall "ghosts" were never observed after membrane dissolution with either of the antibiotics added separately. Since the lateral swelling induced by both antibiotics clearly indicates a weakening of the cell wall mucopeptide which cannot be accounted for by a membrane-directed mechanism and since both antibiotics exert their individual effects in much less time than it takes for one division to occur, it can be concluded that the cell wall is in some way being degraded but that neither of the antibiotics acts directly on the cell wall. This leaves activation of the constitutive autolytic enzyme system via a secondary effect of the membrane disorganization as the only plausible explanation.

In order to ascertain the effect of a known membrane-directed surfactant antibiotic upon *Bacillus* sp. with regard to morphological changes involving both membrane permeability and possible cell wall degradation, log phase cultures were treated with various concentrations of polymyxin B sulfate (7900 units per mg; Sigma Chemical Company). At a concentration of only 1.0 μg/ml, a situation quite similar to that seen with the addition of origin antibiotic exists (Figure 19). Culture turbidity leveled off rapidly after treatment and the cells appeared less phase dense with granules which were more distinct when compared to control cells. At a concentration of 10 μg/ml, polymyxin B exerted precisely the same effect as did the migrating antibiotic. The precipitous

Figure 19. Treatment of Log Phase *Bacillus* sp. Cells with Polymyxin B. Appropriate dilutions of an aqueous polymyxin B stock solution were added to 5 ml aliquots of cells growing in glucose-aspartic acid medium (arrow). ●, control cells; ■, 1.0 µg/ml polymyxin B; ▲ 10 µg/ml polymyxin B.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TIME (HOURS)

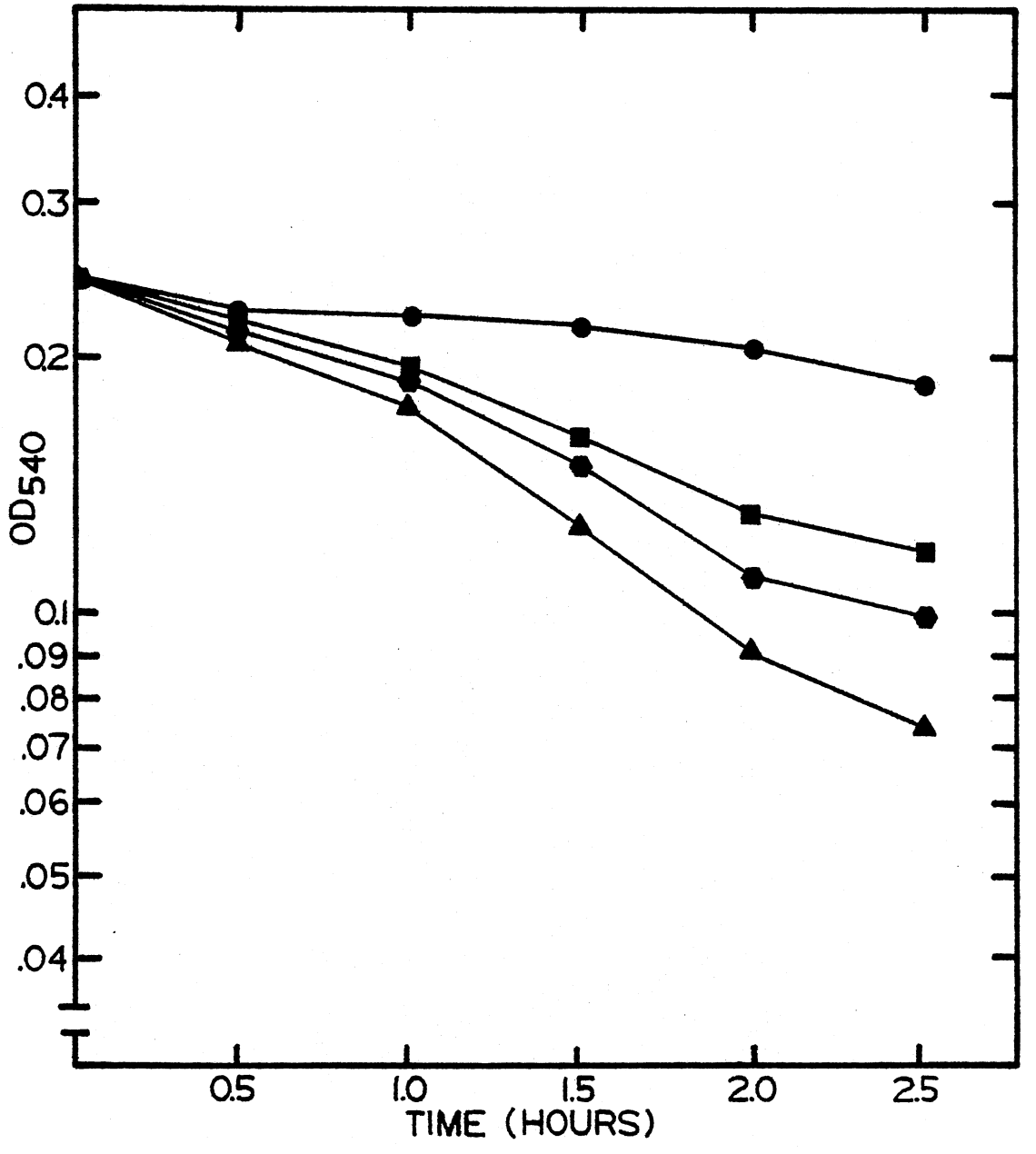
decrease in culture turbidity was accompanied by a rapid loss of phase density with intracellular granules becoming increasingly distinct, vacuolization, lateral swelling and eventual total lysis with no intact cell wall "ghosts" remaining. Cell wall degradation in the presence of polymyxin B, which is known to exert no direct deleterious effect upon the cell wall, lends further support to the hypothesis that the cell wall degradation in the presence of the origin and migrating antibiotics is in fact due to the activation of a cell wall autolytic enzyme system.

Still further evidence for a membrane-directed activity for the antibiotics (with concomitant activation of cell wall autolytic enzymes) was obtained by adding the isolated antibiotics and polymyxin B to non-growing *Bacillus* sp. cultures (Figure 20). Individual treatment with all three antibiotics affected decreases in cultural turbidity proving that cell division is not required for the inhibitory activities to be exerted. All of the morphological changes observed with growing cells were noted in the resting situation and again, no cell wall "ghosts" were evident.

Membrane Phospholipid Composition

The clearly membrane-directed mechanisms by which the *B. bassiana*-produced extracellular antibiotics act upon both growing and nongrowing *Bacillus* sp. cells and the similarity with effects brought about by treatment with the antibiotic polymyxin B prompted experiments to determine the major phospholipid composition of the *Bacillus* sp. cell membrane. Although the precise molecular mechanism of the surfactant-like activity which polymyxin B exerts upon bacterial cells remains unknown (Storm et al., 1977), the binding of the basic molecule to

Figure 20. Treatment of Nongrowing *Bacillus* sp. Cells with Isolated Antibiotics and Polymyxin B. Cells were treated with appropriate antibiotic solutions to yield indicated final concentrations at time zero. ●, control cells; ■, 2.0 mg/ml origin antibiotic; ▲, 8 mg/ml migrating antibiotic; ⬢, 10 μg/ml polymyxin B.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TIME (HOURS)

anionic phospholipids as a necessary initial step in a sequence of events leading to the disorganization of the cell membrane has been well established (Newton, 1954; Teuber and Miller, 1977; and Conrad et al., 1979).

Bacillus sp. membrane phospholipids were obtained using the chloroform-methanol extraction procedure of Folch et al. (1957) and their identifications made through comparisons of R_f values to those of known standards following thin-layer chromatography (Johnson and Grula, 1980). The phospholipids present in the extracts in detectable amounts were determined to be diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG). Small amounts of phosphatidic acid were also extracted.

The major membrane phospholipid constituents of several of the bacteria used in this study to determine the antibacterial spectrum of the antibiotics have been described previously. *Bacillus cereus* contains PG, phosphatidylethanolamine (PE) and phosphatidylcholine (PC; Kates et al., 1962); *Micrococcus lysodeikticus* contains DPG, PG and phosphatidylinositol (PI; Johnson and Grula, 1980); *Erwinia carotovora* contains DPG, PG and PE (Rice, 1967) and *Escherichia coli* contains DPG, PG and PE (Raetz, 1978).

Bacillus sp. and *B. cereus* both have PG as a common major membrane phospholipid and both are sensitive to the origin antibiotic, however, the membranes of *E. carotovora, E. coli* and *M. lysodeikticus* also contain PG and are origin antibiotic resistant. *Bacillus* sp. and *B. cereus* are both sensitive to the migrating antibiotic yet *Bacillus* sp. has DPG and lacks PE and PC while the reverse is true for *B. cereus*. *M. lysodeikticus* contains DPG along with PI which is lacking in all others and is migrating antibiotic sensitive. Finally, *E. carotova* and *E. coli*

possess identical phospholipid profiles yet the former is sensitive to the migrating antibiotic while no sensitivity has ever been demonstrated for the latter in either disc assays, bioautography or liquid culture treatments.

Therefore, no one membrane phospholipid can be implicated as the site necessary for the binding of either antibiotic. If the adsorption of the antibiotics to the cell membrane can be considered similar to that of polymyxin B, which requires electrostatic associations with different anionic phospholipids having various affinities for the molecule (Teuber and Miller, 1977), then a nonspecific interaction must be assumed. Differences in bacterial susceptibility would then be due to differences in permeability of the cell wall in Gram-positive cells or the outer cell membrane in Gram-negative bacteria. An alternative mechanism of binding could involve specific interactions with a membrane protein present in sensitive bacteria.

CHAPTER IV

DISCUSSION

Antibiotics have been defined by Stanier et al. (1976, p. 84) as "organic substances of microbial origin which are either toxic or growth-inhibitory for other organisms". The *B. bassiana* strains being used in a comprehensive study of the mechanism of their entomopathogenicity were first suspected of having the capacity to excrete a diffusible antibiotic factor when it was observed that certain bacterial isolates failed to grow in areas contiguous with fungal colonies on SDA plates. This inhibition of bacterial growth was eventually shown to be due in fact to the excretion of two paper chromatographically distinguishable antibiotics produced by the fungus in the course of normal growth on several different media.

In the subsequent research on the antibiosis exhibited by *B. bassiana*, two major problems were encountered. First, no previous investigations involving extracellular antibiotics other than oosporein and the Kodaira antibiotic (Kodaira, 1961) appear in the literature pertaining to this micro-organism. This necessitated that all of the systems used to both maximize antibiotic production and purify the antibiotic factors be worked out without the benefit of previous investigation. Initial experiments had to be designed to establish procedures with which meaningful data could then be obtained. A somewhat confusing situation exists in that important data were collected using systems that were eventually

replaced by better methods. For this reason, the results presented in the previous chapter were reported in the order that the respective systems were used.

A second problem was encountered in the purification of the antibiotics. The molecules were found to be highly water-soluble yet insoluble in all of the organic solvents tested. Since all of the growth media components and other extracellular products resulting from fungal growth are themselves water-soluble, the antibiotic purification attempts were rendered more difficult than would have otherwise been expected.

The two antibiotics can, however, be separated from each other quite readily by altering fungal growth conditions such that one is produced in a much higher concentration than the other and then eluting the culture filtrate through a Sephadex G-25 gel filtration column. Preparations obtained in this manner contain only one antibiotic, are desalted and lack all extracellular proteins but are contaminated with certain impurities from the growth medium. Using the low pH n-butanol partitioning procedure, it was possible to obtain small amounts of the migrating antibiotic in a truly pure form but this procedure is limited by the slight solubility of the molecule. Therefore, future studies must involve more work in the area of the purification of both antibiotics in quantitative amounts.

A more positive aspect of this type of study exists with the inherent ability of antibiotic molecules to inhibit the growth of bacteria. This biological activity can be exploited making the detection and quantification of the antibiotics a simple matter of biological assay.

In this study, both disc antibiotic assay and the bioautography of paper chromatograms using a bacterium (*Bacillus* sp.) isolated from soil and determined to be highly sensitive to the *B. bassiana*-mediated antibiosis were employed.

The initial phase of this study focused on the production of the antibiotics in laboratory media. It was found that *B. bassiana* S-7 produces both antibiotics in relatively high concentrations when grown in submerged cultures on complex media (SFB, NCB and CASE broth). Antibiotic production during growth on a simpler chemically-defined medium containing glucose, NH_4Cl, three amino acids and trisodium citrate (GAC) was significantly decreased and longer incubation periods were required to obtain maximum production. However, the observation that either of the two antibiotics could be selectively produced by omitting certain substrates of the GAC medium made growth and antibiotic production in the chemically-defined system more desirable overall in that isolation was facilitated and basic biosynthetic information could be obtained.

Growth on the amino acids mixture (consisting of L-alanine, L-phenylalanine and L-valine) in the absence of glucose, NH_4Cl and citrate resulted in the production of the origin antibiotic in relatively large amounts while migrating antibiotic production was minimal. This suggests that these three amino acids or their derivatives are directly involved in origin antibiotic biosynthesis. Growth with glucose, NH_4Cl and citrate in the absence of any amino acid resulted in the preferential production of the migrating antibiotic with the amount synthesized being dependent upon the amount of citrate provided over a certain concentration range. This, along with the inability

of the fungus to utilize citrate as the sole source of carbon and energy, suggests that citrate might provide the fungus with a carbon chain which is readily incorporated into the secondary biosynthesis of the migrating antibiotic.

Using both the disc antibiotic assay and bioautography of crude culture filtrate extracts, it was possible to elucidate certain physical and chemical parameters of the two antibiotics. The existence of titratable groups on either molecule is unclear but the activity of the migrating antibiotic appears to be enhanced by low pH conditions as are its solubility characteristics in solvents other than water. Both antibiotics are rendered inactive by heating (82^0C) for 10 minutes.

Antibiotic activity was antagonized by certain divalent cations in a situation analogous to that of several known surface active antibiotics. The activity of the migrating antibiotic was decreased in the presence of Ca^{2+}, while Sr^{2+}, Ca^{2+} and Ba^{2+} all exhibited antagonistic effects towards the origin antibiotic.

Another characteristic shared by the *B. bassiana*-produced antibiotics and known membrane-directed surfactant antibiotics is their general relative insolubility in solvents other than water and complete insolubility in nonpolar organic solvents. Both antibiotics were found to be quite water-soluble with the migrating antibiotic also being soluble to slightly soluble in lower molecular weight alcohols (depending on the pH).

With regard to the antibacterial spectrum of the antibiotics, it can be concluded that only *Bacillus* sp. and *Bacillus cereus* are appreciably sensitive to the origin antibiotic at the concentrations tested.

The migrating antibiotic, on the other hand, clearly inhibits the growth of *Bacillus* sp., *B. cereus*, *Bacillus thuringiensis*, *Micrococcus lysodeikticus* and *Erwinia carotovora*. Less than clear results were obtained for *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus lactis*, *Corynebacterium equi*, *Pseudomonas aeruginosa* and *Serratia marcescens* which exhibited only "hazy" zones of growth inhibition with the disc antibiotic assay. The Gram-negative bacteria *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris* and *Salmonella typhimurium* were resistant to both antibiotics.

From these data, it would appear that Gram-positive bacteria are, in general, more sensitive to the antibiotics than are Gram-negative bacteria but it is evident that not all Gram-positives are inhibited. The absence of a specific major membrane phospholipid requirement for sensitivity tends to implicate differences in the penetrability of either the cell wall (for Gram-positives), the outer cell membrane (for Gram-negatives) or the presence of specific protein binding sites in the membrane as the reason for the differences in sensitivity. Of the nine best characterized surface active antibiotics (all of which are at least in part peptides), four are primarily active against Gram-positive bacteria, two are primarily active against Gram-negative bacteria and three are equally effective against both (Coleman, 1969).

The final phase of this study has entailed a preliminary investigation into the mechanisms by which the extracellular antibiotics are able to inhibit the growth of the assay *Bacillus* sp. This assessment was accomplished by adding partially purified preparations containing the isolated antibiotics to cells growing in a glucose-aspartic acid

defined medium and to nongrowing cells suspended in the buffered salts and minerals of the same medium in the absence of glucose and aspartic acid.

When added individually to *Bacillus* sp. cultures, both the origin and migrating antibiotics were observed to effect precisely the same consequences upon cell morphology (although to different degrees at the concentrations tested) as were seen with crude culture filtrate extracts which contained the antibiotics together. These morphological effects, which include the rapid loss of cell phase density with the intracellular volutin granules becoming increasingly more distinct, vacuolization, lateral swelling and eventual total lysis without remaining cell wall remnants, occurred regardless of whether the cells were actively dividing or in a "resting" state. These cellular effects were observed spectrophotometrically as a precipitous clearing of culture turbidity by the migrating antibiotic whereas only an initial decrease in optical density followed by the cessation of growth occurred with the origin antibiotic. In view of the similarity between the effects of both antibiotics upon cellular morphology, this disparity is presumed to be due to differences in effective concentrations in the antibiotic-containing preparations.

The observations regarding increased membrane permeability and lysis clearly implicate a membrane-directed surface-active mechanism for both antibiotics, a conclusion which is supported by the fact that gross changes in cellular morphology occur with a rapid onset and by the marked susceptibility of resting cells. The lateral swelling and absence of cell wall "ghosts", which are indicative of cell wall degradation, must be presumed to be due to the activation of the cell

wall autolytic enzyme system as a secondary effect of membrane disorganization since these too occur in less time than it takes for one division to transpire. The fact that polymyxin B, a surfactant antibiotic which causes the same morphological changes as do the *B. bassiana*-produced antibiotics, also affects cell wall degradation in this organism helps to verify this conclusion.

If the ability of the antibiotics to illicit activation of the *Bacillus* sp. cell wall autolytic system is a phenomenon which is capable by itself of inhibiting bacterial growth, then an additional possibility for why certain bacteria exhibit resistance exists. Resistant organisms, in addition to possessing surface structures such that the antibiotics are either denied access to or in some other way are unable to interact with the cell membrane, may possess autolysins which are not activated by antibiotic treatment or they might lack a cell wall autolytic system altogether.

Of the heretofore known membrane-directed antibiotics, the one which most closely resembles those reported here on the basis of both inhibitory activity and physical parameters is a polypeptide of unknown structure called amphomycin (Coleman, 1969). Amphomycin is obtained from the culture broths of several *Streptomyces* strains and is primarily active against Gram-positive bacteria (Heinemann et al., 1953). Other similarities exist in that it is soluble only in water and some lower molecular weight alcohols, is insoluble in nonpolar organic solvents and can be isolated by extracting from water with n-butanol at low pH values and then extracted back into water at neutrality. It exhibits a reaction with ninhydrin only after acid hydrolysis.

When considering the potential practical application of any new

antibiotic, clinical exploitation is usually foremost in the minds of most people. The applicability of such a role for the extracellular antibiotics produced by *B. bassiana* should and will be studied further but it should be noted that other possibilities exist. Woodruff (1980) has stated that the use of antibiotics by basic researchers as reagents, as targets for research and observation and as aids in the study of biochemical pathways has contributed to the importance accorded to the detection and isolation of new natural product antibiotics in recent years. In the case of the *B. bassiana*-produced antibiotics reported here, it is conceivable that the elucidation of the detailed mechanisms by which the molecules interact with the cell membrane could augment current knowledge pertaining to the structure of the bacterial cell membrane. Certainly, these considerations justify continued study of the compounds responsible for the extracellular antibiosis exhibited by *B. bassiana*.

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