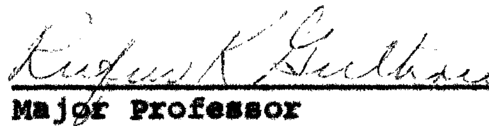
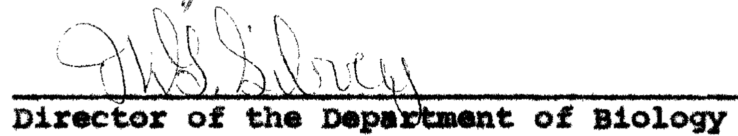
# A SEROLOGICAL STUDY OF THE ANTIGENIC FRACTIONS OF THE AEROBIC ACTINOMYCETES

APPROVED:

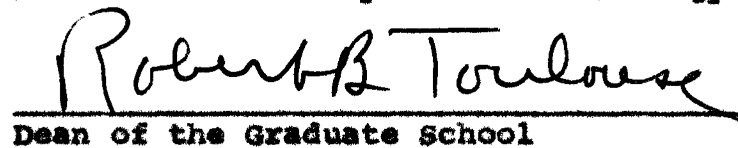


Major Professor

Minor Professor



Director of the Department of Biology



Dean of the Graduate School

# A SEROLOGICAL STUDY OF THE ANTIGENIC FRACTIONS OF THE AEROBIC ACTINOMYCETES

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Josephine Hutcheson, B. S.
Denton, Texas
June, 1964

## TABLE OF CONTENTS


| | | Page |
|---|---|---|
| LIST OF TABLES | | iv |
| Chapter | | |
| I. | INTRODUCTION | 1 |
| II. | MATERIALS AND METHODS | 7 |
| | Antigen Production | |
| | Cell Wall Antigens | |
| | Cytoplasmic Antigens | |
| | Antisera Production | |
| | Production of Antigen for Survey | |
| | Agar Precipitation Survey | |
| III. | RESULTS | 15 |
| | Separation of Antigenic Components | |
| | Cell Wall Antigens | |
| | Cytoplasmic Antigens | |
| | Agar Precipitation Survey | |
| IV. | DISCUSSION | 26 |
| V. | SUMMARY | 32 |

## LIST OF TABLES


| Table | | Page |
|---|---|---|
| I. | Actinomycetes Used for Survey | 12 |
| II. | Serological Groupings--Single Reactions with Single Antiserum | 20 |
| III. | Serological Groupings--Single Reactions with Multiple Antisera | 21 |
| IV. | Serological Groupings--Single and Multiple Reactions | 22 |
| V. | Serological Groupings--Multiple Reactions | 23 |

# CHAPTER I

# INTRODUCTION

The use of serological properties of microorganisms as a taxonimic tool is a device used by microbiologists with many types of organisms. It has only been recently, however, that this technique has been utilized in an effort to classify the *Actinomycetales*. A majority of the work done with this group has been concerned with the anaerobic, sometimes pathogenic species of *Actinomyces* and *Nocardia*. The aerobic actinomycetes, generally considered to be soil and water saprophytes and non-pathogenic, failed to interest many investigators until their antibiotic-producing properties were discovered. However, any resulting attempts of large scale serological study have been thwarted by their apparent failure to stimulate specific antibody production in test animals.

Guthrie, Roach, and Ferguson (3) reported a successful immunization schedule using adjuvant with sonically disrupted cells of *Streptomyces* species. Though the results of this survey demonstrated improved specificy of *in vitro* reactions using gel diffusion, multiple bands occurring in both homologous and heterologous reactions prevented any extensive attempt to group the species serologically.

Common antigenic components were reported by Cummins (1) to be present in all strains of Corynebacterium, Mycobacterium and Nocardia which had arabinose and galactose as their principle cell wall sugars. The antigens were not present in Actinomyces and Arthrobacter. In all cases, however, the preparations used were poorly antigenic and the investigator used as antigens whatever was effective for the particular strain --whether it was cell walls, whole cells or partially disintegrated cells.

Slack, Winger, and Moore (9) established four serological groups of Actinomyces, Corynebacterium and anaerobic diptheroid species by means of fluorescent antibody technique using reciprocal adsorption. There was no correlation between habitat, species designation and serological grouping. Tests of group antisera with Nocardia and Streptomyces revealed no apparent cross reaction.

Cummins and Harris (2) utilized the chemical composition of cell walls as shown by chromatographic technique to group Nocardia with Mycobacterium rather than with Actinomyces while Kwapinski and Snyder (7) found cytoplasmic fractions of Actinomyces and Mycobacterium to be antigenically related.

Kwapinski (6), in working with fifty-two chemically obtained fractions of Actinomyces and Nocardia, found

cytoplasmic materials were very closely related serologically, genus-specific antigens being located in the cell walls.

King and Meyer (5) extracted cell-free culture media with acetone in which species of Actinomyces and anaerobic diphtheroids had been grown for fourteen days and then removed the precipitate by centrifugation. The precipitate obtained by this extraction was used to stimulate antibody production in rabbits. Further serological tests revealed that Actinomyces israelii was serologically distinct from Actinomyces bovis while both species cross reacted with Actinomyces naeslundii. No cross reactions were shown between the anaerobic diphtheroids and any of the Actinomyces species.

Pirtle and Rebers (8) likewise used cell-free culture media of Actinomyces bovis, obtaining a nitrogen-containing complement-fixing antigen by precipitation with ethanol. Agar diffusion tests with rabbit antiserum prepared by intravenous injection of viable organisms showed single lines indicating some degree of homogeneity. No taxonomic applications were attempted here.

Guthrie, Roach, and Ferguson (4) reported the effects of growth medium and culture age on antigen levels in aerobic actinomycetes. The number and titer of antigens present were shown to be determined somewhat by the nutritive requirements.

Regardless of the medium used, antigenic components present in largest amounts reached a peak titer in the mycelium at two weeks of age followed by subsequent appearance in the medium. Standardization of conditions of growth medium and culture age increased the accuracy and specificity of serological reactions.

Numerous cross reactions and multiplicity of homologous reactions between strains of the aerobic actinomycetes have resulted in confusion and insurmountable difficulty in attempts to establish serological groups. It is the purpose of this investigation to alleviate this confusion by first testing means of separating the antigenic components of the aerobic actinomycetes--obtaining, if possible, a monospecific antigen. Antisera produced from these monospecific antigens will then be tested for their possible use in the serological grouping of the aerobic actinomycetes.

CHAPTER BIBLIOGRAPHY

1. Cummins, C.S., "Chemical Composition and Antigenic Structure of Cell Walls of Corynebacterium, Mycobacterium, Nocardia, Actinomyces, and Arthrobacter," Journal of General Microbiology, XXVIII (1962), 35-50.

2. Cummins, C.S. and Harris, H., "Studies on the Cell-Wall Composition and Taxonomy of Actinomycetales and Related Groups," Journal of General Microbiology, XVIII (1958) 173-189.

3. Guthrie, R.K., Roach, A.W., and Ferguson, J.K., "Serology of Aerobic Aquatic Actinomycetes. I. Factors Involved in Antiserum Production and In Vitro Antigen-Antibody Reactions," Journal of Bacteriology, LXXXIV (1962), 313-317.

4. Guthrie, R.K., Roach, A.W., and Ferguson, J.K., "Serology of Aerobic Aquatic Actinomycetes. II. Effects of Growth Medium and Culture Age on Antigen Levels," Journal of Bacteriology, LXXXVI (1963), 173-178.

5. King, S. and Meyer, F., "Gel Diffusion Technique in Antigen-Antibody Reactions of Actinomyces Species and Anaerobic Diphtheroids," Journal of Bacteriology, LXXXV (1963), 186-190.

6. Kwapinski, J.B., "Antigenic Structure of Actinomycetales. VI. Serological Relationships Between Antigenic Fractions of Actinomyces and Nocardia," Journal of Bacteriology, LXXXVI (1963), 179-186.

7. Kwapinski, J.B., and Snyder, M.L., "Antigenic Structure and Serological Relationships of Mycobacterium, Actinomyces, Streptococcus, and Diplococcus," Journal of Bacteriology, LXXXII (1961), 632-639.

8. Pirtle, B.C. and Rebers, P.A., "A Nitrogen-Containing Carbohydrate Antigen from Actinomyces bovis," Abstracts of the 63rd Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, Braun-Fromfield, Inc.,(1963).

9. Slack, J.M., Winger, A., and Moore, D.W., "Serological Grouping of Actinomyces by Means of Flourescent Antibodies," Journal of Bacteriology, LXXXII (1961), 54-65.

## CHAPTER II

## MATERIALS AND METHODS

### Antigen Production

Organisms used in the production of antigen were *Streptomyces griseus* (ATC #11429), *Streptomyces antibioticus* (ATC #8663), and *Streptomyces parvus* (ATC #11796). All cultures were maintained on liquid starch medium as reported by Williams and McCoy (3) in tubes containing slants of 1.0 per cent ion agar. The starch medium was modified to contain the following: 10 grams soluble corn starch, 5 grams of casamino acids, 0.5 grams of dibasic potassium phosphate, 0.5 grams of sodium chloride, and 0.1 grams of ferrous sulfate in one liter of distilled water. Flasks containing fifty milliliters of liquid starch medium were inoculated from stock culture and placed on shaker at room temperature. The contents of the flask, actively growing after twenty-four hours, were transferred to a one liter flask containing 500 milliliters of liquid starch medium which was placed on the shaker at room temperature until secondary surface mycelium appeared--usually four to seven days. The large flasks were then set aside. At two weeks of age, the

cultures were harvested by centrifugation and/or filtration and washed thoroughly with deionized water. The organisms at this point were either utilized immediately or were frozen for later study.

Organisms were subjected to sonic disintegration until of uniform consistency by a Branson Sonifier (2000 cycles). The homogenate was centrifuged at 3000 to 4000 rpm to deposit cell wall fractions. Further separation of antigen components was performed with the cell wall fraction and the cytoplasmic supernatant.

All tests for the determination of antigenicity of the fractions were by means of agar diffusion precipitation. Titers were determined by antigen dilution against antisera. Petri plates were prepared with 1.0 per cent ion agar containing phenol in a final concentration of 0.5 per cent. Wells were cut in the agar using a "Feinburg Agar Gel Cutter" (Consolidated Laboratories, Inc., Chicago Heights, Illinois) in a pattern of one large central well surrounded by six smaller symmetrically arranged wells. After application of antigen and antiserum, the plates were maintained in a humidifying chamber at room temperature for four days before results were recorded.

## Cell Wall Antigens

(1) Cell wall fragments were washed thoroughly with deionized water and tested for antigenicity.

(2) The washed cell wall fragments were then treated with purified trypsin after the method of Cummins and Harris (1). Fragments were incubated with crystalline trypsin (0.5 mg/ml) at 37° C for four hours, recentrifuged (4000 rpm) and both fragments and supernatant tested for antigenicity.

## Cytoplasmic Antigens

Each test was run separately. The numbered sequence following does not indicate a series.

(1) Cytoplasmic material was subjected to electrophoretic separation with a Shandon cellulose-acetate electrophoresis apparatus at five milliampheres for six to twelve hours.

(2) Cytoplasmic material was heated at 60° C for four hours. Coagulant was removed by centrifugation at 2000 rpm and the supernatant tested for antigenicity.

(3) A portion of the cytoplasm was digested with crystalline trypsin (0.5 mg/ml) for four hours at 37° C, centrifuged (4000 rpm) and supernatant tested for antigenicity.

(4) Cytoplasmic material was treated with streptomycin sulfate (final concentration of 1.0 per cent). The precipitate was washed thoroughly with phosphate buffer (pH 6.9) after

removal by centrifugation at 4000 rpm and resuspended in buffer. Both precipitate and supernatant were tested for antigenicity.

(5) Portions of cytoplasmic material were treated with sodium sulfate in concentrations varying from 26 per cent to 40 per cent. The precipitate thus obtained was removed by centrifugation at 4000 rpm and resuspended in physiological saline (0.85 per cent). The precipitate and the supernatant, after dialysis, were tested for antigenicity.

(6) Trichloroacetic acid, in final concentrations of 4.0 per cent was added to cytoplasmic material. The resulting precipitate was removed by centrifugation at 4000 rpm, resuspended in saline (0.85 per cent), and tested for antigenicity.

(7) Cytoplasmic material was placed in high speed, refrigerated International centrifuge and subjected to differential centrifugation at speeds of 3000, 5000, 7000, 10,000, 13,000, 15,000, and 17,500 rpm. Fractions obtained at these speeds were resuspended in saline and tested along with the supernatant for antigenicity. The supernatant remaining after centrifugation at 12,000 rpm was the fraction utilized in the subsequent production of antisera.

## Antisera Production

Antigenic fractions were mixed in 1:1 proportions with Freund's complete adjuvant and injected subcutaneously according to the schedule reported by Guthrie, Roach, and Ferguson (2); *i.e.*, three injections of two milliliters each every other day, one week of rest, a repeated injection schedule, and another week of rest. Antisera, in this manner, was produced first to whole cell homogenate of *S. griseus*, *S. parvus*, and *S. antibioticus*, and later to the antigenic fractions isolated from each of these species. Rabbits were bled each week for three weeks and the like antisera from at least two immunized rabbits were pooled. Phenol was added to the serum in a final concentration of 0.5 per cent and the serum preserved by freezing.

## Production of Antigen for Survey

Actinomycete cultures used in the serological survey were certain named species as shown in Table I, and the available marine actinomycete isolates of a numbered "H" series. The named species, as indicated in Table I, were obtained from either American Type Culture or through the courtesy of Dr. Elwood B. Shirling, Ohio Wesleyan University, Delaware, Ohio. The "H" series were organisms isolated by this laboratory.

TABLE I

ACTINOMYCETES USED FOR SURVEY

| | Actinomycete Species and Source |
|---|---|
| Antiserum Production | *Streptomyces griseus* (ATC #11429)<br>*Streptomyces antibioticus* (ATC #8663)<br>*Streptomyces parvus* (ATC #12433) |
| Antigens for Survey | *Streptomyces abikoensis* (ATC #12766)<br>*Streptomyces antibioticus* (ATC #8663)<br>*Streptomyces coelicolor* (ATC #10147)<br>*Streptomyces fradiae* (Shirling)<br>*Streptomyces gougerotii* (ATC #10975)<br>*Streptomyces griseolus* (ATC #11796)<br>*Streptomyces griseus* (ATC #11429)<br>*Streptomyces lavendulae* (ATC #8664)<br>*Streptomyces netropsis* (Shirling)<br>*Streptomyces parvus* (ATC #12433)<br>*Micromonospora chalcea* (ATC #12452) |

All cultures were grown in eight ounce screw top bottles containing a slant of 1.0 per cent ion agar and thirty milliliters of liquid starch medium. Both submerged and surface mycelium were harvested after two weeks growth, washed thoroughly with deionized water, sonicated, and frozen for later use.

## Agar Precipitation Survey

An extensive survey was run comparing antisera which had been produced in response to whole cell homogenate and antisera built to the antigenic fraction of those cells. In the first section of the survey, the antigen used consisted of the whole cell homogenate and in the second section, the antigen was the supernatant remaining after centrifugation of the whole cell homogenate at 12,000 rpm. In all cases, the antigen was placed in the wells surrounding the center well which contained the antisera. All reactions were read after four days at room temperature in a humidifying chamber. All named species and numbered isolated were grouped according to the number of precipitation bands produced in common with each of the three antisera.

CHAPTER BIBLIOGRAPHY

1. Cummins, C.S. and Harris, H., "The Chemical Composition of the Cell-Wall in Some Gram-Positive Bacteria and Its Possible Value as a Taxonomic Character," Journal of General Microbiology, XIV (1956), 583-600.

2. Guthrie, R.K., Roach, A.W., and Ferguson, J.K., "Serology of Aerobic Aquatic Actinomycetes. I. Factors Involved in Antiserum Production and In Vitro Antigen-Antibody Reactions," Journal of Bacteriology, LXXXIV (1962), 313-317.

3. Williams, A.M. and McCoy, E., "Degeneration and Regeneration of Streptomyces griseus," Applied Microbiology, I (1953), 307-313.

# CHAPTER III

# RESULTS

## Separation of Antigenic Components

Most of the procedures utilized for the separation of the whole cell homogenate into its various antigenic components did not prove satisfactory. The chemical processes for the most part resulted in either the loss or the alteration of antigenic properties.

## Cell Wall Antigens

(1) It was found that after thorough washing of the cell walls, most of the antigenicity was located in the washings. The cell walls themselves were only slightly antigenic after washing.

(2) The slight antigenicity remaining with the cell walls after washing was almost totally removed by digestion with trypsin. After centrifugation, the supernatant was found to contain some of the antigenicity originally present with the cell walls, but some precipitation bands had disappeared altogether.

## Cytoplasmic Antigens

(1) It was not possible to demonstrate any separation of components using electrophoresis.

(2) After heating cytoplasmic material at 60° C for four hours, it was found that many of the precipitin bands, both homologous and heterologous, were destroyed.

(3) Similarly, digestion with trypsin resulted in the removal of the majority of heterologous and homologous precipitin bands.

(4) Cytoplasmic material treated with streptomycin sulfate yielded a precipitate which, when suspended in buffer, did not exhibit any antigenic properties. The supernatant retained most of its antigenicity.

(5) The sodium sulfate treatment, of all the chemical procedures, showed the most promise as a possible means of fractionation of the actinomycete cytoplasmic material. The precipitate obtained after treatment with the salt, after resuspension in saline, exhibited considerable antigenic activity. Those bands, not appearing in the precipitate fraction, which had been originally present were retained in the supernatant. A series of treatments with varying concentrations of the salt would have appeared to be an ideal

method for separating the components of the cytoplasmic fractions; however, the procedure did not have universal application. In two of the actinomycete species tested, S. griseus and S. parvus, addition of the salt even to the saturation point failed to produce any type of precipitate.

(6) Trichloroacetic acid proved to be too drastic a treatment. Possibly the heaviest precipitate of all was obtained by this method, but the antigens were denatured completely. No antigenic activity was demonstrable by this method.

(7) Simple mechanical separation of the antigenic components was found to be possible by differential centrifugation. Most of the antigenic activity was removed with the heavier debris at 3000 rpm. Each fraction obtained at speeds above 3000 rpm up to 12,000 rpm exhibited limited antigenic activity which was relatively indistinguishable. None of the material deposited at speeds above 12,000 rpm appeared to be antigenic. The supernatant, remaining after 17,500 rpm, retained a limited amount of antigenicity. When checked by antisera produced to the whole cell homogenate, the supernatant appeared to be monospecific.

## Agar Precipitation Survey

The first section of the survey consisted of a comparison of antisera built to whole cell homogenate and antisera produced to the cytoplasmic fraction checked against the whole cell homogenates of the "H" series isolates and ATC species. The results of this survey did not show a reduction in cross reactions and multiplicity of reactions as was expected. Reactions were, however, considerably sharpened. For example, what appeared to be a broad, blurred precipitin band with the whole cell sera was actually shown to be several sharp, definite, narrow bands with the fraction sera. Multiplicity and number of reactions still prevented any attempt to group the actinomycetes.

The second section of the survey consisted of the same comparison of the whole cell sera and the fraction sera but the antigen in this case was the supernatant remaining after centrifugation of the whole cell homogenate at 12,000 rpm. The results of this survey demonstrated a marked decrease in the multiplicity of both homologous and heterologous reactions. This simplification of antigenic response made it possible to arrange the named species and the marine isolates in groups displaying similar serological properties as shown in Tables II through V. Most of the actinomycetes fell into five

large groups with fourteen other isolates making up twelve minor categories. The five main groups consisted of (1) those actinomycetes which did not react with any of the three antisera--including H72, H13, H48, H29, H65, H66, H61, H74 (all similar in pigmentation--buff-grey with no soluble exopigment); H11, H9, H10, H7, H129 (all white with no soluble exopigment); and one named species which did not fit into the three pigmentation categories, Micromonospora chalcea. (2) The second group consisted of those actinomycetes which reacted only with the S. parvus antiserum to produce a single precipitin band--including H128, H101, H82, H93, H85, H80 (all buff-grey with no soluble exopigment). (3) The third group consisted of those actinomycetes which produce a multiple reaction (two or more precipitin bands) with both S. parvus and S. griseus sera and no reaction with S. antibioticus serum --including H104 (buff-grey with no soluble exopigment); H121, H123, H135, H113 (buff with dark exopigment); H127, H115, H120 (white with no exopigment); and one name species which did not easily fit into the pigmentation categories, Streptomyces fradiae. (4) The fourth group consisted of the actinomycetes which produced multiple reactions with the S. parvus and S. griseus sera and a single precipitin band with the S. antibioticus serum--including H126, Streptomyces

TABLE II

SEROLOGICAL GROUPINGS--SINGLE REACTION WITH SINGLE ANTISERUM

(Antisera Produced to 12,000 rpm supernatant of *Streptomyces griseus*, *S. parvus*, & *S. antibioticus*)

| ANTIGEN COLOR GROUP | No reaction | Single parvus | Single griseus | Single antibioticus |
|---|---|---|---|---|
| Buff-grey (No sol. pig.) | H72 H13<br>H48 H29<br>H65 H66<br>H61 H74 | H128 H85<br>H101 H80<br>H 82<br>H 93 | H138 | H17 |
| Buff (With sol. pig.) | . . . | . . . | . . . | . . . |
| White (No sol. pig.) | H11 H129<br>H10 H 9<br>H 7 | . . . | . . . | . . . |
| Other | *Micromonospora chalcea* | . . . | . . . | . . . |

TABLE III

SEROLOGICAL GROUPINGS--SINGLE REACTION WITH MULTIPLE ANTISERA

(Antisera Produced to 12,000 rpm supernatant of *Streptomyces griseus*, *S. parvus*, & *S. antibioticus*)

| ANTIGEN COLOR GROUP | Single parvus<br>Single griseus | Single griseus<br>Single antibioticus | Single parvus<br>Single griseus<br>Single antibioticus |
|---|---|---|---|
| Buff-grey (No sol. pig.) | H30<br>H99 | . . . | . . . |
| Buff (With sol. pig.) | . . . | H124 | . . . |
| White (No sol. pig.) | . . . | . . . | . . . |
| Other | . . . | . . . | . . . |

TABLE IV

SEROLOGICAL GROUPINGS--SINGLE AND MULTIPLE REACTIONS

(Antisera Produced to 12,000 rpm supernatant of *Streptomyces griseus*, *S. parvus*, & *S. antibioticus*)

| ANTIGEN COLOR GROUP | Single parvus Multiple griseus | Single parvus Mul. antib. | Mul. griseus Single parvus Single antib. | Mul. griseus Mul. parvus Single antib. | Mul. griseus Single parvus Mul. antib. |
|---|---|---|---|---|---|
| Buff-grey (No sol. pig.) | . . . | H81 | . . . | H126<br>*S. griseolus* | . . . |
| Buff (With sol. pig.) | H137 | . . . | H108<br>H 91 | H 90<br>H106<br>*S. netropois*<br>*S. lavendulae* | H102 |
| White (No sol. pig.) | . . . | . . . | . . . | *S. parvus* | . . . |
| Other | . . . | . . . | . . . | . . . | . . . |

TABLE V

SEROLOGICAL GROUPINGS--MULTIPLE REACTIONS

(Antisera Produced to 12,000 rpm supernatant of *Streptomyces griseus*, *S. parvus*, & *S. antibioticus*)

| ANTIGEN COLOR GROUP | Multiple griseus | Mul. antib. | Mul. parvus<br>Mul. griseus | Mul. griseus<br>Mul. antib. | Mul. griseus<br>Mul. parvus<br>Mul. antib. |
|---|---|---|---|---|---|
| Buff-grey (No sol. pig.) | . . . | H56 | H104 | . . . | . . . |
| Buff (With sol. pig.) | S. abikoensis | . . . | H121<br>H123<br>H135<br>H113 | *S. antibioticus* | H118 H84<br>H105 H95<br>H131 H73<br>*S. coelicolor* |
| White (No sol. pig.) | . . . | . . . | H127<br>H115<br>H120 | . . . | H107<br>*S. gougerotii*<br>*S. griseus* |
| Other | . . . | . . . | *S. fradiae* | . . . | . . . |

griseolus (buff-grey with no soluble exopigment); H90, H106, Streptomyces lavendulae, Streptomyces netropsis (buff with dark soluble exopigment); and Streptomyces parvus (white with no soluble exopigment). (5) The fifth large group consisted of those actinomycetes which exhibited multiple reactions with all three antisera--including H118, H105, H131, H84, H95, H73, Streptomyces coelicolor (buff with dark soluble pigment); H107, Streptomyces gougerotii, Streptomyces griseus (white with no soluble pigment).

The minor categories included one or two isolates each: (1) single precipitin band with S. griseus serum--H138 (buff-grey with no soluble exopigment); (2) single precipitin band with S. antibioticus serum--H17 (buff-grey with no soluble exopigment); (3) single precipitin band with both S. griseus and S. parvus sera--H30 and H99 (buff-grey with no soluble exopigment); (4) single precipitin band with both S. griseus and S. antibioticus sera--H124 (buff with dark exopigment); (5) single precipitin band with all three antisera--H3 (buff-grey with no soluble exopigment); (6) multiple reaction with S. griseus serum--Streptomyces abikoensis (buff with dark exopigment); (7) multiple reaction with S. antibioticus serum--H56 (buff-grey with no soluble exopigment); (8) multiple

reaction with *S. griseus* serum and a single precipitin band with *S. parvus* serum--H137 (buff with dark exopigment); (9) multiple reaction with *S. antibioticus* serum and a single precipitin band with *S. parvus* serum--H81 (buff-grey with no soluble exopigment); (10) multiple reaction with both *S. griseus* and *S. antibioticus* sera--*Streptomyces antibioticus* (buff with dark exopigment); (11) multiple reaction with *S. griseus* serum and single precipitin bands with both *S. parvus* and *S. antibioticus* sera--H108, H91 (buff with dark exopigment); and (12) multiple reactions with both *S. griseus* and *S. antibioticus* sera and a single precipitin band with *S. parvus* serum--H102 (buff with dark exopigment).

# CHAPTER IV

## DISCUSSION

The primary obstacle in approaching the problem of separation of the antigenic components of the actinomycete cell was that the nature of these antigens and their location in the cell was unknown. The two principle portions of the cell, the cell wall and the cytoplasm, were subjected to standard chemical methods used to isolate various components of cellular material. Most of these chemical procedures did not prove satisfactory. Since the nature of the antigens was unknown, it was impossible to determine if antigenic activity retained after treatment had in any way been altered. The use of sodium sulfate to induce precipitation of protein material showed promise in that both precipitate and supernatant appeared to retain most of the antigenicity of the original cytoplasmic material. However, this technique failed to apply to all species investigated. In two of the actinomycete species tested, S. griseus and S. parvus, addition of the salt, even to the saturation point, failed to produce any type of precipitate. It is possible that the antigens demonstrable in

these species either were not protein in nature, or were protein bound to other types of compounds in such a manner as to prevent denaturation. The effects of such chemical compounds as trichloroacetic acid, streptomycin sulfate, and trypsin indicate that at least a portion of some of the antigenic components are protein. The results of heat application to cytoplasmic material would seem to indicate that the most likely explanation is that some of the antigens are protein in nature and the remaining antigens are of a different composition--probably polysaccharide in nature. The failure to obtain separation using electrophoresis could be explained in this manner, as polysaccharides would not be expected to migrate in an electric field.

The actinomycete antigens appear not only to be present in the cytoplasm of the cell, but are also bound in some fashion to the cell wall. The antigens apparently are bound by peptide linkage, as trypsin accomplishes their release. In opposition to the findings of Kwapinski (1) investigating the Nocardia and Actinomyces species, the cell walls, after washing to remove cytoplasmic material trapped in the debris, were essentially antigenically inert. The cell wall removal eliminated many of the cross reactions among the isolates indicating that the strain specific antigens are in the cytoplasmic material.

The best method of separation of the antigenic components of the actinomycetes proved to be that of a simple mechanical process-centrifugation. It was found that high speed centrifugation eliminated the majority of the antigens from cytoplasmic material leaving behind what appeared to be a monospecific antigen in the supernatant (12,000 rpm). It was later found that the monospecific antigen in reality stimulated antibody which produced a multiple homologous reaction. It is presumed that the cellular debris, which was contained in the whole cell homogenate used to produce the original antisera, masked these cytoplasmic antigens, thus preventing the formation of their specific antibodies. Therefore, the supposedly monospecific antigen actually consisted of several antigens which were not demonstrable with the whole cell serum.

Since the antigens used to stimulate antibody production were not monospecific, the results of the first survey were quite confusing from the standpoint of multiplicity of reactions. The precipitin bands were, however, considerably sharper and more definite. Again one assumes that the cellular debris used in production of the whole cell serum masked many antigens resulting in less specific reactions. This would tend to produce one large, broad band which would actually

consist of several narrow, sharp bands. In order to obtain workable results from a taxonomic viewpoint, the second survey utilized the reactions between the cell fraction antigen and the cell fraction antisera. The results showed a marked decrease in multiplicity of reactions and five large serological groups containing a total of forty-six isolates were demonstrated. Fourteen other isolates fell into twelve minor categories.

Two of the actinomycetes used to produce the grouping sera, S. parvus and S. griseus, were found to be almost serologically identical. It is significant to note that even with two sera which are so closely related, it is possible to obtain the serological separation that is reported in this investigation. Further expansion of this survey to include sera of other isolates would strengthen the validity of these serological groups and indicate the importance of each of the twelve minor categories. In no case was there any cross reaction between sera produced to the Streptomyces species and the actinomycete designated as belonging to the genus Micromonospora.

The actinomycetes are classified by Bergey according to their type of pigmentation. This investigator grouped all the "M" seried marine isolates and the named species using

their characteristic pigmentation into three general morphological groups: (1) the buff-grey group, none of which produced a soluble exopigment; (2) the buff group, all of which produced a dark exopigment; and (3) the white group, none producing an exopigment. It is to be observed in the data, that there was very little correlation between serological grouping and pigmentation. Only the group which reacts with a single precipitin band to *S. parvus* antiserum formed a homogeneous group in pigmentation--all were buff-grey with no soluble pigment. It may be concluded from this data that serological grouping of the actinomycetes is possible using the methods described in this investigation. This grouping shows very little correlation with the groups formed by present taxonomic criteria of pigmentation and gross morphological characteristics.

## CHAPTER BIBLIOGRAPHY

1. Kwapinski, J.B., "Antigenic Structure of *Actinomycetales*. VI. Serological Relationships Between Antigenic Fractions of *Actinomyces* and *Nocardia*," *Journal of Bacteriology*, LXXXVI (1963), 179-186.

# CHAPTER V

## SUMMARY

Mechanical means of separation of the antigenic components of the actinomycete cell were found to be desirable over that of chemical separation.

The use of cellular fractions of actinomycetes to stimulate antibody production produces antisera with highly improved specificity over that of antisera produced in response to whole cell homogenate.

The fraction antiserum, when tested in survey with antigens consisting of similar fractions of named species and marine isolates, demonstrated a drastic reduction in the number and multiplicity of homologous and heterologous reactions. This simplification of reaction permitted the serological grouping of sixty actinomycetes into five large groups containing forty-six isolated with fourteen other isolates forming twelve minor categories.

This grouping showed very little correlation with the groups formed by present taxonomic criteria of pigmentation and gross morphological characteristics.

## BIBLIOGRAPHY

### Articles


Cummins, C.S., "Chemical Composition and Antigenic Structure of Cell Walls of *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Actinomyces*, and *Arthrobacter*," *Journal of General Microbiology*, XXVIII (1962), 35-50.

Cummins, C.S. and Harris, H., "The Chemical Composition of the Cell-Wall in Some Gram-Positive Bacteria and Its Possible Value as a Taxonomic Character," *Journal of General Microbiology*, XIV (1956), 583-600.

Cummins, C.S. and Harris, H., "Studies on the Cell-Wall Composition and Taxonomy of *Actinomycetales* and Related Groups," *Journal of General Microbiology*, XVIII (1958), 173-189.

Guthrie, R.K., Roach, A.W., and Ferguson, J.K., "Serology of Aerobic Aquatic Actinomycetes. I. Factors Involved in Antiserum Production and *In Vitro* Antigen-Antibody Reactions," *Journal of Bacteriology*, LXXXIV (1962), 313-317.

Guthrie, R.K., Roach, A.W., and Ferguson, J.K., "Serology of Aerobic Aquatic Actinomycetes. II. Effects of Growth Medium and Culture Age on Antigen Levels," *Journal of Bacteriology*, LXXXVI (1963), 173-178.

Kang, S. and Meyer, F., "Gel Diffusion Technique in Antigen-Antibody Reactions of Actinomyces Species and Anaerobic Diphtheroids," *Journal of Bacteriology*, LXXV (1963), 186-190.

Kwapinski, J.B., "Antigenic Structure of *Actinomycetales*. VI. Serological Relationships Between Antigenic Fractions of *Actinomyces* and *Nocardia*," *Journal of Bacteriology*, LXXXVI (1963), 179-186.

Kwapinski, J.B., and Snyder, M.L., "Antigenic Structure and Serological Relationships of Mycobacterium, Actinomyces, Streptococcus, and Diplococcus," Journal of Bacteriology, LXXXII (1961), 632-639.

Pirtle, E.C. and Rebers, P.A., "A Nitrogen-Containing Carbohydrate Antigen from Actinomyces bovis," Abstracts of the 63rd Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, Braun-Brumfield, Inc., (1963).

Slack, J.M., Winger, A., and Moore, D.W., "Serological Grouping of Actinomyces by Means of Fluorescent Antibodies," Journal of Bacteriology, LXXXII (1961), 54-65.

Williams, A.M. and McCoy, E., "Degeneration and Regeneration of Streptomyces griseus," Applied Microbiology, I (1953), 307-313.