# DEVELOPMENT OF A DIFFERENTIAL MEDIUM FOR THE ISOLATION AND STUDY OF SOIL BACTERIA IN THE FAMILY RHIZOBIACEAE

by

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# INTRODUCTION

The group of bacteria commonly called "nodule bacteria" and classified under the generic name of Rhizobium has warranted considerable agronomic and bacteriological interest. Symbiotic nitrogen fixation by the rhizobia is of practical use in green manuring of soils prior to growing non-leguminous as well as leguminous crops. This symbiotic action converts the free nitrogen of the air into nitrogenous compounds in the plant. The importance of this group of soil bacteria is suggested (15, 24) by its use as a commercial inoculum for the Leguminosae. Mass cultivation and sale of rhizobia play a significant role in trade and agriculture. The distribution of rhizobia in the soil, longevity under field conditions, and the chemical activity of this flora in the vicinity of leguminous and other plants are problems about which little is known.

According to the classification set forth in Bergey's Manual of Determinative Bacteriology (9) three genera have been described in the family Rhizobiaceae. Only one genus, Rhizobium, can fix nitrogen when growing symbiotically in the roots of leguminous plants. The remaining two genera, Agrobacterium and Chromobacterium, do not fix nitrogen. Agrobacteria are plant pathogens and saprophytes, associated with the rhizosphere of plants. Chromobacteria are free-living, saprophytic soil and water forms.

Many aspects of the rhizobia have been investigated. Allen and Allen (2) state that few groups of bacteria have been so thoroughly investigated as the rhizobia. No direct and accurate method, however,

has been devised for enumeration and isolation of these bacteria from other soil microorganisms. The need for a truly selective medium has been emphasized by Fred, Baldwin and McCoy (16), Allen and Allen (2), and Katznelson, Lockhead and Temorin (21).

This paper presents evidence that the use of selective agents incorporated into a basal medium for the selective isolation of bacteria in the family Rhizobiaceae from soil is practical. A study of the groups of soil bacteria which grew on the devised media is presented.

# LITERATURE REVIEW

## Heterotrophic Non-Spore Forming Bacteria in the Soil.

Comparison of Major Groups. The rhizobia may be included in the general group of soil microorganisms known as heterotrophic non-spore forming rods, which are considered to be the predominate group of bacteria in the soil (13, 34). General characteristics of these bacteria are regarded as follows: sometimes coccoid, Gram-negative and producing carbonic acid from sugars (12). Included in this group are *Rhizobium*, *Pseudomonas* ("fluorescens" group), Crown Gall bacteria (*Agrobacterium* species), *Bacterium globiforme*, and *Alcaligenes*. Among these genera the "fluorescens" group (*Pseudomonas*) is especially abundant as shown by Hiltner and Stormer (original not seen, 34) and Conn (13). Stoklassa and Doerell (original not seen, 34) found that the organisms in the rhizosphere of plants consisted largely of non-spore formers.

According to Waksman (34) the study of heterotrophic non-spore forming bacteria in the soil has been neglected. If a soil bacteria did not have a role in nitrification, sulfur oxidation, nitrogen fixation, cellulose decomposition or produce ammonia they were assumed to be unimportant in the soil.

Information about the physiology, nutritional requirements and agronomic role of the heterotrophic non-spore forming bacteria in the soil is scant. The *Pseudomonas* group is known to decompose soil organic matter and is among the most strongly proteolytic types in the soil (13). The rapid liquefaction of gelatin distinguishes them easily from other soil bacteria. In addition, the formation of a blue pigment and the

fact that they generally accompany nitrogen-fixing organisms facilitates rapid identification (34). In this group of heterotrophs Bacterium caudatum, also named Pseudomonas caudatum and Flavobacterium reginse, flourishes in large numbers in soil and may be readily recognized by its orange color (34).

Conn (12) has suggested that Alcaligenes, Phytomonas, Rhizobium and Chromobacterium be placed in the same family, Rhizobiaceae, because they seem to possess the same general characteristics. In a recent paper Sguros and Harksell (32) reported that the achromobacteria appear to represent a strongly aerobic group of bacteria physiologically similar in many respects to such non-fermentative types as Alcaligenes, Phytomonas and Pseudomonas.

Bryan's work (10) using a medium consisting of one part of congo red per 20,000 parts of medium used for distinguishing Rhizobium from Phytomonas, Azotobacter and Achromobacterium indicates that these genera are essentially the types of organisms that will develop in the presence of an acid dye. Thus the foregoing information indicates in a general way that physiological similarities exist among the genera Phytomonas, Pseudomonas, Rhizobium, Agrobacterium, Azotobacter and Alcaligenes.

Nutritional and Cultural Requirements of Rhizobium.

Nitrogen Source. The rhizobia do not require an organic source of nitrogen for growth (2). As early as 1888 Beijerinck found that meat-peptone agar was not well adapted for culturing rhizobia (original not seen, 16). He isolated some of these bacteria on a medium of leguminous plant extract, sucrose, asparagine and gelatin. In 1890 Proznoski noted again the inability of the rhizobia to grow on meat-

extract-peptone-gelatin agar (16). He emphasized what he thought to be an ability of rhizobia to grow on a nitrogen-free medium. Fred, Baldwin and McCoy (16) state that the rhizobia can live and multiply in a medium almost free of combined nitrogen, and yet the nature of the nitrogen source is an important factor and determines to a large degree the rate of growth. The best sources are extracts of yeast, malt and plants. Further, it is known that nitrate and ammonium salts can be utilized.

This literature review would give the impression that rhizobia require certain nitrogen sources. Another interpretation is that the form in which the nitrogen is supplied is not so important as the oxidation-reduction potential poised by these substances. One growth stimulating effect of yeast extract has been shown by Allyn and Baldwin (6) to be caused by poising the oxidation-reduction potential in a range suitable for growth. These investigations also showed that a medium highly oxidized by addition of nitrate would allow growth only when sufficiently reduced by adding thioglycollic acid. This property of the rhizobia is certainly an outstanding cultural characteristic for consideration as a factor in an isolation medium for the rhizobia.

Allen and Allen (2) state that there is considerable versatility in the utilization of nitrogenous compounds by rhizobia. All the rhizobia can live and multiply sparingly in a synthetic medium composed of purified chemicals with and without added nitrogenous compounds (34). Their rates of respiration and multiplication are greatly improved by increasing the available nitrogen up to 108 ppm (35, 36). Laird and West (23) claim that the rhizobia require five to ten ppm of nitrogen for optimum growth.

Carbon Source. Nearly all the rhizobia can utilize mannitol, mono- and disaccharides and to a lesser degree tri- and polysaccharides, alcohols and sugar acids (2). According to Waksman (34) the best carbon sources are sucrose, glucose, maltose and mannitol. Mannitol is the best carbon source for growth (16).

The slow growing group of rhizobia, Rh. japonicum, Rh. meliloti, and the cowpea organism, are more specific in their carbon and nitrogen requirements. Arabinose is preferred to mannitol and other sources (2). Xylose has been deemed the most effective sugar for isolation of Rhizobium japonicum from soil (2). Although various carbon sources have been used successfully in culture media for the rhizobia, the compound most currently used and recommended is mannitol (2, 16).

Inorganic Sources. According to Allen and Allen (2) the inorganic requirements of the rhizobia have not been completely worked out probably because of difficulty of interference by the plant extracts used for growing them. Iron is essential for growth but not stimulatory (2), i.e., in the sense that increased amounts would improve growth. The mineral elements present in yeast extract provide a lead to the inorganic requirements because of the stimulatory effect of yeast extract. It contains iron, calcium, magnesium, strontium, sodium, potassium and lesser amounts of barium, manganese, copper, aluminum, lead, vanadium and silicon.

Special Nutritive Factors. A great deal has been accomplished in establishing the special growth requirements of the rhizobia. The stimulatory effect of plant extracts has long been known. Nutritive

and growth factors from peptone, seeds and seedlings, mold tissue, extracts of Azotobacter, Rhizobium species, nodules, yeast and sauerkraut have been reported (2).

Rh. trifolii Wisconsin strain 205 cannot synthesize biotin which is essential for its growth (2, 40). This strain also requires thiamine, riboflavin, pyridoxine, B-alanine, nicotinic, p-aminobenzoic acids and pantothenic acid. Slow growing rhizobia do not respond to biotin as do the fast growing strains.

Allen and Allen (2) sum up some of the more reasonable explanations for the stimulatory effect of yeast extracts: (1) the availability of various essential protein degredation products, (2) the presence of certain substrates favorable for respiration, (3) the stimulatory effect of vitamins and accessory growth factors, (4) the presence of certain trace elements, and (5) the poising of the oxidation-reduction potential in the range suitable for the growth of rhizobia. The stimulating effect of yeast extract is in direct proportion to its concentration in the medium (original not seen, 2). Biotin has been designated by Wilson (40) as the major growth factor although thiamin and riboflavin stimulate growth.

Media Employed for Culturing Rhizobia. Since Beijerinck's leguminous plant extract medium reported in 1888 many workers have reported other culture media for the rhizobia (16), and while not discussed here they were none the less important in contributing to what has culminated in a standard medium currently employed. The composition of this medium, yeast-mannitol-mineral salts, is taken from Fred, Baldwin and McCoy (16) and Allen (4).

| | |
|---|---|
| Mannitol | 10 gm. |
| $K_2HPO_4$ | 0.5 gm. |
| $MgSO_4$ | 0.2 gm. |
| NaCl | 0.1 gm. |
| $CaCO_3$ | 3.0 gm. |
| Yeast Extract 10% | 100 ml. |
| Dist. $H_2O$ | 900 ml. |
| Agar | 15 gm. |

To Prepare Yeast Extract: Steam 100 gm. of starch-free pressed yeast 3-4 hours in 1,000 ml. of water. Allow to stand one week. The straw-colored supernatant liquid should be siphoned, bottled in 100 ml. quantities and sterilized at 15 pounds pressure for 45 minutes.

The advantages of this medium over many others are: It (1) supports very good growth of all species of rhizobia, (2) possesses simplicity of preparation and sterilization, and (3) is uniform in composition and thus may be depended on for uniform results.

The recent reports on a medium developed by Albrecht and McCalla (1, 2) deserve special attention. The use of sauerkraut juice provides special stimulating growth factors and addition of calcium gluconate affords a soluble form of calcium. This form of calcium has advantages over calcium carbonate in that there is no precipitate to confuse colony counts on plates and the gluconate lowers the surface tension at the cell-medium interface and thus may furnish a supply of this nutrient.

Additional Biochemical Characteristics of Rhizobium. Rhizobia have been classified into two divisions on the basis of growth rate (2, 16). Fast-growing rhizobia are Rhizobium meliloti, Rh. trifolii, Rh. leguminosarum and Rh. phaseolus since they produce turbidity in broth and grow well on the surface of agar in five to seven days. The slow-growers require nine to twelve days or longer for growth and include Rh. japonicum, Rh. lupini, Rh. species and others.

Rhizobium species are aerobic, yet can grow under reduced oxygen tension (2, 16).

Certain strains produce slow reduction of litmus milk followed by slight proteolysis and formation of a serum zone believed to be the result of settling of suspended particles (16). Others do not reduce litmus at all.

The optimum temperature of growth for the rhizobia is 29-31°C. except for Rh. meliloti which has an optimum at 35°C. (2).

Gelatin is liquefied very slowly if at all (2, 16) and in general occurs at the top of the media with no liquefaction in one to two weeks. Some liquefaction may occur in two to three months. This action is never as fast as observed with many of the common soil organisms (16). The alfalfa group is particularly active in gelatin liquefaction (16).

The rhizobia are definitely not gas formers and usually produce little acid, their fermentation of sugars usually proceeding to carbon dioxide. Fred, Baldwin and McCoy (16) report that sucrose and maltose are fermented by most rhizobia but not by Agrobacterium radiobacter. All species of Rhizobium have about the same tolerance to alkalinity. Rh. meliloti is the most sensitive to acidity with a limit of pH 5.0. Rh. lupini and Rh. japonicum are the most tolerant with limits at pH 3.2-4.0.

Previously Devised Methods for Isolation of Rhizobiaceae from the Soil.

Methods and media developed for the purpose of isolating bacteria in the family Rhizobiaceae are outlined in the following paragraphs. In addition, statements have been made concerning their suitability or unsuitability for enumerating and isolating the Rhizobiaceae. Methods have been classified as indirect and direct. Indirect methods involve

selective isolation based on the ability of the rhizobia to invade the roots of leguminous plants; direct methods involve the selective isolation directly on agar plates.

A. Indirect Method.

1. An indirect method of isolating bacteria in the genus Rhizobium from the soil is based on the principle that some strains of these organisms will invade the leguminous roots causing the formation of nodules. This method has been used by Wilson (39) to determine the legume bacterial population in soils. Sterile seeds of leguminous plants are treated with soil suspensions and become inoculated by the rhizobia present in the soil. Using a serial dilution of the soil samples a relation may be obtained between the number of nodules that develop and the number of rhizobia in the dilutions. Therefore, estimates of numbers can be made and the bacteria may be isolated from the nodules that develop. This method is useful for isolation but is inaccurate for estimation of numbers of bacteria in the soil because of the elaborate technique and indirect procedure involved. The correlation between the number of nodules that develop on the roots and the number of bacteria in the soil dilutions lacks definite proof because nodule formation does not depend entirely upon the number of bacteria present. The possibility of error by this method is great.

B. Direct Methods.

1. A satisfactory method for the direct isolation of rhizobia from soil was devised by Budinov (original not seen, 16, 34).

Capillary tubes were filled with a sterile yeast-mannitol medium and placed in a soil suspension for a period of one hour. The contents of the tube were then plated on a brom-thymol blue-yeast-mannitol agar. On the poured plates incubated for 12 hours about 90% of the colonies were rhizobia. This method may be used to increase the growth and multiplication of rhizobia in the capillary tubes but these bacteria would not necessarily develop in direct proportion to the actual population in the soil.

2. Soil previously sterilized and inoculated with a strain of Rhizobium from alfalfa nodules was plated by Kellerman and Leonard (22) on the Grieg-Smith medium which is composed of levulose, asparagine, sodium citrate and potassium citrate. While growth of Rhizobium was favored on this medium, selection of Rhizobium meliloti from sterile soil inoculated with this organism did not occur.

3. Two media were developed by Lipman and Fowler (25). One medium was composed of maltose phosphate and sulfate salts with minute quantities of NaCl, $FeCl_3$, $MnSO_4$, $CaCl_2$, agar and distilled water. Neither of the two media eliminated growth of fungi and bacteria as contaminants on plates of rhizobia.

4. A method for direct isolation of cultures from soil was described by Allen and Baldwin (3) who repeated the Budinov method by which root nodule bacteria can be readily isolated from the soil. A yeast-water-mannitol medium was the most selective basal medium for isolation of Rhizobium meliloti

and Rhizobium japonicum. This medium cannot be used to estimate numbers of the bacteria in soil because these organisms are not increased in proportion to their actual population in the soil.

5. Pohlman (30) used a yeast-mannitol agar with and without the addition of dyes. He found that rosaniline hydrochloride, acid fuchsin, congo red and pholoxine red were the least inhibitory dyes to rhizobia. However, he reported results which indicated that none of these dyes were active selective agents. He concluded that no suitable medium had been developed for the direct isolation of rhizobia.

6. Bryan (10) reported that the use of congo red in concentration of one part per 20,000 parts of the nitrogen-free medium of Ashby could be used for distinguishing rhizobia directly from other bacteria in the soil. Agar plates were inoculated and capped with a second layer of agar to assure subsurface colonies. Achromobacteria and rhizobia colonies were white, whereas Phytomonas and Azotobacter were red and pink, respectively. Thus, the total count of achromobacteria and rhizobia could be made by counting the white colonies. Similar counts could be made of achromobacteria in a medium at pH of 11 on which rhizobia will not grow; the difference in the above counts gave estimates of the numbers of rhizobia.

7. A determination of Agrobacterium radiobacter in the soil was proposed by Hofer (19). His medium was composed of calcium gluconate, diethyl potassium phosphate, $KNO_3$, NaCl, $MgCl_2$,

$MgSO_4$, agar and distilled water adjusted to a pH of 8. Ninety per cent of colonies which developed on these plates were identified as Agrobacterium radiobacter, yet many fluorescent colonies grew.

8. Anderson (7) found that one part of crystal violet dye in 7,500 parts of medium was useful for isolation of Rhizobium leguminosarum but not for plate counts because of the strong killing action of this dye. He indicated that brilliant green and malachite green exhibited some selective action for the alfalfa and bean organisms. He found that safranine, rose bengal and eosin Y were too weak to be useful below a concentration of one part of dye in 1,000 parts of medium.

The methods described above were devised for isolation of either rhizobia or agrobacteria. The medium of Hofer is considered quite suitable for the isolation and estimation of Agrobacterium radiobacter in soil. Those devised for rhizobia have not met with success as a practical direct method for isolation. As yet, no special medium has been developed to isolate the entire group of Rhizobium species.

Recent Experimental Work Leading to this Investigation. Smith and Dawson (33) developed a rose bengal medium specifically for the fungi in the soil. The effect of the rose bengal dye is to inhibit growth of the bacteria and to confine the fungal colonies in order that they may be counted easily on agar plates. These investigators, however, observed the presence of a few "soft, raised glistening colonies that would not be confused for fungal colonies." Gamble and Orcutt (18) reported that these bacteria appeared to be members of the family Rhizobiaceae. They

also suggested the possibility of using rose bengal dye for the differential isolation of bacteria in this family. That these colonies were, in fact, members of this family was not completely tested.

# THE INVESTIGATION

## Object of the Investigation.

The object of this study was to investigate the possibility of developing a suitable medium for the differential isolation of bacteria in the family Rhizobiaceae from soil.

## Plan of Approach.

The plan of approach to this problem is outlined below:

A: To determine what bacteria would grow on the rose bengal agar of Smith and Dawson (33).

1. Pure cultures of Rhizobiaceae were inoculated on the unmodified medium to determine which of these bacteria would develop.
2. All cultures that developed on this medium from soil were classified.

B: To select a basal medium and modify it in various ways in an attempt to devise a specific medium for isolating and enumerating bacteria in the family Rhizobiaceae. Possible modifications were based on the following studies.

1. To determine the bacteriostatic activity of various dyes on representative organisms.
2. To determine the relative inhibition of certain dyes on pure cultures of rhizobia and the most common contaminants on rose bengal agar.
3. To determine the effect of adjusting the pH to a more alkaline range.
4. To determine whether the oxidation-reduction potential can be poised to allow growth of rhizobia and not of the contaminants.

5. To test certain antibiotic substances for the ability to select rhizobia.
6. To test the most promising combinations of dye, basal medium, pH, oxidation-reduction potential, and antibiotic to obtain the most suitable medium for the selective isolation of rhizobia.
7. To test the newly developed medium by isolation of bacteria from nodules of leguminous plants and from soils seeded with known cultures of rhizobia.

Experimental Procedures.

Determination of Growth on Agar Slants. Agar slants of rose bengal agar were inoculated with pure cultures of Rhizobium leguminosarum, Rh. trifolii, Rh. meliloti, and Rh. japonicum. Growth was estimated after incubation at 30°C. for three weeks and the results were recorded as abundant growth, good growth, slight growth, and no growth.

Isolation of Pure Cultures of Bacteria. Soil samples were selected from the rhizosphere of various leguminous plants and deflocculated with sodium metaphosphate (17, 30). Dilutions were made and plated out on rose bengal agar of Smith and Dawson (33). Bacterial colonies were selected at random from the plates in order to obtain several representative types of organisms. Inocula of these colonies were transferred to slants of yeast extract-mannitol agar. These cultures were than plated out by the pour plate method for isolation of pure cultures until all the colonies appearing on the plates were identical. This colonial uniformity was used as the criterion of pure culture.

In the isolation procedure the dilutions were agitated vigorously for fifteen minutes to enhance separation of various types of organisms, if they were present.

Identification Procedure. The scheme for identification of bacteria followed the outline in Bergey's Manual of Determinative Bacteriology (9) and Skerman's "A Mechanical Key for the Generic Identification of Bacteria" (31).

The cultures, for the most part, were classified as to family. Certain organisms were chosen to represent the bacteria in the families as physiological groups. These organisms were identified in the generic classification status.

Preliminary Evaluation of the Bacteriostatic Effect of Certain Dyes. For this preliminary work the presence or absence of growth on poured agar plates containing given dye concentrations and seeded with a given amount of inoculum served as the criterion of inhibition. The basal medium was yeast extract-mannitol mineral salts medium of Fred, Baldwin and McCoy (16). Yeast extract was used in a concentration of 0.5% as obtained in dehydrated form from DIFCO Laboratories, Inc.

Solutions were made up with 1% ethyl alcohol to insure adequate solubility and distribution of the dye throughout the medium. After sterilization separately the dyes were added to the melted agar medium.

The inoculum consisted of 12-48 hour cultures depending on the period of incubation required by each organism to attain visible growth. 0.5 ml. of this suspension was introduced into sterile petri dishes with melted agar. Temperature of incubation was 30°C.

For each experiment three petri plates were poured for the dye and three for the control medium without dye. Three separate experiments were conducted to minimize errors of technique.

Plate Count Method for Determination of Dye Sensitivity of Bacteria. The purpose of this procedure was to determine the extent of the bacteriostatic effect of dyes on various bacterial strains. The extent of bacteriostatic action was based on relative plate counts which converted into percentage of inhibition.

For each organism tested at least five plates were poured for controls and five plates for each dye concentration. Each individual count was tested to insure that it was within the standard deviation from the mean. Three individual series were conducted for each concentration of dye tested. Plate counts were then averaged and calculated as percentage of inhibition.

The basal medium used was a yeast extract-mineral salts-soil extract medium. The quantity of inoculum was adjusted so that plate counts on the control medium were between 30 and 300. An attempt was made to adjust these counts as close to 300 as possible since it was desired to use the largest possible inocula.

The organisms were grown in a 0.25% yeast-glucose broth until growth was visible (from 12-48 hours depending on the organism). 0.2 ml. of a 1-10,000 dilution was used for Rhizobium trifolii and Pseudomonas and 0.2 ml. of a 1-100,000 dilution was necessary for Flavobacterium. Temperature of incubation was at 30° C. for a period of two weeks.

Effect of Hydrogen-Ion Concentration on the Growth of Bacteria. Preliminary tests showed that the production of turbidity in tubes of liquid media as determined both by the Klett-Summerson Photoelectric Colorimeter and by visual observation of turbidity could be used as a criterion of growth of bacteria when subjected to various hydrogen ion concentrations. While the turbidimetric measurements were more accurate, the visual observation of turbidity was found to be more convenient and less involved, and this method was used.

The simple addition of acid or base to the medium proved insufficient in maintaining continued constant pH. A NaOH - $K_2HPO_4$ buffer was effective in maintaining the pH.

The control consisted of the basal medium with no dye at pH 6.8. The basal medium was a yeast extract-mannitol-mineral salts-soil extract medium. Ten ml. of medium were pipetted into each tube. The pH of the media was adjusted by addition of $K_2HPO_4$ up to 5 gm/liter and the addition of 0.5 M NaOH. Tubes were inoculated with a standard loop from 24-hour cultures and checked for growth at four and seven days.

Effect of Oxidation-Reduction Potential on the Growth of Bacteria. The oxidation-reduction potential of the media was controlled by addition of $KNO_3$. The criterion of effect was the production of visible turbidity as an indication of growth. The experiment was conducted at several pH's.

Determination of Bacterial Sensitivity to Antibiotics. The method as outlined below is a modification of the method of Clancy (11). The purpose of this method was to test the sensitivity of bacteria to various

concentrations of antibiotic. The method is essentially that of inoculation of a constant quantity of a suspension of the organism in question into a given quantity of the desired medium containing the known amount of antibiotic. Sensitivity was recorded as the smallest quantity of antibiotic that completely inhibited growth.

Three tubes of 0.5 ml. of liquid yeast-mannitol-mineral salts medium were sterilized and to these were added 0.1 ml. of the desired concentration of the antibiotic taken from a stock solution prepared so as to contain a known amount of the antibiotic. Inoculations were made and the cultures were incubated for one week or longer at a temperature of 30°C. To check for possible contamination where the antibiotic was not autoclave sterilized, extra controls of antibiotic treatments were not inoculated.

The inoculum consisted of a 12-48 hour broth culture diluted to 1-100 or to a point where no visible turbidity was present. The antibiotic was added after sterilization of the tubes of media. The stock solutions of antibiotic were made up immediately before introduction into the media.

Source of Nodules and of Soils Inoculated with Rhizobium. Nodules and soils were obtained in two ways. In one case seeds of alfalfa, clover, pea and soybean were inoculated with known species of rhizobia from pure cultures of the Virginia Polytechnic Institute Bacteriology Laboratory and from the commercial inoculum, "Nitragin." Seeds were then planted in untreated soil in pots and a suspension of the bacteria sprinkled on the surface of the soil. Plants were grown in the greenhouse.

Most of these plants grew well and developed nodules. Thus, a source of leguminous plant nodules and soils into which known cultures of rhizobia had been introduced served as the test inoculum for selective media.

In the second case, intact plants of alfalfa and clover were removed from the field and placed in pots and tended in the greenhouse. This technique provided a natural source of nodules and rhizosphere soil.

Confinement of Fungi. The techniques for confining fungal growth and methods for preliminary testing of fungicides are listed below:

1. Anaerobic plates. The agar was introduced into deep petri dishes after inoculation with soil and, after mixing, the bottom half of the dish was sealed with scotch tape and modeling clay to another half petri dish poured with agar and streaked with Bacillus subtilis or E. coli. This method was based on the principle that aerobic bacteria either with a high rate of oxygen consumption or a rapid growth consume the oxygen available in a sealed petri dish, leaving insufficient oxygen for growth of other aerobic microorganisms.

2. Application of Mineral Oil. The plates were inoculated and poured in the usual manner and after the agar had solidified a heavy layer of sterile mineral oil was applied. This was based on the contention that the oxygen might be confined to a point where fungal growth would be limited.

3. Capping the Media with Plain Agar-Water. The inoculated agar plates were capped with inert sterile agar to limit the supply of oxygen and to prevent both the physical spreading and massive mycelial growth of fungi (38).

4. Use of Fungicides. Certain fungicides were selected on the basis of their effect on fungi and on bacteria. Some fungicides which were reported in the literature (14, 27) to be fungistatic but to have little effect on bacteria seemed to be most promising. These compounds were incorporated into the medium and inoculated with soil for preliminary information about effect on fungal growth.

Cultural and Physiological Tests Employed for Identification of Rhizobium. The ability of the rhizobia to form nodules on the roots of leguminous plants and the subsequent fixation of nitrogen are the only reliable criteria for their identification (2). Such determinations are not only time-consuming but require special equipment outside the laboratory. Many isolates of rhizobia strains are divergent from the established prototypes described in Bergey's Manual (9) probably because these types were confined to a small number of strains of rhizobia.

In this investigation rapid screening tests were essential because of the large number of isolated cultures to be identified. It was desirable to select as few tests as possible and yet make use of the most prominent distinctive physiological and cultural characteristics for identification. Selected tests are listed below and are qualified on the basis of the general characteristics of the groups of bacteria concerned.

(1) Action on litmus milk was used not only for distinguishing rhizobia from Agrobacterium radiobacter but also for a general distinction of rhizobia from other soil organisms. The action of Agrobacterium radiobacter on litmus milk is usually a slow reduction of the litmus followed by a browning of the milk in

which a pellicle and serum zone develop (9, 20). Rhizobium either does not reduce litmus or may do so very slowly and usually produces a serum zone depending upon the species (9, 16). Groups of soil bacteria other than rhizobia in the nodule or associated with the rhizosphere would be expected to reduce litmus. A very slow reduction of litmus followed by formation of a serum zone in the case of Rhizobium trifolii, Rh. phaseoli, and Rh. leguminosarum would indicate the presence of these types (2, 9, 16). Species of Pseudomonas possessing a high degree of proteolytic activity will rapidly reduce litmus and form a serum zone. Also many of these types will impart a green or blue color to the serum zone. Achromobacter will not greatly alter litmus milk but a reduction and slight acid to alkaline reaction may ensue (9). Alcaligenes may or may not peptonize milk and usually reacts alkaline (9).

(2) The action on gelatin was employed as a general classification test for Pseudomonas because of the usual rapid liquefaction by these types as compared to no or very slow liquefaction by rhizobia and Agrobacterium (9, 34). Achromobacter and Alcaligenes may or may not liquefy gelatin (9).

(3) The action of soil bacteria on carbohydrates is of some use as a differential test among rhizobia, Pseudomonas and Achromobacterium. Rhizobium will grow well on mono- and disaccharides and will utilize these sugars without formation of acid or gas (9, 16). This is also true of Agrobacterium radiobacter, yet all sugars, glycerol and mannitol are utilized. The

achromobacteria may form acids from hexoses but no gas and are generally characterized by feeble power of attacking carbohydrates. Alcaligenes usually produces no acids from hexoses. Species of Pseudomonas frequently ferment glucose, sometimes with visible gas formation, but are inactive in the fermentation of lactose.

(4) The characteristics of growth on calcium glycerophosphate agar are useful for distinguishing between Rhizobium and Agrobacterium radiobacter. The Agrobacterium radiobacter turns brown or has a brown halo with a surrounding white precipitate (9, 20) but Rhizobium does not. Agrobacterium radiobacter is a common contaminant of Rhizobium in the nodule when isolations are made on agar plates and the two are similar in general appearance and cultural characteristics. Many of the tests devised for distinguishing between these organisms are listed by Allen and Allen (2).

Other useful tests for detecting the type organism isolated from soil are the characteristics of colonial growth and growth on agar slant (9).

Results.

Growth of Rhizobium japonicum, Rh. leguminosarum, Rh. meliloti and Rh. trifolii on the rose bengal agar of Smith and Dawson. Experiments were set up to determine if several species of Rhizobium would grow on the rose bengal agar of Smith and Dawson (33). Pure cultures of these organisms were inoculated on the surface of rose bengal agar slants

and the amount of growth was recorded after prolonged incubation. The bacterial cultures were obtained from the Bacteriology Department of the Virginia Polytechnic Institute.

In Table I the results of the relative amounts of growth of Rhizobium japonicum, Rh. leguminosarum, Rh. meliloti and Rh. trifolii on rose bengal agar with soil extract are shown. Rhizobium trifolii grew well at all dye concentrations. Although the other species grew at a dilution of 15,000 of rose bengal the quantity was considerably less.

The results of this experiment are that the four species of Rhizobium were able to grow on the rose bengal agar of Smith and Dawson at a dilution of 15,000 of rose bengal.

Further experiments were conducted to determine if these organisms would grow on rose bengal agar without soil extract. In all cases no growth developed.

These results justified an investigation of the identity of the bacteria which would develop on rose bengal agar when inoculated with soil.

Soil Bacteria that Develop on Rose Bengal Agar. Since Rhizobium, the organism to be isolated, grew well on rose bengal agar, it followed that the identity of the groups of bacteria in the soil which would grow on this medium should be elucidated so that further steps could be taken to eliminate the undesired groups.

Several isolates were taken from plates of rose bengal agar inoculated with soils from different sources. These organisms were then classified according to family. Bacterial colonies isolated from

Table I

GROWTH OF FOUR SPECIES OF RHIZOBIUM ON ROSE BENGAL AGAR[1] INCUBATED FOR THREE WEEKS AT 30°C.

| | Dye Concentration parts dye/ parts medium | | | |
|---|---|---|---|---|
| Organism | 0 | 1/10,000 | 1/15,000 | 1/20,000 |
| Rh. japonicum | ✓ ✓ | - | ✓ | ✓ |
| Rh. leguminosarum | ✓ ✓ | - | ✓ | ✓ |
| Rh. meliloti | ✓ ✓ ✓ | - | ✓ ✓ | ✓ ✓ |
| Rh. trifolii | ✓ ✓ ✓ | ✓ ✓ ✓ | ✓ ✓ ✓ | ✓ ✓ ✓ |

✓ ✓ ✓ abundant growth, ✓ ✓ good growth, ✓ slight growth

\- no growth

The data represent the average results obtained from two replications of four slants each.

[1] Rose Bengal Agar of Smith and Dawson (33)

| | |
|---|---|
| Glucose | 10.0 gm. |
| $NaNO_3$ | 1.0 gm. |
| $K_2HPO_4$ | 1.0 gm. |
| Agar | 15.0 gm. |
| *Soil extract | 1000.0 ml. |

*Soil extract is prepared by autoclaving 500 gm. of good field soil in 1,200 ml. water for one hour and filtering.

the rhizosphere soil of several leguminous plants were raised, translucent, glistening, wet and entire. These colonies were noted in one to two days when incubated at a temperature of 30°C. The bacterial growth was extensive when the inoculum was 1-100 or 1-10 dilution of soil. After four days the plates became overcrowded with fungi which exhibited a strong antibiotic effect on the bacterial colonies.

The results of identification of twenty-four organisms selected at random from several plates are presented in Table II. Most of the colonies were identical in colonial appearance. These were later identified in the families Pseudomonadaceae and Achromobacteriaceae. The only other types among the isolates were one culture of Azotobacter and two cultures of Bacillaceae from 30 different plates examined. Ten of the twenty-four organisms were in the family Pseudomonadaceae and eleven were in the family Achromobacteriaceae. Therefore, 87% of all the organisms isolated and identified were in one of these two groups.

Certain organisms were selected which seemed to represent the predominate groups of bacteria that developed on rose bengal agar and were tentatively identified as Pseudomonas and Flavobacterium.

Preliminary Evaluation of the Bacteriostatic Effect of Certain Dyes on Growth of Flavobacterium, Pseudomonas and Rhizobium trifolii. Experiments were conducted to ascertain which of certain dyes was more inhibitory for Flavobacterium and Pseudomonas than for Rhizobium trifolii. It was believed that in this way the investigation could be confined to only a few dyes; and, that an idea might be obtained as to which dye could be combined with rose bengal to eliminate growth of Pseudomonas

Table II

SOIL BACTERIA RECOVERED FROM ROSE BENGAL AGAR PLATES

| No. of Isolates | Source of Inoculum | Family | Genus |
|---|---|---|---|
| 1-6 | rhizosphere soil of Clover | Achromobacteriaceae | Flavobacterium |
| 7-8 | rhizosphere soil of Clover | Pseudomonadaceae | |
| 9-14 | rhizosphere soil of Alfalfa | Pseudomonadaceae | Pseudomonas |
| 15 | rhizosphere soil of Bean | Bacillaceae | |
| 16-17 | rhizosphere soil of Bean | Achromobacteriaceae | |
| 18-19 | rhizosphere soil of Bean | Pseudomonadaceae | |
| 20 | rhizosphere soil of Peanut | Bacillaceae | |
| 21 | rhizosphere soil of Peanut | Azotobacteriaceae | |
| 22 | rhizosphere soil of Peanut | Pseudomonadaceae | |
| 23-24 | rhizosphere soil of Bean | Achromobacteriaceae | |

and Flavobacterium while allowing Rhizobium trifolii to grow. Dyes which have been reported in the literature to show some degree of selectivity for rhizobia from other soil organisms were used.

The results of the bacteriostatic action of crystal violet are shown in Table III. The toxicity of this dye for Rhizobium trifolii, even at a dilution of 1/500,000, was great, for only a few colonies developed on the plates. Pseudomonas did not grow well at any concentration of crystal violet, while Flavobacterium grew abundantly at all concentrations tested.

The comparative bacteriostatic effect of rose bengal on the three test organisms is given in Table IV. In all cases concentrations of 1/5,000 to 1/20,000 allowed abundant growth of Rhizobium trifolii and Pseudomonas. There was little difference in the tolerance of these organisms to rose bengal. Flavobacterium grew only slightly at concentrations of 1/12,000 and less, never approaching good growth.

Safranine O (Table V) was less inhibitory to Flavobacterium than to the other organisms; abundant growth occurred at concentrations from 1/20,000 to 1/500,000. This dye was more toxic for Pseudomonas and Rhizobium with only slight growth development at dilutions of 1/500,000 and 1/100,000, respectively.

Rhizobium trifolii and Flavobacterium grew abundantly in the presence of 1/20,000 congo red, whereas Pseudomonas grew only slightly at 1/40,000 as represented in Table VI.

Table VII demonstrates that growth of Rhizobium trifolii was favored over the other two organisms on brilliant green at concentrations less than 1/15,000 but no growth occurred at 1/5,000 for any of the organisms.

Table III

EFFECT OF CRYSTAL VIOLET ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Dye Concentration Parts Dye/ Parts Media | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1/20,000 | 1/50,000 | 1/80,000 | 1/100,000 | 1/500,000 |
| *Flavobacterium* | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| *Pseudomonas* | ++++ | + | + | + | + | ++ |
| *Rhizobium trifolii* | ++++ | - | - | + | + | + |
| *Bacillus subtilis* | ++++ | - | - | - | - | - |

++++ abundant growth; ++ good growth; + slight growth; - no growth

Plates incubated for 18 days at 30°C. Data represent the average results of three replications of three plates.

Table IV

EFFECT OF ROSE BENGAL ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS*, AND *RHIZOBIUM TRIFOLII*

| Organism | Dye Concentration Parts Dye/ Parts Media | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1/5,000 | 1/10,000 | 1/12,000 | 1/15,000 | 1/20,000 |
| ***Flavobacterium*** | ++++ | - | - | + | + | + |
| ***Pseudomonas*** | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| ***Rhizobium trifolii*** | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| ***Bacillus subtilis*** | ++++ | - | - | - | - | - |

++++ abundant growth; +++ good growth; + slight growth; - no growth

Plates incubated for 14 days at 30°C. Data represent the average results of three replications of three plates.

Table V

EFFECT OF SAFRANINE O ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Dye Concentration Parts Dye/ Parts Media | | | |
|---|---|---|---|---|
| | 0 | 1/20,000 | 1/100,000 | 1/500,000 |
| *Flavobacterium* | + + + | + + + | + + + | + + + |
| *Pseudomonas* | + + + | - | - | + |
| *Rhizobium trifolii* | + + + | + | + | + + |

+ + + abundant growth; + + good growth; + slight growth; - no growth

Plates incubated for 14 days at 30°C. Data represent the average results of three replications of three plates.

Table VI

EFFECT OF CONGO RED ON GROWTH OF FLAVOBACTERIUM, PSEUDOMONAS, AND RHIZOBIUM TRIFOLII

| Organism | Dye Concentration Parts Dye/ Parts Media | | |
|---|---|---|---|
| | 0 | 1/20,000 | 1/40,000 |
| Flavobacterium | + + + | + + + | + + + |
| Pseudomonas | + + + | - | + |
| Rhizobium trifolii | + + + | + + + | + + + |

+ + + abundant growth; + + good growth; + slight growth; - no growth

Plates incubated for 14 days at 30°C. Data represent the average results of three replications of three plates.

Table VII

EFFECT OF BRILLIANT GREEN ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Dye Concentration Parts Dye/ Parts Media | | | | |
|---|---|---|---|---|---|
| | 0 | 1/5,000 | 1/10,000 | 1/15,000 | 1/20,000 |
| *Flavobacterium* | + + + | - | + | + | + |
| *Pseudomonas* | + + + | - | + | + | + |
| *Rhizobium trifolii* | + + + | - | + | + + | + + |

+ + + abundant growth; + + good growth; + slight growth; - no growth

Plates incubated for 14 days at 30°C. Data represent the average results of three replications of three plates.

The results of the comparative effects of other dyes did not require tabular form and are easily described here. All three organisms showed about the same sensitivity to rosaniline hydrochloride. Pseudomonas was able to tolerate acid fuchsin better than the other organisms. Only a few colonies of Rhizobium trifolii grew on plates containing acid fuchsin. Phloxine B exhibited about the same effect on Rhizobium trifolii and Flavobacterium but was more toxic to Pseudomonas. Little difference was shown in the case of malachite green which was fairly toxic at 1/10,000 for all organisms. Finally, it is seen that brilliant green was considerable more inhibitory for Pseudomonas and Flavobacterium than for Rhizobium trifolii. Phloxine B and congo red were more selective in bacteriostatic action for Rhizobium trifolii and Flavobacterium and exhibited a greater degree of inhibition for Pseudomonas. Little selectivity for Rhizobium trifolii and Pseudomonas was shown by use of rose bengal.

According to the results of this experiment further inquiry might be confined to brilliant green, congo red, and phloxine B since these dyes were the only ones tested which were inhibitory for either Pseudomonas or Flavobacterium and not active against Rhizobium trifolii.

Relative Dye Sensitivity at Various Concentrations of Dye on Flavobacterium, Pseudomonas and Rhizobium trifolii. The factor of concentration of dye was considered in this experiment as a means of differential inhibition. Preliminary experiments indicated very strongly the differential action of brilliant green; and that rose bengal would be more toxic to Flavobacterium than to Rhizobium trifolii. The percentage of inhibition of these dyes was obtained from several replicate plate counts. The individual plate counts were examined statistically to determine that

they were within the standard deviation from the mean. Arithmetical averages of dye plate counts were divided by control plate counts to calculate the percentage inhibition.

It can be seen from Table VIII that at concentrations of 1/5,000 to 1/20,000 of rose bengal the percentage of inhibition of Pseudomonas and Rhizobium trifolii was not significantly different. Flavobacterium was inhibited only about five per cent more than Rhizobium trifolii at 1/20,000.

The results in Table IX indicate a distinctly significant difference in the percentage of inhibition of brilliant green among the test organisms. Rhizobium trifolii was inhibited 78% but Pseudomonas and Flavobacterium were inhibited 100% at all concentrations tested.

Therefore, the statements are made that (1) rose bengal could not eliminate growth of Pseudomonas and/or Flavobacterium differentially and yet allow growth of Rhizobium trifolii on the basis of concentration, and (2) that the toxicity of brilliant green to Pseudomonas and Flavobacterium was much greater than for Rhizobium trifolii.

Effect of Combination of Dyes on the Growth of Rhizobium trifolii, Pseudomonas and Flavobacterium. Foregoing results showed that Pseudomonas and bacteria in the family Achromobacteriaceae were the predominate soil bacteria which would grow on rose bengal agar. The percentage of inhibition of organisms which were selected to represent these groups in the presence of brilliant green was 100% whereas Rhizobium trifolii was much less inhibited. In view of this, it was inferred that the combination of brilliant green with rose bengal would provide a means of selection of rhizobia from soil should those dyes act independently.

Table VIII

PERCENTAGE INHIBITION OF VARIOUS CONCENTRATIONS OF ROSE BENGAL ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Parts Dye/ Parts Media | | | | |
|---|---|---|---|---|---|
| | 0 | 1/5,000 | 1/10,000 | 1/15,000 | 1/20,000 |
| *Flavobacterium* | 0 | *92.6 | 86.6 | 86.1 | 81.5 |
| *Pseudomonas* | 0 | *90.9 | 81.7 | 81 | 76.9 |
| *Rhizobium trifolii* | 0 | 90.8 | 85.2 | 81.1 | 78.1 |

Data represent the average of three replications of five plates each.

* Averages of two replications.

Table IX

PERCENTAGE INHIBITION OF VARIOUS CONCENTRATIONS OF BRILLIANT GREEN ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Parts Dye/ Parts Media | | | |
|---|---|---|---|---|
| | 0 | 1/5,000 | 1/10,000 | 1/20,000 |
| *Flavobacterium* | 0 | 100 | 100 | 100 |
| *Pseudomonas* | 0 | 100 | 100 | 100 |
| *Rhizobium trifolii* | 0 | 85.2 | 83.6 | 76.6 |

Data represent the average of three replications of five plates each.

When the dyes were incorporated into a common medium both Pseudomonas and Flavobacterium grew profusely at concentrations of 1/20,000 for each dye. Combination of these dyes caused a change in the reaction of the medium intermediate between the pH of each dye medium taken separately. Rose bengal in the yeast extract-mannitol medium had a pH of 5.9. With brilliant green alone the pH was 6.8 The pH of the medium when both dyes were added was 6.38. The medium without dye had a pH of 6.9.

In previous experiments it was found that a concentration of 1/20,000 of congo red inhibited growth of the Pseudomonas test organism. It was assumed that congo red and rose bengal combined would eliminate not only the growth of most bacteria in soil on such a medium but also prevent growth of Pseudomonas as well.

The results of combining these dyes indicated that Pseudomonas was more confined, while Rhizobium trifolii and Flavobacterium grew profusely. Concentrations of 1/20,000 rose bengal and 1/40,000 congo red were used and plates were incubated for two weeks at 30°C.

Further tests were made using a soil inoculum. The colonies that developed were numerous and varied. Many were flat, hammered, yellowish colonies. Some were raised, glistening, watery, and translucent. Isolates tested on litmus milk caused reduction in 24 hours; some with strong proteolysis and others with coagulation. A few slants which appeared typical of rhizobia were contaminated with a lemon yellow-pigmented organism. Many colonies were typical of Pseudomonas and Achromobacteriaceae.

Effect of Hydrogen Ion Concentration. The extremely high pH limit for growth of rhizobia offered another possible factor for investigation. This experiment was carried out to determine the effects of pH on growth of Pseudomonas, Flavobacterium and Rhizobium trifolii. Broth media in tubes were adjusted to pH of 6.8, 9 and 10 and inoculated with a constant amount of inoculum. The effect on growth of adjusting the reaction of the medium at different pH values is shown in Table X. The only difference was observed at pH 10 where in four days little, if any, growth developed in tubes of Pseudomonas and Flavobacterium, but abundant growth was observed in nearly all cases for Rhizobium trifolii.

Preliminary Tests of the Effect of Oxidation-Reduction Potential. Initial respiratory reactions of bacteria require reducing conditions to the extent that compounds with sulphydryl groups are necessary for activating enzymes in the first steps of oxidation of foods for energy. The peculiar requirement of Rhizobium (6) for a more highly oxidized chemical environment was believed useful for selective action. A medium poised at suitable oxidation-reduction potential for growth of Rhizobium might limit growth of Flavobacterium and Pseudomonas.

Preliminary experiments concerning the effect of oxidation-reduction potential served only to indicate that Flavobacterium and Pseudomonas were able to adjust to a wider range of oxidation-reduction potential than Rhizobium trifolii. $KNO_3$ was used to poise the oxidation-reduction potential. No further investigation of this factor was made.

Antibiotic Sensitivity of Rhizobium trifolii, Flavobacterium and Pseudomonas to Bacitracin Chloromycetin and Neomycin. The possibility

Table X

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | pH 6.8 | pH 9.0 | pH 10 | |
|---|---|---|---|---|
| | 4 Days | 4 Days | 4 Days | 7 Days |
| *Flavobacterium* | + + + | + + + | ± | + + |
| *Pseudomonas* | + + + | + + + | ± | + + |
| *Rhizobium trifolii* | + + + | + + + | + + + | + + + |

+ + + abundant growth; + + good growth; + slight growth; - no growth

Data represent the average of three replications of three tubes each.

that an antibiotic might inhibit Pseudomonas and Flavobacterium at concentrations not toxic to Rhizobium was considered. Antibiotics were selected on the basis of known inhibitory concentrations for Pseudomonas and Flavobacterium taken from Baron (8). Other criteria were solubility in water, stability at neutral pH, high temperature, and the availability of the antibiotic commercially.

The bacitracin obtained from Commercial Solvents Corporation had an assay of 49 units per mg. Preliminary inhibitory concentrations were based on bacitracin assayed at 66 units per mg. at maximum activity by serial dilution with Streptococcus hemoliticus and the cylinder plate and turbidometric assay with Staphlococcus aureus. The inhibition concentration given for Flavobacterium was .0025 units/ml. (8).

The chloromycetin assay was given at 630 streptomycin units/mg. with Bacillus subtilis (8). Inhibition concentrations were based on 10-200 mg./ml. for Pseudomonas aeruginosa.

Neomycin was obtained as 50% crude hydrochloride form from the Research Laboratories of Merck and Company, Incorporated. Preliminary inhibitory concentrations were based on 12.5-25 units per ml. for Pseudomonas aeruginosa (8). The assay for neomycin by the agar plate dilution and cylinder plate method with E. coli and B. subtilis was 500-700 units/mg. for purified neomycin (8). For the crude 50% pure form 250 units/mg. was established as the standard assay of the neomycin used in this experiment.

The results of the antibiotic sensitivity of Flavobacterium, Pseudomonas and Rhizobium trifolii are shown in Tables XI , XII and XIII.

Table XI

EFFECT OF CHLOROMYCETIN ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Concentration ug/ml | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 5 | 10 | 20 |
| *Flavobacterium* | ≠ | ≠ | ≠ | - | - | - |
| *Pseudomonas* | ≠ | ≠ | ≠ | ≠ | - | - |
| *Rhizobium trifolii* | ≠ | ≠ | ≠ | - | - | - |
| No Inoculum | - | - | - | - | - | - |

≠ growth; - no growth

Data represent average of three replications of three tubes each.

Tubes were incubated at 30°C. for 7 days.

Table XII

EFFECT OF BACITRACIN ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | Concentration | | | |
|---|---|---|---|---|
| Units/ml | 0 | 1 | 10 | 20 |
| ug/ml | 0 | 20.4 | 204 | 408 |
| *Flavobacterium* | ≠ | ≠ | ≠ | - |
| *Pseudomonas* | ≠ | ≠ | ≠ | ≠ |
| *Rhizobium trifolii* | ≠ | ≠ | ≠ | ≠ |
| No Inoculum | - | - | - | - |

≠ growth; - no growth

Data represent the average of three replications of three tubes each.

Tubes were incubated at 30°C. for 7 days.

Table XIII

EFFECT OF NEOMYCIN ON GROWTH OF *FLAVOBACTERIUM*, *PSEUDOMONAS* AND *RHIZOBIUM TRIFOLII*

| Organism | units/ml. | Concentration 0 | 1 | 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|---|---|
| *Flavobacterium* | | ≠ | ≠ | ≠ | - | - | - |
| *Pseudomonas* | | ≠ | ≠ | - | - | - | - |
| *Rhizobium trifolii* | | ≠ | ≠ | ≠ | ≠ | - | - |
| No Inoculum | | - | - | - | - | - | - |

≠ growth; - no growth

Data represent three replications of three tubes each. Tubes were incubated at 30°C. for 7 days.

Chloromycetin inhibited Rhizobium trifolii and Flavobacterium at 5 ug./ml. and Pseudomonas at 10 ug./ml. Bacitracin did not inhibit Pseudomonas and Rhizobium trifolii at concentrations as high as 20 units/ml. Above this concentration the quantity of bacitracin becomes too great to be of practical value and, therefore, is a limiting factor. However, Flavobacterium was inhibited at 20 units/ml.

The effect of neomycin was a clear cut differential inhibition among the three organisms. Pseudomonas was inhibited at five units/ml. and Flavobacterium at ten units/ml., whereas Rhizobium trifolii was inhibited only at 15 units/ml. Moreover, other isolates of bacteria in the families Achromobacteriaceae and Pseudomonadaceae were inhibited at a concentration of ten units/ml. in further tests.

Preliminary Characterization of the Bacteria that Develop on Selective Media. Preliminary findings about the general identify of bacteria which would develop on media fortified with dyes and neomycin, potentially differential for isolation of Rhizobium were considered in this experiment. On the basis of such data further investigation was restricted to only a few dyes.

The basal media used in this experiment are shown in Table XIV. A yeast extract-mannitol-mineral salts medium was used since it would support growth of rhizobia effectively. The most clear-cut selective agent, neomycin, which had been shown to inhibit Pseudomonas and Flavobacterium at a concentration not toxic for Rhizobium trifolii was incorporated into the basal medium. Colonial characteristics of the bacteria which grew on four dye media are recorded in Table XIV.

None of the characteristics of bacterial growth produced evidence that the colonies were rhizobia when any of the four dyes were included

in a glucose-mineral salts medium. The lack of fungal growth on plates of brilliant green agar suggested that it had an advantage over the other dyes. The addition of neomycin and brilliant green to a yeast extract-mannitol soil extract medium eliminated growth of all organisms but one type from bacterial flora of the soil. The colonial characteristics of this organism were described as white, viscous, opaque, raised in center and growing slowly but profusely on agar slants. When this medium was inoculated with a nodule suspension the resulting colonies appeared to be typical of rhizobia and/or Agrobacterium radiobacter.

Congo red-neomycin agar allowed growth of colonies, some of which appeared to be rhizobia. However, most of the isolated bacteria reduced litmus milk.

The rose bengal-neomycin agar supported growth of colonies typical of rhizobia with both nodule and soil inocula, and none of the isolates reduced litmus milk. However, fungal growth overcrowded the plates inoculated with soil. Neomycin was apparently effective in eliminating bacteria in the families Achromobacteriaceae and Pseudomonadaceae.

These results showed that rose bengal-neomycin agar was the superior medium of those tested for isolation of rhizobia from soil.

Additional tests in Table XV with rose bengal-neomycin agar were conducted using a combined mixture of crushed nodules and soil suspensions as inocula. The bacterial colonies which developed were considered typical of rhizobia on the basis of colonial characteristics, growth on agar slants and action on litmus milk. The extent of fungal growth depended upon the proportion of soil in the inoculum mixture.

Table XIV

COMPARISON OF THE SOIL ORGANISMS RECOVERED FROM MEDIA CONTAINING VARIOUS DYES

| Dye | Concentration | Medium A[1] | Medium B[2] |
|---|---|---|---|
| Brilliant green | 1/15,000 | Colonies raised, translucent, glistening and reduce litmus milk--typical of Pseudomonas. Other colonies confined, white, viscous, raised in center and grow slowly. | Colonies white, opaque, viscous, confined, raised in center and grow slowly. |
| Congo Red | 1/20,000 | Colonies raised, translucent, glistening and reduce litmus milk. Many flat hammered colonies. | Colonies large, watery, raised, translucent and glistening. Many reduce litmus milk and some do not. |
| Rose Bengal | 1/10,000 | Colonies raised, translucent, glistening and watery, reduce litmus milk. | Colonies raised, translucent, glistening. Do not reduce litmus milk and form a serum zone in milk. Agar slants typical of rhizobia. |

[1]Medium A

| | |
|---|---|
| Glucose | 10 gm. |
| $K_2HPO_4$ | 1. gm. |
| $NaNO_3$ | 1. gm. |
| Agar | 15. gm. |
| Soil extract | 1,000 ml. |

[2]Medium B

| | |
|---|---|
| Mannitol | 10 gm. |
| $K_2HPO_4$ | 0.5 gm. |
| $MgSO_4 \cdot H_2O$ | 0.2 gm. |
| NaCl | 0.1 gm. |
| $CaCO_3$ | 3.0 gm. |
| Yeast extract | 5.0 gm. |
| Soil extract | 1,000 ml. |
| Neomycin hydrochloride | 0.04 gm./liter (10 units/ml.) |

Table XV

CHARACTERIZATION OF BACTERIAL GROWTH ON ROSE BENGAL-NEOMYCIN AGAR INOCULATED WITH CRUSHED CLOVER NODULES AND SOIL

| Dilution of Nodule suspension | 1/100 | 1/100 | 1/100 |
|---|---|---|---|
| Dilution of Soil suspension | 1/100 | 1/500 | 1/1,000 |
| | Numerous bacterial colonies. Raised, translucent, glistening, mucilagenous. Do not reduce litmus milk. Agar slants typical of *Rhizobium* or *Agrobacterium radiobacter*. | Same. Few fungal colonies. | Same. Fewer fungal colonies. |
| Dilution of Nodule suspension | 1/1,000 | 1/1,000 | 1/1,000 |
| Dilution of Soil suspension | 1/100 | 1/500 | 1/1,000 |
| | Primarily fungal growth. Scattered bacterial colonies, raised, translucent, glistening and mucilagenous. Do not reduce litmus milk. Agar slants typical of *Rhizobium* or *Agrobacterium radiobacter*. | Fewer fungal colonies. More bacterial colonies. | Many bacterial colonies. Few fungal colonies. |

Groups of Bacteria that Develop on Selective Media as Distinguished by Rapid Identification Tests. The purpose of this experiment was to acquire more detailed information about the identity of groups of soil bacteria which would grow on the neomycin-yeast extract-mannitol medium in the presence of congo red, brilliant green or rose bengal. Additional information was desired concerning the relative abundance of the various groups.

Preceding experiments were indicative of the specific selective action of rose bengal-neomycin agar for rhizobia. Congo red was deemed to be selective from preliminary tests and by its common use for isolation of rhizobia from soil and nodules (10, 16). Brilliant green was included in this experiment because it exhibited stronger bacteriostatic action on Pseudomonas and Flavobacterium than on Rhizobium, and when nodule suspensions were inoculated on brilliant green-neomycin medium some colonies which developed were typical of Rhizobium.

Isolates from bacterial colonies selected at random from the agar plates were tested for action on litmus milk, gelatin, glucose broth, growth on calcium glycerophosphate and growth on agar slants. The basal medium was yeast extract-mannitol-mineral salts-soil extract with 10 units/ml. of neomycin. The dyes were added to this medium.

In Table XVI are the percentages of groups of bacteria isolated from neomycin-yeast extract-mannitol medium to which congo red, brilliant green or rose bengal were added. The inoculum consisted of a suspension of unsterile, mashed, clover nodules. This data is presented only as a qualitative representation of the selectivity of the dye-neomycin media. No attempt was made to resolve it into accurate quantitative data.

Treatments with rose bengal and congo red showed high percentages of rhizobia colonies identified. Agrobacterium radiobacter grew on both these media. A larger proportion of the organisms isolated from rose bengal-neomycin agar were rhizobia than when congo red was substituted for rose bengal in the medium. Most of the organisms (61.5%) isolated from brilliant green agar were identical in appearance. Practically all of the bacteria that grew on rose bengal-neomycin agar were identified as Rhizobium and Agrobacterium radiobacter.

In another experiment, differing from the preceding only in the source of inoculum used, rhizosphere soil from clover plants was diluted 1/100 and inoculated into plates of the dye-neomycin agar. Results are shown in Table XVII. All isolated organisms from the congo red treatment were of the "Pseudomonas" type, producing acid and gas from glucose and rapidly liquefying gelatin.

Ninety-five per cent of bacterial isolates from plates containing the rose bengal and neomycin were identified as rhizobia.

A large number of isolates from brilliant green-neomycin agar were bacteria that could not be identified as Rhizobium or Agrobacterium. These organisms were identical and similar to some of those isolated from unsterile nodules. Yet, when the medium was inoculated with unsterile nodule suspensions, some agrobacteria and rhizobia were isolated.

The results of this experiment are in close agreement with the foregoing one; rose bengal-neomycin agar is by far the most selective medium for Rhizobium.

Table XVIII shows the results of isolates identified from rose bengal-neomycin agar inoculated with soils treated six months before with species

of Rhizobium. In all cases the isolates were either rhizobia or Agrobacterium radiobacter. The percentages of each varied considerably, but no statistical treatment was made and thus the results could only be preliminary data.

The important result shown here is that virtually all the isolates identified were in the family Rhizobiaceae.

Confinement of Fungal Growth. The unrestricted growth of fungi on rose bengal-neomycin agar was detrimental to isolation and enumeration of the bacterial colonies developing on plates inoculated with soil. Although the bacteria did not appear to be much affected by antibiosis, subsurface colonies could not be easily isolated or distinguished for colony counting.

Preliminary attempts to confine the growth of fungi without affecting the bacterial growth were made and the results are given. Rose bengal-neomycin agar was used in all cases.

Crystal Violet. Crystal violet at a dilution of 1/1,000,000 was added to rose bengal-neomycin agar and plates were inoculated with soil. The colonies observed after incubation were typical of rhizobia, being wet, raised, glistening and translucent. Isolates of these organisms reduced litmus milk in two to three days. Contaminants of a yellow-pigmented organism grew on most slants. Only one or two fungal colonies grew on the plates.

Tetramethylthiuramdisulfide. Addition of tetramethylthiuramdisulfide at a dilution of 1/160,000 and at 1/20,000 did not reduce mold growth, whereas bacterial colonies grew at the higher dilution but not at 1/20,000. The bacterial colonies appeared to be rhizobia.

Table XVI

PERCENTAGE OF GROUPS OF BACTERIA ISOLATED FROM VARIOUS DYE-NEOMYCIN MEDIA INOCULATED WITH UNSTERILE CLOVER NODULES

| Dye | Concentration | Percentage of Organisms Identified | | | |
|---|---|---|---|---|---|
| | | Rhizobium | Agrobacterium radiobacter | Pseudomonas | Other Bacteria |
| Congo red | 1/20,000 | 55 | 25 | 20 | 0 |
| Brilliant green | 1/15,000 | 13.3 | 33.3 | 0 | *53.3 |
| Rose bengal | 1/20,000 | 82 | 15 | 3 | 0 |

Data was obtained from 15 to 20 isolated cultures for each dye treatment. The basal medium used for isolation purposes was medium B given on Table XIV.

*These organisms were apparently in the family Achromobacteriaceae.

Table XVII

PERCENTAGE OF GROUPS OF BACTERIA ISOLATED FROM VARIOUS DYE-NEOMYCIN MEDIA INOCULATED WITH RHIZOSPHERE SOIL FROM CLOVER

| Dye | Concentration | Percentage of Organisms Identified | | | |
|---|---|---|---|---|---|
| | | Rhizobium | Agrobacterium radiobacter | Pseudomonas | Other Bacteria |
| Congo red | 1/20,000 | 0 | 7.2 | 92.8 | 0 |
| Brilliant green | 1/15,000 | 13.3 | 13.3 | 60 | 13.3 |
| Rose bengal | 1/20,000 | 94.2 | 0 | 5.8 | 0 |

Data was obtained from 15 to 20 isolated cultures for each dye treatment. The basal medium used for isolation purposes was medium B given on Table XIV.

Table XVIII

PERCENTAGE OF GROUPS OF BACTERIA ISOLATED FROM ROSE BENGAL-NEOMYCIN AGAR INOCULATED WITH SOILS TREATED WITH RHIZOBIUM

| Soil Inoculum | Percentage of Organisms Identified | | | |
|---|---|---|---|---|
| | Rhizobium | Agrobacterium radiobacter | Pseudomonas | Other Bacteria |
| Rh. trifolii | 82 | 18 | 0 | 0 |
| Rh. meliloti | 23.5 | 76.5 | 0 | 0 |
| Rh. japonicum | 22.2 | 77.8 | 0 | 0 |

Data was obtained from 15 to 20 isolated cultures for each dye treatment. The basal medium used for isolation purposes was medium B given on Table XIV.

Chlorothymol. At a concentration of ten ppm of chlorothymol the numbers of fungi were reduced and the bacterial growth did not appear to be inhibited.

2, 4, 5 Trichlorophenoxyacetic acid. This compound at a concentration of ten ppm tended to confine fungal growth without affecting the bacterial growth. The indication is that this compound would be useful for confining fungal growth.

Effect of Capping the Agar Plates. Plates were prepared as before except that a water-agar mixture was poured over the surface after the medium had become solid. The subsurface bacterial colonies grew well and were lenticular and lens-shaped with smooth, even edges. After three to four days mold mycelium developed on these plates, yet the growth was sufficiently confined so that observation of subsurface bacterial colonies was possible.

Anaerobic Plates. The plates inoculated with soil and sealed to a second plate inoculated with E. coli or B. subtilis were incubated for long periods. No fungal growth or bacterial colonies appeared on plates sufficiently sealed.

Layering with Mineral Oil. The layering of sterile mineral oil on the surface of the inoculated agar plates was effective in confining aerial and mass growth of fungi. Mycelium but not heavy fungal colonies covered plates under the oil surface. Bacterial colonies grew well in the medium and could be easily distinguished since the fungal mycelium did not obscure colonies of bacteria.

Chlorothymol and 2, 4, 5 trichlorophenoxyacetic acid were capable of confining fungal growth without obvious effect on the bacteria. These bacteria/ would be

rhizobia or Agrobacterium radiobacter since preceding experiments showed that rose bengal-neomycin was highly specific for these genera.

The layering of seeded agar with the inert sterile agar or with sterile mineral oil were useful aids in making plate counts. When sufficient light was transmitted through the agar plates the bacterial colonies could be easily distinguished.

Discussion of Results.

The evidence that several species of Rhizobium could grow on the rose bengal agar of Smith and Dawson implied that (1) the dye, rose bengal, was not highly bacteriostatic to Rhizobium, (2) the nutrients of the medium were sufficient to support growth and (3) either the oxidation-reduction potential was in the range necessary for growth or the inoculum was large enough to overcome this effect. Thus, it was believed that cells of rhizobia present in the soil might grow on this medium in the presence of rose bengal.

Bacteria that Develop on Rose Bengal Agar. When organisms isolated from the soil on rose bengal agar were identified none of them were Rhizobium. The predominate groups of bacteria isolated from this medium were in the families Pseudomonadaceae and Achromobacteriaceae. The absence of Gram-positive organisms from media containing this acid dye was conspicuous.

The fact that rhizobia had not developed may have been caused by an insufficient number of cells in the soil inoculum since the dilutions of inocula were 1-10 and 1-100. Necessary growth factors may not have been present since Rhizobium requires biotin, thiamine and other growth

substances usually supplied in yeast extract. These substances may have been supplied when pure culture inocula were transferred to rose bengal slants in sufficient quantity to support growth but were not sufficient in the soil inoculum. Yet, it would seem that these nutrients were present in the soil extract since Rhizobium did not grow on rose bengal agar except when soil extract was added.

The effect of soil extract on Rhizobium had been attributed by McCalla (1, 29) to the colloidal clay fraction and an accessory growth factor. Presumably, the action of the soil extract in the presence of dye would seem to be a "protective action" by the soil colloids. Another presumption would be that the soil extract was able to poise the oxidation-reduction potential in a range suitable for growth. This would seem to be more logical since the potential of soil extract at pH 7.8 is nearly the same as the yeast-extract mannitol-mineral salts medium.

It was assumed that the groups of bacteria which grew on rose bengal agar were Pseudomonadaceae, Achromobacteriaceae and Rhizobium. Further investigation was conducted based upon this assumption. Various steps were taken to prevent growth of bacteria in families Pseudomonadaceae and Achromobacteriaceae without inhibiting growth of Rhizobium by modifying the rose bengal agar.

To simplify the investigation organisms tentatively identified as Pseudomonas and Flavobacterium were compared with Rhizobium trifolii when treated with various selective agents and under altered growth conditions. Experiments were conducted to test the effects of dyes, concentration of dyes, pH, oxidation-reduction potential and antibiotic sensitivity.

Bacteriostatic Effect of Dyes. Brilliant green was the most selective of the dyes tested, strongly inhibiting Pseudomonas and Flavobacterium but exhibiting a weaker action on Rhizobium trifolii. Rose bengal exhibited no selection between Pseudomonas and Rhizobium trifolii even at different concentrations. Safranine O inhibited Pseudomonas considerably but was not significantly inhibitory to Flavobacterium and Rhizobium trifolii.

The action of brilliant green, safranine O, and congo red inferred that these dyes might be combined in a medium with rose bengal and thus allow only growth of Rhizobium. Such a conclusion would depend on whether the dyes would act independently.

Tests on the combination of brilliant green and rose bengal resulted in a mutual precipitation and completely altered the pH and the original effects of both dyes. On a medium in which congo red and rose bengal were incorporated Pseudomonas was more inhibited than were Flavobacterium and Rhizobium trifolii. However, when inoculated with soil many contaminants developed on the agar plates which showed that the combined dyes did not act independently.

Effect of pH and Oxidation-Reduction Potential. Another factor, that of effect of pH on growth, was tested. When the reaction of broth medium was increased to a pH of 10 both Pseudomonas and Flavobacterium were strongly inhibited but the growth of Rhizobium trifolii apparently was not affected. At this increased pH one would also suspect that fungal growth would be inhibited when rose bengal agar was inoculated with soil. Attempts to limit the growth of Pseudomonas and Flavobacterium by poising

the medium at a high oxidation-reduction potential level were of little consequence because Flavobacterium and Pseudomonas could adjust to a wider range of oxidation-reduction potential than could Rhizobium trifolii.

Sensitivity to Antibiotics. The sensitivity of the test organisms to bacitracin, chloromycetin and neomycin indicated a favorable selective action for Rhizobium trifolii only in the case of neomycin. The same concentration found to inhibit growth of Pseudomonas and Flavobacterium in broth cultures was found to be effective when used in a rose bengal-yeast extract-mannitol medium inoculated with soil. The quantity of this antibiotic necessary for selective action is only 0.04 gm. per liter of 50% pure neomycin hydrochloride. Neomycin is thermostable in neutral solutions (8) and, therefore, can be sterilized with the medium.

Bacteria Developing in Presence of Combined Selective Agents. It appears that only those bacterial strains of Pseudomonas and Achromobacteriaceae which can develop on rose bengal media are completely inhibited by neomycin. Other strains in the "Pseudomonas" group can grow in the presence of neomycin when used with congo red and safranine. Whether this is true with phloxine B is not known. In a preliminary determination where phloxine B and rose bengal were incorporated into the neomycin-yeast extract-mannitol medium and inoculated with soil the resulting bacterial colonies were typical of rhizobia. This is to be expected because these dyes differ only in that four iodine atoms of the rose bengal molecule are replaced by bromine on the fluorane molecule in the molecular structure of phloxine B. The suggestion is made that further work be done using phloxine B even though this dye was apparently more bacteriostatic to

rhizobia than was rose bengal. Its potential advantage over rose bengal was a more powerful bacteriostatic effect toward the Pseudomonas organism tested.

The colonial characteristics of bacterial colonies that developed on selective media and the reactions on litmus milk were used to indicate generally if the organisms were rhizobia. In this way it was possible to determine which combination of selective agents was differential enough to warrant further study.

The rose bengal-neomycin combination was determined to be the most selective for rhizobia. Some rhizobia were isolated when congo red or brilliant green were substituted for rose bengal. Further evidence of the selective action of rose bengal-neomycin medium for rhizobia was obtained by experiments in which the inoculum was a suspension of mascerated clover nodules and soil. All colonies were typical of rhizobia and the growth on agar slants and the reaction on litmus milk were again typical.

The reactions on litmus milk and on calcium glycerolphosphate agar may be open to question where differentiation of Rhizobium and Agrobacterium radiobacter is desired; yet, these tests have been described (9, 16) as valid means for distinguishing this common contaminant from Rhizobium. Although the final identification test for Rhizobium is the production of nodules on leguminous plants with resulting fixation of nitrogen, there is no reason to believe that the tests used for identification under the conditions of this investigation are of less significance. The identification of this investigation was actually reduced to deciding among Rhizobium, Pseudomonas, Agrobacterium radiobacter and Achromobacteriaceae. Explanation of this is that pure cultures of rhizobia were

shown to grow on rose bengal agar; moreover, that the most predominate groups of soil bacteria which could grow in the presence of rose bengal were known.

Information obtained by identifying a number of organisms isolated from plates of the basal neomycin-yeast extract-mannitol medium in which different dyes were added proved that rose bengal was the most selective dye. The data of the percentages of the bacterial isolates in the different groups can only be held as qualitative information. Nevertheless, the fact remains that in all instances in which rose bengal-neomycin was used for isolation virtually all the organisms were identified in two genera, Rhizobium and Agrobacterium radiobacter.

Confinement of Fungal Growth. The growth of fungi impairs the ease of isolating cultures directly from agar plates. This may be overcome by transfer of colonies not directly in the mycelial mass or by streaking the inoculum on sterile agar plates of yeast extract-mannitol-mineral salts medium. Heavy fungal growth obscured the colonies when plate counts of the bacteria were attempted. Preliminary attempts were made to control fungal growth for this reason.

Chlorothymol and 2, 4, 5 trichlorophenoxyacetic acid proved to deter fungal growth without effect on bacteria isolated on rose bengal-neomycin agar. Techniques of layering the poured agar plates with inert agar on sterile mineral oil were particularly useful in confining fungal growth sufficiently to allow plate counts to be made.

Possible Uses and Applications of the Devised Medium. What, then, are the advantages and possible uses of this medium? Current methods

for isolation of rhizobia from nodules involve careful sterilization of the intact nodule before it is mascerated and inoculated with yeast extract-mannitol-mineral salts medium and in some cases with the addition of congo red dye. The rose bengal-neomycin medium could be used without the previous sterilization of the nodules since there is little reason to expect any contaminants except Agrobacterium radiobacter.

For direct soil isolations the chemotrophic method of Budinov and Bryan's congo red medium are probably the best available techniques at this time. The difficulties involved by these techniques have been discussed in the literature review section of this paper. The devised medium has several advantages over these methods. The soil may be inoculated directly into the plates mixed with rose bengal-neomycin agar and after incubation the bacteria may be transferred from colonies on the agar. Of course, further tests to distinguish between Rhizobium and Agrobacterium radiobacter would be necessary.

Estimation of the number of rhizobia and Agrobacterium radiobacter in soils may be obtained by use of the rose bengal-neomycin medium. It would be necessary to estimate the proportion of Rhizobium to Agrobacterium radiobacter. A sufficient number of plates could be sectored so that the colonies within a sector would not exceed about 20 in number. After testing these isolates on litmus milk and calcium glycerophosphate agar calculation of the number of each group would be possible, since both the total count and the relative numbers of Rhizobium and Agrobacterium would be known.

To relate plate counts to the actual numbers in the soil would require multiplying the counts by a predetermined factor, correcting for the

percentage inhibition of the organisms by the rose bengal and neomycin.

This medium is not difficult to prepare. All the components are easily obtainable, and it appears to give reproduceable results.

Conclusions.

Pure cultures of Rhizobium japonicum, Rh. leguminosarum, Rh. meliloti, and Rh. trifolii were able to grow on rose bengal agar of Smith and Dawson. Soil extract was found to be a necessary constituent for growth in the presence of rose bengal. Other information obtained by identification of the soil bacteria which grew on rose bengal agar did not support the contention that they were in the family Rhizobiaceae. The predominate groups of bacteria which developed on this medium were in the families Achromobacteriaceae and Pseudomonadaceae. None of the organisms isolated could be classified in the family Rhizobiaceae.

Organisms which were used to represent bacteria in the families Achromobacteriaceae and Pseudomonadaceae as physiological groups were identified tentatively in the genera Flavobacterium and Pseudomonas.

Conclusions of the effects of bacteriostatic activity of dyes, pH, oxidation-reduction potential and antibiotic sensitivity on Flavobacterium, Pseudomonas and Rhizobium trifolii are given below.

(1) Brilliant green was less active in its bacteriostatic action on Rhizobium trifolii than on Flavobacterium and Pseudomonas. Congo red and safranine O were more inhibitory for Pseudomonas than for Flavobacterium and Rhizobium trifolii. Other dyes tested were acid fuchsin, crystal violet, malachite green, phloxine B and rosaniline hydrochloride and rose bengal. These dyes were no more bacteriostatic to Flavobacterium and

Pseudomonas than to Rhizobium trifolii. Flavobacterium was highly tolerant for crystal violet and safranine O.

(2) Inhibitions of the test organisms at dilutions of 1/5,000 to 1/20,000 of rose bengal were not markedly different. Brilliant green, on the other hand, was highly bacteriostatic to Pseudomonas and Flavobacterium, and under the conditions of the experiment these organisms were inhibited 100% at concentrations which inhibited Rhizobium trifolii 77%.

(3) Combinations of brilliant green, congo red or safranine O with rose bengal agar failed to limit the growth of Flavobacterium, Pseudomonas and other contaminants in the soil inocula.

(4) Flavobacterium and Pseudomonas were sensitive at a pH of ten where Rhizobium trifolii would grow. Growth lag phase of Flavobacterium and Pseudomonas was prolonged.

(5) Flavobacterium and Pseudomonas were able to adjust to a wider range of oxidation-reduction potential than could Rhizobium trifolii. This was concluded on the basis of preliminary experiments in which the oxidation-reduction potential was increased by addition of $KNO_3$.

(6) Pseudomonas was more resistant to chloromycetin than were the other organisms. All test organisms were resistant to high concentrations of bacitracin. Flavobacterium was the most sensitive. Pseudomonas and Rhizobium trifolii were not inhibited at a concentration of 408 ug./ml., the highest concentration tested.

Pseudomonas was inhibited at a concentration of five units per ml., Flavobacterium at ten units per ml. and Rhizobium trifolii at 15 units

per ml. of neomycin hydrochloride. This selection by neomycin was the most promising among the selective agents tested.

Bacteria that developed on neomycin-yeast extract-mannitol medium supplemented in each case with brilliant green, congo red and rose bengal indicated that rose bengal-neomycin agar was the most selective combination for Rhizobium attempted.

Bacteria isolated from yeast extract-mannitol-mineral salts medium supplemented with rose bengal, neomycin and soil extract inoculated with soil and nodules of leguminous plants were identified in the genera Rhizobium and Agrobacterium radiobacter.

Chlorothymol and 2, 4, 5 trichlorophenoxyacetic acid were effective in confining fungal growth at concentrations not inhibitory to bacteria. Confinement of fungal growth by covering the poured agar with a thick layer of sterile inert agar was useful where it was desired to make plate counts.

Summary.

An investigation of the possibility of developing a differential medium for isolation of soil bacteria in the family Rhizobiaceae was conducted. Although the observation was made that pure cultures of Rhizobium were able to grow on the rose bengal agar of Smith and Dawson, bacteria in the family Rhizobiaceae did not develop when soil was used as the inoculum. The predominate groups of bacteria which developed on this medium from soil were in the families Achromobacteriaceae and Pseudomonadaceae. Organisms chosen to represent the groups of bacteria in the families Achromobacteriaceae and Pseudomonadaceae were identified

tentatively in the genera Flavobacterium and Pseudomonas, respectively.

The effects of dyes, pH, oxidation-reduction potential and antibiotics on growth of Rhizobium trifolii, Pseudomonas and Flavobacterium are included. The most suitable combination of selective agents for isolation of Rhizobium and Agrobacterium radiobacter were rose bengal and neomycin. These compounds when incorporated into a yeast extract-mannitol-mineral salts medium fortified with soil extract prevented growth of virtually all bacteria except Rhizobium and Agrobacterium radiobacter when inoculated with soil and crushed nodules of leguminous plants.

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