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Lysis of the Rhizobiaceae

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LYSIS OF THE RHIZOBIACEAE
(TITLE)

BY

CRAIG RICHARD BAUMRUCKER

THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

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IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1971
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I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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ABSTRACT

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The following members of the Rhizobiaceae were tested for their lytic response to various treatments: Agrobacterium tumefaciens, Chromobacterium violaceum, Rhizobium phaseoli, Rhizobium trifolii, Rhizobium lupini, and Rhizobium japonicum. Lysis was observed in spent growth media and as a function of saline concentration, pH, buffer composition, disodium (ethylenedinitrilo) tetraacetate (EDTA) concentration, and lysozyme.

Cells were harvested by centrifugation, washed, and resuspended in various treatment solutions. The lytic response was followed spectrophotometrically.

The different responses of the six cultures was applied to a summation of reclassification proposals evolved through modern taxonomic techniques such as DNA homology and percent guanine and cytosine nitrogen base composition.

Average percent lysis of the tested bacteria formed three groups. These were Agrobacterium tumefaciens, Rhizobium phaseoli, and Rhizobium trifolii; Rhizobium lupini and Rhizobium japonicum; and Chromobacterium violaceum. These groupings lend further support to the need to reclassify these organisms according to the suggested proposals.

The presence of a "typical" mucopeptide in Agrobacterium tumefaciens, Chromobacterium violaceum, Rhizobium phaseoli, and Rhizobium trifolii was demonstrated by their lytic response to EDTA-lysozyme treatment. Furthermore, the results depended upon the addition of EDTA. This suggests the presence of a "typical" gram-negative cell wall membrane (lipoprotein).

Rhizobium lupini and Rhizobium japonicum exhibited little or no lysis in all tests. This suggests the lack of a mucopeptide, a lysozyme resistant mucopeptide, different bonding of the cell wall membrane, and/or an additional accessory layer such as the capsule. The author suspects the last possibility.

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INTRODUCTION

Historically, walls have been of interest and importance to man. In many cases, the walls of caves proved life saving to early man seeking safety. Walls also provided him with shelter from the ensuing weather. He realized that there must be an opening to gain access to the protection and by which he could leave when the danger was past.

Walls are no less important to man today. One of these structures is the biological cell wall. It too allows access, but still retains its characteristic strength. One of the differences between plants and animals is that the former have cell walls. This appears to be the sole criterion for classifying bacteria in the plant kingdom.

When bacterial cell walls are studied, they are found to be intricately designed. Although this adds to the difficulty of understanding, it also opens new avenues of study. Since complex cell wall components are found to vary among bacterial species, these differences can be used as a key to classification of similar organisms.

By far, the cell wall has been the foremost structure in terms of bacterial taxonomy. From the first major characterization by Gram Stain and morphological studies to today's approaches to ultra-structure and function, the cell wall has been the focus of attention of bacteriologists. As Rogers (1970) wrote, "there are yet mysteries about bacteria worth solving and in the solving may contribute to general biology." This certainly applies to bacterial cell walls.

Of extraordinary interest is the cell wall of the family Rhizobiaceae. Principle nitrogen-fixing bacteria are placed within this family. Fixation of free nitrogen is accomplished only while in a symbiotic relationship with the higher plant family, Leguminosae. During this time, odd bacterial shapes are observable and can be directly correlated with the nitrogen-fixation process. Since the shape of a bacterium is dependent upon the cell wall, the wall seemingly plays a part in this important process.

HISTORICAL

The Taxonomy of the Rhizobiaceae

By noting similarities between individuals and arranging them as groups that have certain common characteristics, a natural organization is achieved. This organization is called systematics.

Thus, the organisms within the family Rhizobiaceae have been grouped together using the following characteristics. They are gram negative rods without endospores; they may or may not be flagellated; they may or may not produce a pigment; they grow aerobically on glucose media; and they can be saprophytes, symbionts, or plant pathogens. It is interesting to note that other family characteristics are similar to these, such as the Pseudomonadaceae. Thus, the family Rhizobiaceae seems very loosely bound.

The family Rhizobiaceae is composed of three genera. The first to be considered is Chromobacterium. This genus is distinguished by the production of an alcohol soluble pigment called violacein (Breed et al., 1957). Species are determined by such physiological characteristics as growth or no growth at 37C and gelatin stab reactions.

The genus Agrobacterium includes plant pathogens that do not fix free nitrogen, although some can produce nitrites from nitrates. Species are separated by host-specificity according to the groups of plants that they parasitize such as angiosperms and gymnosperms.

The genus Rhizobium is by far, the most important genus in this family. The organisms in the genus enter into a symbiotic relationship with members of the plant family Leguminosae and, as a result, fix free atmospheric nitrogen

to ammonia. This characteristic has tremendous economic and agricultural significance. Because of this, Rhizobium has been one of the most frequently studied bacterial genera. Certainly it is not without reason that the family was named Rhizobiaceae.

Since this author is also cognizant of economic and practical significance, the major portion of this paper will deal with this genus.

As is the case for Agrobacterium, species of Rhizobium are distinguished by host-specificity. As Fred, Baldwin, and McCoy (1932) wrote about root nodule bacteria, "In spite of the known exceptions to the ordinary list of cross inoculations, it seems true that the ability of the organism to infect certain plants and not others is as fixed and definite as any phase of the physiology of the organisms. In view of its relative stability and practical importance, we feel justified in regarding it as the prime character in species differentiation." To reflect another view, Allen and Allen (1950) stated "the ability to invade the roots of leguminous plants and stimulate the production of nodules is the sole criterion for the existence of the genus Rhizobium." Thus, the current edition of Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) reflects this viewpoint although it does not regard such classifications as final in any sense, but only temporary progress leading to a future classification, as this paper will demonstrate.

Faults, however, were found with host-specificity groups by a number of individuals. Appleman and Sears (1942) and Wilson (1944) rejected the cross inoculation basis of Rhizobium classification because their evidence demonstrated that the cross inoculation groups were, in fact, not fixed. Species cross-inoculation between hosts was reported. Some authors assert that there is little relationship between Rhizobium and Agrobacterium.

Pre'vot (1961) put Agrobacterium between such unrelated groups as the Nitrobacteriaceae and the Thiobacteriaceae. Thus, the classification of these organisms was, and still seems to be, a matter of personal reference and not necessarily the reflection of a true relationship.

As bacterial taxonomy matures, elaborate techniques and instrumentation allow taxonomists to apply criteria other than host-specificity to group these organisms. The development of these new techniques will very likely have profound effects upon the present classification of the Rhizobiaceae. Before this author discusses these results it seems appropriate to discuss the new techniques.

The bacterial taxonomist, being dependent upon the laboratory, has become more of an experimentalist whose imagination creates new methods to cope with taxonomic inadequacies. These methods are providing supplements for morphological and host-specificity characteristics. After all, specific morphological characteristics are only expressions of a genotype. It is the complete genotype of an organism that determines its relationship to another organism. It seems reasonable, then, not merely to use a single phenotypic characteristic as the sole criterion for classification as Fred, Baldwin, and McCoy suggested in 1932, but rather to use some basis that reflects all or as much as possible of the genotype. The following methods follow this theme.

Deoxyribonucleic acid (DNA) base compositions are becoming more widely used in taxonomic studies and it seems evident that such information will be included in future species descriptions. The best reference at this time is a publication by Hill (1966) which indexes a great number of organisms.

The method is based upon the hypothesis that the DNA of related organisms have similar base compositions. Thus, if base composition can be computed

and compared, a similarity factor can be expressed. The computation of similarity is based upon the percent of guanine (G) and cytosine (C) base complements when compared to the entire DNA base total. The early procedure (DeLey and Van Muylen, 1963) was a direct estimation by DNA hydrolysis and base separation by paper chromatography, followed by ultraviolet spectrophotometry. The quantity of each base was determined by the integration of absorption peaks.

Chemical analysis of DNA nitrogen bases has been replaced in most cases by methods that require less time and material and are less tedious. Such methods include thermal denaturation (Marmur, 1962) and buoyant density in a cesium chloride gradient (Schidkraut, 1962).

Nitrogen base compositions are expressed as moles-percent of guanine and cytosine (%G+C) of the moles of total bases. DeLey (1969) calculated the percent G+C that can occur between two organisms that share a number of almost identical cistrons. He estimated that 20-30% difference in the G+C ratio would mean practically no nucleotide sequences in common. This forms, therefore, a firm basis for the observation that highly similar organisms must have a very similar G+C ratio and, if the percent is substantially different, then the base sequence is different. DeLey also reported that a G+C percent difference of 5 usually implies at least a species differentiation, whereas a 2% difference is seldom of significance.

It is known that related detached strands of purified DNA "recognize" each other and under certain conditions will reunite. This recombination has supplied this century's greatest molecular tool for studying the relationship between organisms.

Techniques such as those of McCarthy and Bolton (1963) measure the reassociation of single-stranded DNA from two different organisms. Although

there are a number of detection methods for determining percent of DNA pairing, they all depend upon physically detecting the difference between single and double stranded DNA. The double strand stability is proportional to the number of nucleotides that match, thus percent of homology is computable.

There are a number of uncertainties concerning this method. Are the reassociated sequences recombining in short regions or over the entire length of the DNA molecule? What is the degree of overlapping of strands? The problems of this method are slowly being solved and as a result the promise for even greater taxonomic information is certain.

A somewhat similar approach to DNA homology is that of transformation. Transformation is the transfer of hereditary properties (genes) from one bacterium to another. Molecular DNA is taken up by the recipient cell and is "absorbed" into its chromosome where the transferred genetic character is expressed phenotypically. Such an "absorption" indicates a similarity of genotypes for the transferable trait. This type of study is a useful tool for the bacterial taxonomist allowing him to study species interrelationships. Avery et al. (1944) were the first to review this subject and indicate its taxonomic significance. A more up to date review can be found in The Bacteria (Schaeffer, in Gunsalus and Stanier, 1964). Specific Rhizobium transformations are reported by Balassa (1963).

Similarly, transduction can demonstrate species interrelationships. It involves the entrance of a small piece of genetic material into a recipient cell by the action of bacteriophages.

It is appropriate at this time to discuss still another taxonomic method even though the method ostensibly strays from the genotype theme.

The use of bacteriophage host-specificity as reported by Conn et al. (1945) and Stocker (1955) is of interest because a specific phage will attack and lyse only a characteristic and limited range of bacterial strains. It is believed that this susceptibility is dependent upon host cell wall structure. Thus, those strains that are susceptible to the same phage are always somewhat related.

There are a great number of molecular studies that have contributed to modern bacterial taxonomy. However, these studies are only more elaborate means of detecting specific phenotypic expressions. Such methods as biochemical analysis, serological techniques, immunization and immunofluorescence are a few among many new techniques. An interesting review of a number of these subjects is reported by Marmur et al. (1963).

Because of the tremendous amount of different information being accumulated about each strain of bacterium, bacterial taxonomy has reached a point at which there is need for mechanical aid to sort taxonomic data. Lange (1961) suggests that to be effective as a classification method, the system must be composed of non-biased, over-all information concerning everything that is known about that organism. He suggested the application of numerical taxonomy to the Rhizobiaceae.

The development of numerical systematics has brought considerable attention to the procedures involved in taxonomy. Sneath and Sokal (1962) present an excellent discussion of numerical taxonomy. Numerical methods for estimating phenotypic relationships are based upon observations of many characteristics of many strains. Each characteristic is usually given equal weight, indicated as a plus or minus, although under special conditions, different characteristics get different weight. The organisms are related by matching coefficients. Either all matches of similarity are divided by the total tests

or positive matches are divided by the total tests minus the negative matches. Using both of these coefficients as an average, a final similarity ("S" value) is determined. However, it is still impossible to know what portion of the genotype is being expressed by the many phenotypic characteristics. Although weaknesses are known, this system of classification has widespread support since it covers, in the most ideal application, a reflection of all that is known about the genetics, morphology, ecology, biochemistry, and serology of the organism.

Although numerical taxonomy can be accomplished without mechanical aid, the tremendous volume of data makes it a very tedious task. Thus, by demand, the application of computers to numerical taxonomy (Sneath, 1957) has greatly reduced the time and effort of this process.

Recent studies with DNA homology, nitrogen base composition, transformation, transduction, and numerical computerized taxonomy has resulted in a number of suggested changes in the taxonomy of the family Rhizobiaceae. In order to minimize confusion, each genus within the family will be discussed separately. However, overlapping cannot be prevented since the suggestions often cross generic boundaries.

Chromobacterium is considered a member of the family Rhizobiaceae because of the "gummy" colony growth that is characteristic of many species found in the Rhizobiaceae. In addition, the organisms show similar carbohydrate metabolism and they possess the same type of flagellation. The main genus distinction is that they produce a violet pigment. However, there seems to be a question as to the placement of Chromobacterium in this family. Indeed, Pre'vot (1961) classifies Chromobacterium in a family separate from Rhizobiaceae.

After conducting an Adansonian Analysis of the Rhizobiaceae, Moffett and Colwell (1967) concluded that there is strong support for the separation

of Chromobacterium from the other genera of the Rhizobiaceae. Chromobacterium was shown to be more similar to Pseudomonas aeruginosa (57% S value) than to either Rhizobium (48% S value) or Agrobacterium (46% S value). Heberlein et al. (1967) reported that Chromobacterium was far removed genetically from the bulk of the Agrobacterium-Rhizobium strains. They suggest that Chromobacterium be reclassified in some manner that will not exaggerate its actual relationship to the Rhizobiaceae. The G+C% as reported by DeLey et al. (1966) is in the 65-67% range, whereas Agrobacterium and Rhizobium strains range approximately 5-8% lower. According to DeLey, this suggests a species or even a genus difference.

Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) presently recognizes four species within the genus Chromobacterium. They are characterized by growth temperatures, gelatin stab reactions, and the ability to grow in various salt concentrations. The results of Adansonian Analysis (Sneath, 1956; 1957) suggests that there be only two species, Chromobacterium violaceum and Chromobacterium lividum, separated on the basis of optimum growth temperature. DeLey et al. (1966) concurs on the basis of G+C%. He finds G+C values for C. violaceum and C. lividum, 65% and 67% respectively. Further evidence of these results was reported by Heberlein et al. (1967). Thus, it seems conclusive that only two species of Chromobacterium should be recognized regardless in which family they are placed.

There does not seem to be much question as to the relationship of Agrobacterium and Rhizobium using molecular methods of taxonomy. DeLey et al. (1966) reported that all strains of Agrobacterium have a corresponding G+C% of 59-62. This actually overlaps the Rhizobium G+C% of 55-62, indicating a very close genetic relationship. Further evidence by Heberlein et al. (1967) demonstrates that Agrobacterium and Rhizobium have a 60-90% DNA homology.

Bergey's Manual of Determinative Bacteriology (Breed et al., 1957)

lists seven species of Agrobacterium. They are A. tumefaciens, A. ~~ty~~so-
philae, A. pseudotsugae, A. rhizogenes, A. rubi, A. radiobacter, and A.
stellulatum. Species are characterized by nitrite production from nitrates
and by host-specificity. The following are proposals that have been made
concerning species relationships within the agrobacteria.

Graham (1964) reported that the "S" values for A. tumefaciens and A.
radiobacter were almost identical. This is supported by bacteriophage
cross-lysis between these species (Roslycky, 1960). Transformation studies
by Klein and Klein (1953) not only support this conclusion, but include
A. rubi in this relationship. Similar results were reported by DeLey et al.
(1966) with their studies on a number of factors including G+C%. The G+C%
of A. tumefaciens and A. rubi were indistinguishable. Heberlein et al.
(1967) reported similar DNA homologies between the species A. tumefaciens,
A. radiobacter, and A. rubi plus Rhizobium leguminosarum. This Agrobacterium-
Rhizobium relationship was also reported by transformation studies of Klein
and Klein (1953), G+C% reported by DeLey and Rassel (1965), and Adansonian
Analysis reported by Graham (1964).

A very close relationship between A. tumefaciens, A. radiobacter, and
A. rhizogenes has been reported by DeLey et al. (1966) based on G+C%. Further-
more, DNA homology (Heberlein et al., 1967) has shown a close relationship
between A. rhizogenes and R. leguminosarum. DeLey et al. (1966) have sug-
gested that A. tumefaciens, A. radiobacter, A. rubi, and A. rhizogenes are
varieties under the species type A. radiobacter. In light of the cross-genus
link, all of these species have been suggested as varieties of the genus
Rhizobium within the species R. leguminosarum (Heberlein et al., 1967).

This last arrangement is mildly supported by t'Mannetje (1967) but she suggests further study before any reclassification is made.

A. stellulatum is included with Agrobacterium because of its ability to form star-shaped clusters, one of the characteristics of Agrobacterium, Chromobacterium, Rhizobium, and Pseudomonas. Except for this feature, A. stellulatum does not display any of the characteristics of Agrobacterium. DeLey et al. (1966) report a G+C% of 56 as compared to an Agrobacterium average of 60%. This strongly suggests the removal of the species from Agrobacterium with possible placement in the genus Pseudomonas.

A. gypsophilae is included in the genus Agrobacterium because of its ability to produce tumors (Breed et al., 1957). However, its G+C% of 56 (as compared to 60% for Agrobacterium) and other characteristics indicate little genetic relationship to Agrobacterium (DeLey et al., 1966). This idea was supported by Moffett and Colwell (1967) by Adansonian Analysis. They suggest placement in the Enterobacteriaceae.

A. pseudotsugae has been found by DeLey et al. (1966) and Moffett et al. (1967) to be quite unrelated to Agrobacterium and both groups suggest a separate generic designation.

The taxonomy of the genus Rhizobium is also in a turmoil. The first jolts of change were indicated when Wilson (1944) rejected the still-standing six species based upon cross-inoculating groups. Graham (1964) shocked bacterial taxonomists with his Adansonian Analysis of Rhizobium. He proposed the following relationships and classification changes. He reported that R. leguminosarum, R. phaseoli, and R. trifolii formed a homogeneous groups with "S" values of 80-97%. Therefore, he suggested the single species R. leguminosarum. He also found a 70-90% "S" value between R. japonicum and R. lupini and likewise made a reclassification proposal. He suggested that

a new genus called Phytomyxa, species P. japonicum be created due to the low "S" value (55-65%) between these two type species, P. japonicum and R. leguminosarum. Further suggestions include the merging of A. tumefaciens and A. radiobacter into the species Rhizobium radiobacter because of the high "S" values (75-85%) between these organisms and Rhizobium species. In conclusion, Graham (1964) decided to retain the species R. meliloti although it is similar to R. radiobacter.

Since Graham's (1964) proposal, modifications of this plan have been reported. Deley and Russell (1965) and Deley et al. (1966) favor combining R. leguminosarum, R. meliloti, and certain agrobacteria into the species R. leguminosarum. They also agree with the combination of R. japonicum and R. lupini into the species R. japonicum. However, they believe that there is a close relationship, based upon G+C%, between the R. leguminosarum and R. japonicum groups. Therefore, the genus Phytomyxa should not be formed.

t'Mannetje (1967) and Moffett and Colwell (1967) repeated Graham's Adansonian Analysis but used different clustering techniques and found a closer "S" value between the groups R. leguminosarum and R. japonicum.

One of the most recent proposals (Heberlein et al., 1967) concurred with the inclusion of R. leguminosarum, R. trifolii, and R. phaseoli into the species R. leguminosarum. R. meliloti was retained as a species and R. japonicum and R. lupini were combined as the species R. japonicum. The summation of classification proposals is presented in Table 1.

Thus, available evidence suggests three species within the genus Rhizobium. Whether R. meliloti is retained as a separate species or not, one thing seems to be agreed upon by most bacterial taxonomists. Rhizobium seems to be composed of two major species groups: the fast growers (R. leguminosarum) and the slow growers (R. japonicum). As reported by Norris (1957),

TABLE 1
Proposed Classification Changes

CLASSIFICATION ACCORDING TO BERGEY'S MANUAL	SUMMATION OF PROPOSED RECLASSIFICATION
FAMILY: Rhizobiaceae	FAMILY: Pseudomonadaceae?
Genus: <u>Chromobacterium</u>	Genus: <u>Chromobacterium</u>
<u>C. violaceum</u>	<u>C. violaceum</u>
<u>C. amethystinum</u>	<u>C. violaceum</u>
<u>C. janthinum</u>	<u>C. lividum</u>
<u>C. marismortui</u>	<u>C. lividum</u>
	FAMILY: Rhizobiaceae
Genus: <u>Agrobacterium</u>	Genus: <u>Agrobacterium</u> or <u>Rhizobium</u>
<u>A. tumefaciens</u>	<u>? radiobacter</u>
<u>A. radiobacter</u>	<u>? radiobacter</u>
<u>A. rubi</u>	<u>? radiobacter</u>
<u>A. rhizogenes</u>	<u>? radiobacter</u>
<u>A. pseudotugae</u>	unknown ?
<u>A. stellulatum</u>	Enterobacteriaceae
<u>A. gypsophilae</u>	<u>Pseudomonas</u>
Genus: <u>Rhizobium</u>	Genus: <u>Rhizobium</u>
<u>R. leguminosarum</u>	<u>R. leguminosarum</u>
<u>R. trifolii</u>	<u>R. leguminosarum</u>
<u>R. phaseoli</u>	<u>R. leguminosarum</u>
<u>R. meliloti</u>	<u>R. meliloti</u>
<u>R. japonicum</u>	<u>R. japonicum</u>
<u>R. lupini</u>	<u>R. japonicum</u>

"a logical approach to the problem would be to eliminate all existing species [of Rhizobium] and then to erect new species on the basis of slow growing and fast growing types."

Rhizobium-Leguminosae Nodulation

Interest in the family Rhizobiaceae lies primarily in the genus Rhizobium with its ability to enter into a symbiotic relationship with legumes in which atmospheric nitrogen is fixed. Nitrogen fixation is of great agronomic importance because it provides a supply of available nitrogen used by major commercial crops.

Rhizobia are soil bacteria which utilize materials secreted into the soil by plant roots and possibly microorganisms. The ability of rhizobia to exist in soils can be deduced from the establishment of the organisms, without artificial inoculation, when crops of native legumes are grown. The organisms have been found to cluster several cells deep around the legume root surface (Dart and Mercer, 1964) and eventually, some bacteria enter the root and cause nodulation.

The site of infection by Rhizobium is most often the root hairs (Dixon, 1969). The first visible indication of the infection is the elongation and deformation of the root hairs (Thornton and Nicol, 1936). This effect has been attributed to the influence of indoleacetic acid (IAA) as reported by Kefford et al. (1960). IAA is formed within the bacterium from excreted plant materials. Although root hair elongation seems to be caused by indoleacetic acid there is some question as to the cause of root hair deformation (Haak, 1964 in Dixon, 1969).

The entrance of the bacterium into the plant root involves a softening and breakdown of cell wall substances by indirect regulation of RNA and protein synthesis. Key et al. (1967) have shown that IAA softens cell walls.

Once inside, the polysaccharide coat of the bacterium seems to induce the formation of an enzyme (Ljunggren and Fahraeus, 1959). The enzyme alters the metabolism of the root hairs so that normal plant growth substances are then used in the infection process.

After the bacteria have successfully entered the root hair they are enclosed in a cellulose tube called the infection thread. The infection thread grows through the root hair into the cortex and in some way activates the division of cortical cells to form the nodule.

The bacteria move to the periphery of the infection thread. They push out the surrounding membrane which then pinches in and encloses the bacteria in an envelope by the process of endocytosis (Dixon, 1967).

After being enclosed in the membrane and released into the cytoplasm of the host, the bacteria increase in size and may change in shape. In some hosts, the bacteria divide within the membrane container until there are as many as six in each. In others, the membrane divides with the bacterium so that each is enclosed separately (Goodchild and Bergersen, 1966).

At this point the bacteria undergo a number of drastic morphological and physiological changes. These changes coincide with the ability to fix nitrogen and, when no changes occur, no nitrogen is fixed. The morphological changes are quite bizarre and at first the cells were believed to be protein bodies called "bacteroids" (bacteria-like) a term which, although inappropriate, is in common use today. These bacteroids are frequently club shaped or branched into "X" and "Y" forms. They appear in great numbers within the nodule and only rarely in culture media. The bacteroid shape appears to represent an abnormal or disrupted synthesis of the cell wall material. Since the supporting component of the bacterial cell wall is a layer called the mucopeptide (Salton, 1964) the bacteroid shape must be the

result of at least some mucopeptide change. However, if the mucopeptide is affected, it is only altered in certain places and not along the entire length of the bacterium. Bacteria devoid of the mucopeptide layer take the characteristic spherical shape of a protoplast or spheroplast.

The initiation of bacteroid formation and the rapid increase in nitrogen fixation has been directly correlated (Bergersen, 1957). Since the extraction of bacteroids from the nodule leaves them unable to fix nitrogen it seems likely that the process is a joint effort between the host and the pathogens. However, it has been speculated that nitrogen fixation is set in motion by the plant alone in an effort to isolate and destroy the invading rhizobia (Jordan, 1962).

Concurrent with morphological change, biochemical alterations are also happening either as related or independent events. One change is the production of leghaemoglobin. The amount of leghaemoglobin in root nodules has been correlated with the amount of nitrogen fixed (Jordan, 1962). Yocum (1964) reported that leghaemoglobin is present within the membrane envelope surrounding the bacterioids, thus suggesting bacterial origin. Although it was believed that this substance was directly involved in nitrogen fixation, it has been shown by Bergersen and Turner (1968) that it is possibly only an oxygen carrier of some vast, complicated process.

Further biochemical changes are seen in the cytochromes. Some bacteroids contain no cytochrome a, a₃, or o while all cytochromes are present in free-living forms (Appleby and Bergersen, 1958). Thus, drastic changes in the respiratory enzymes to initiate nitrogen fixation is indicated.

The changes that take place in the bacteria during bacteroid transformation are obviously many since both metabolism and morphology are altered. While some of these changes have been induced or happen spontaneously in

culture under various conditions, the sum of all the changes has never been approached outside of the symbiotic relationship.

Thus, a great deal is left unanswered about the nitrogen-fixing ability of legume root nodules. It should be realized that a complete understanding of the process may eventually lead to non-nodule rhizobial nitrogen fixation in the laboratory. The economic significance of this is overwhelming. Although impractical at present, it still represents a desirable goal worth scientific inquiry.

Bacterial Cell Wall Structure

An early discovery that there were two major categories of bacteria and that they could be identified by differential staining resulted from the work of Gram in 1884 (in Brock, 1961). These two major groups of bacteria are characterized as being either gram-positive or gram-negative (no crystal violet retention). Since this time, great strides have been made to further characterize the differences between these bacteria. The knowledge gained from these investigations has given new insight into the structural and biochemical processes in living things.

To those interested in bacterial origin, these discoveries have substituted for the lack of fossil records. The bacterial cell wall, which is the "hardest" of all the cellular components, is likely to have survived for relatively short geological periods. Thus, through comparative anatomy and physiology, the bacterial taxonomist has obviated bacterial paleontology.

The extensive work of Cummins and Harris (1956) established the usefulness of physiological and biochemical criteria in the taxonomy of bacteria. Although the taxonomic characters of related organisms can be determined by observing the structure and chemical constitution of their cell walls, there are no clear lines of evolution that can be seen from one group to another.

The most interesting feature of biochemical studies has been the discovery of several structures which are unique to the bacterial cell wall. The isolation and identification of compounds such as muramic acid, N-acetylglucosamine, and teichoic acid can now be used in defining bacterial cells and a related group, the blue-green algae.

From the following discussion of the layers and chemical composition of the bacterial cell wall it should be apparent that the task of determining bacterial species by chemical analysis is relatively easy. This is true because the cell wall layers are chemically the most individualistic components of the bacterial cell.

Excellent chemical and structural reviews on bacterial cell walls are presented by Salton (1964) and Rogers and Perkins (1968). A brief outline of the main components of the cell wall is discussed here to clarify subsequent descriptions of these structures.

The component chiefly responsible for the strength of the cell wall has been called mucopeptide, murein, murein sacculus, rigid layer, and glycopeptide. It is absent from only a few halobacteria and from bacterial protoplasts, some sphereoplasts and L forms. Otherwise it is found in all bacteria so far studied. It forms a relatively small portion, 5-10% of the cell wall in gram-negative bacteria (Salton, 1964) and often a much larger proportion, 80-90%, in gram-positive bacteria (Rogers and Perkins, 1968).

In addition to the mucopeptide, the walls of gram-negative bacteria usually contain layers of protein, lipids, lipoproteins, and lipopolysaccharide. The lipopolysaccharide chemistry has been extensively reviewed by Horecker (1966).

In contrast, gram-positive genera show various quantities of non-murein components in their cell walls. These may include the teichoic acids and

polysaccharides which have antigenic properties. Although Salton and Horne (1951) reported that cell walls of gram-positives contain no protein, it may be more prevalent in the cell wall than previously believed, since wall analysis generally involves the use of proteolytic enzymes. Another distinguishing characteristic is that gram-positive bacteria contain little or no lipid in their cell wall. This difference is discussed by Salton (1964) as the cause of the differential Gram stain.

Although the bacterial cell wall gives mechanical strength to the organism, better enabling it to withstand high osmotic pressures, it is by no means indispensable for the biological and biochemical continuity of the cell. A recent publication by Guze (1967) illustrates the extent to which microorganisms can tolerate a decrease or loss in cell wall structure. A stable existence without certain components of the cell wall is recognized in a large number of bacteria at the present time. Under conditions of only partial loss of the cell wall, the bacterial cell has been known to revert to the normal form. However, bacteria devoid of their whole wall have not been reported to revert (Guze, 1967). Thus, possession of the cell wall is not an absolute requirement for bacterial survival.

As to the biochemical functions that may be an integral part of the cell wall, little can be added. The continued survival due to biochemical activities of protoplasts makes it unlikely that the wall itself plays a major role in biochemical processes of gram-positives. However, the great complexity and the localization of enzymes (Strominger, in Gunsalus and Stanier, 1962), chromatophores (Gunsalus and Stanier, 1960), and lipids (Horecker, 1966) in the cell wall of gram-negative bacteria suggests biochemical function.

In conclusion, the bacterial cell wall is obviously responsible for the shape and morphological characteristics of the cell. Its involvement

in the Gram stain reaction is definite, since bacterial protoplasts devoid of cell wall, are gram-negative (Gerhardt et al, 1956). Furthermore, the presence of certain components in the wall also governs the sensitivity of the bacterium to bacteriophages and enzymes (Stocker, 1955). However, all of these properties are related more to the chemistry and structure of the wall rather than to its biochemical function.

Generally, the functional status of the bacterial cell wall seems to be essentially that of a rigid framework, supporting and protecting the biochemically active parts of the cell. However, present studies with bacteria such as Rhizobium seem to indicate that this is not universally true. That biochemical function may be associated with the cell wall in these bacteria as well as in other gram-negatives seems reasonable.

The functions of the cell wall are attributable to certain cell wall layers and their chemical components. If one could start on the inside of a gram-negative bacterial cell wall and work outward, he would pass through a series of layers. From the inside out they would be the mucopeptide, protein, lipopolysaccharide, and lipoprotein. Although gram-positive bacterial walls differ from this arrangement, the author will use this path as a guide for discussing the chemical structure of both gram-negatives and gram-positives.

In most species of bacteria studied to date the mucopeptide consists of polysaccharide chains of alternating residues of two amino sugars. N-acetylglucosamine (AcGN) and N-acetylmuramic acid (AcMA) are joined by B-1,4 bonds just as are glucose residues in cellulose (Rogers, 1970). This is considered the "backbone" of the mucopeptide layer (Figure 1). The polysaccharides are joined by chains of rather short peptides consisting of a limited number of amino acids varying among the different species

of bacteria. The amino acids always consist of D-glutamic acid and D-alanine with frequent occurrences of L-alanine and either L-lysine or meso-diaminopimelic acid (DAP). According to the amino acids and linkages present, five groups of mucopeptides have been recognized by Ghuyssen (1968). She reports that three of these five are the peptide subunits found in most bacterial mucopentides (Figure 2). The first amino acid residue of the subunit is always attached by its N-terminal end to the carboxylic acid residue of muramic acid.

Strength is added to the complex by cross-linkages between peptide subunits called bridges. Almost all bridges known are extended between the second amino group of L-lysine or DAP of one peptide subunit to the carboxyl group of D-alanine of a second subunit. However, according to the species, bridges vary in chemical composition. Some bridges are direct peptide bonds between the subunits, whereas others are composed of several other amino acid units.

In gram-negatives, bridges can extend between adjacent peptide subunits from the same amino sugar backbone to peptide subunits of other backbones (Figure 3). These many links add strength and rigidity to the mucopentide.

In contrast, gram-positive bacteria can bridge adjacent subunits of the same backbone molecule but do not directly link peptides of other backbones. Instead, adjacent backbone complexes link to a common substance called teichoic acid (Figure 4). Two types of teichoic acids are known. One is composed of polyglycerophosphate (P-glycerol) and the other of polyribitolphosphate (P-ribitol). In addition, a variety of other sugars and amino acids are substituents. These compounds are confined to gram-positive bacteria.

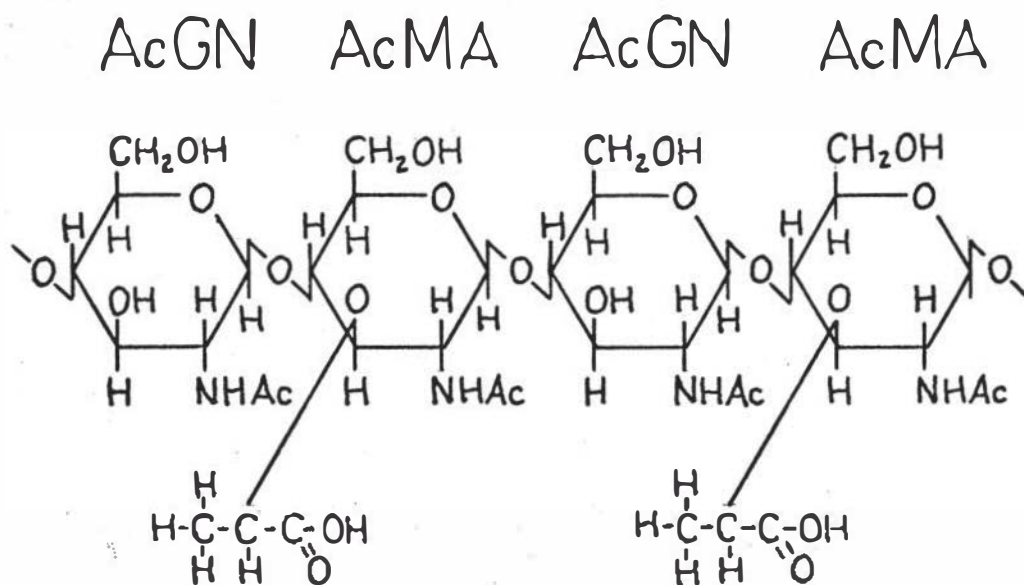


FIG. 1.

MUCOPEPTIDE "BACKBONE"

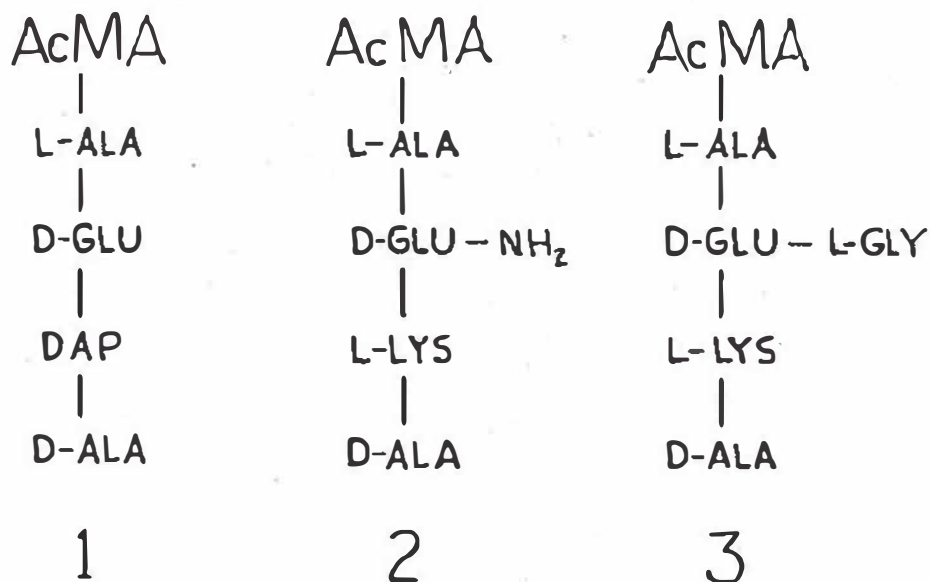
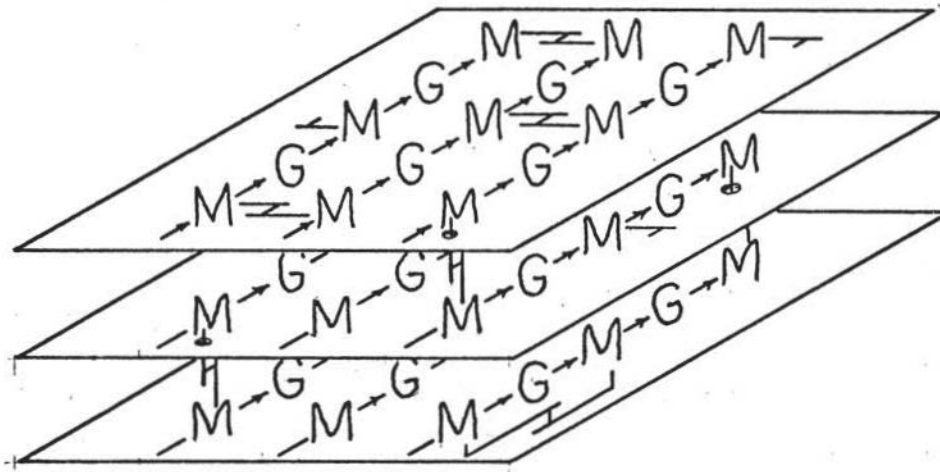


FIG. 2.

PEPTIDE SUBUNITS

GRAM-NEGATIVE MUCOPEPTIDE BONDING

FIGURE 3



G N- acetylglucosamine

M N- acetylmuramic acid

R-Ⓟ-R P- ribitol (teichoic acid)

— peptide subunit

→ B-1,4 linkage

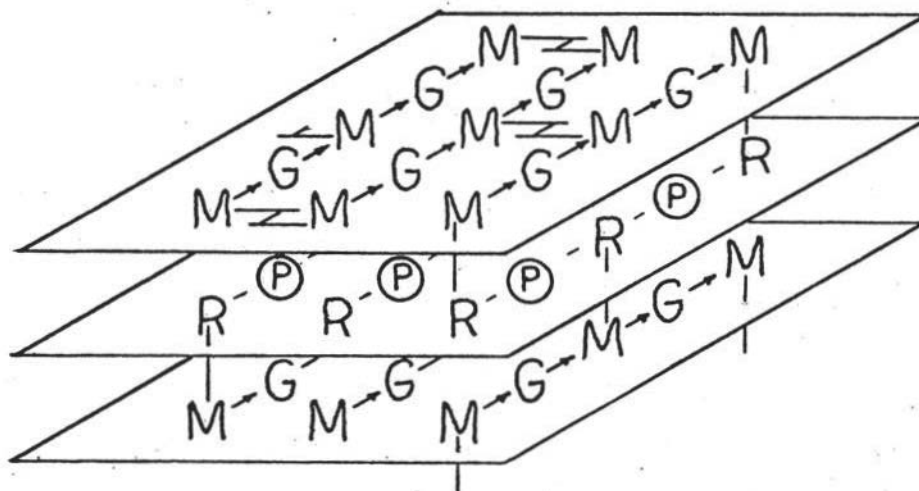


FIGURE 4

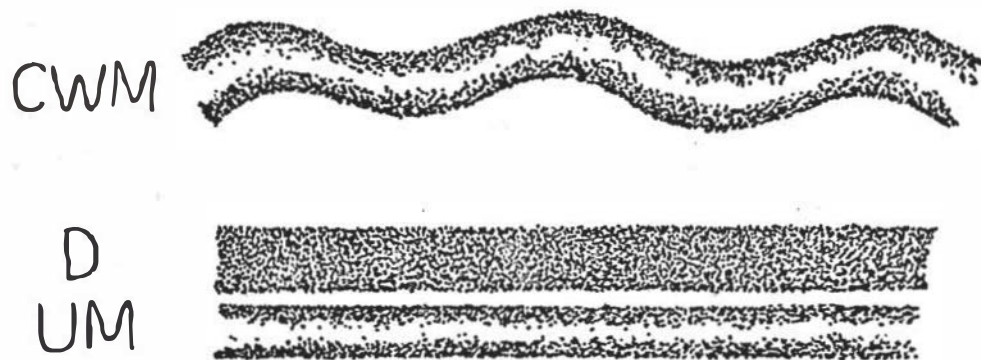
GRAM-POSITIVE MUCOPEPTIDE BONDING

It is interesting to note that both D and L forms of amino acids are used by the cell wall biosynthetic mechanisms. This is rare, since most life forms use only the L amino acid isomer.

Since the mucopeptide is the supporting layer for the bacterial cell wall, it is the breakdown of this layer that causes the cells to burst or lyse in hypotonic solution or to form protoplasts or spheroplasts in isotonic solution. Natural or spontaneous degradation (autolysis) of the mucopeptide seems to happen as the cell ages. Autolysis can be accelerated by using a variety of bacteriolytic enzymes (heterolysis). The principle enzyme used in conjunction with the mucopeptide is lysozyme. Lysozyme has been thoroughly studied and its specific action upon the mucopeptides of bacterial cell walls is well established. Lysozyme has been shown by Phillips (1966) to break the linkages between the carbon one of N-acetylglucosamine and the carbon four of N-acetylmuramic acid. Although most gram-positives are sensitive to lysozyme, gram-negatives are usually resistant and require special pretreatment to render them susceptible to the enzyme. Most researchers agree that this is correlated with the lipid and protein content in such layers as the lipoprotein and lipopolysaccharide. These layers may act as a protective cover for the underlining mucopeptide (Grula and Hartsell, 1957).

Electron microscopy of gram-positive bacteria reveals one fairly homogeneous, dense layer, D, (Salton, 1964). In contrast, the cell wall of a gram-negative bacterium shows two electron-dense layers. Digestion with lysozyme removes part or all of the inner dense layer, D, suggesting that it is the site of the mucopeptide (Murray et al, 1965). Immediately outside the mucopeptide layer is a wide, electron-opaque layer followed by another dense layer, CWM (Figure 5).

GRAM - NEGATIVE



GRAM - POSITIVE

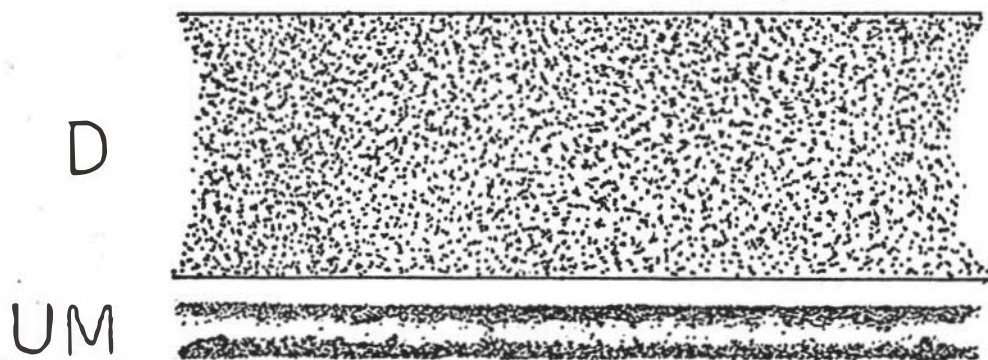


FIG. 5.

THE BACTERIAL CELL WALL AS SEEN IN ELECTRON MICROGRAPHS
(as reported by Glauret and Thornley, 1969)

Little is visible in the "space" between the two electron-dense layers, although de Petris (1965;1967) has described globular structures which correspond to the protein granules described by Weidel et al., (1960). Treatment of this layer with proteolytic enzymes removes the protein granula which results in a wider electron-opaque layer, suggesting that the granula provide a cohesive link between the two electron-dense areas.

Chemical analyses of these globular proteins reveal a full range of amino acids present (Salton, 1964). Researchers speculate (Rogers, 1968) that this protein layer consists of enzymes which perform vital cell wall functions since partial breakdown of the surrounding layers causes a "leaking out" of this protein material (Rogers, 1968). If it were structural protein, it probably would be more stable.

Also found in the space between the two electron-dense layers is lipopolysaccharide. These complex macromolecules are responsible for many of the biological properties of the cell surface. They appear to be O antigen determinants, bacteriophage receptor sites, and are endotoxic.

When isolated, the lipopolysaccharide is composed of a polysaccharide linked covalently to a glucosamine-containing lipid called Lipid A. Lipid A contains N-acetylglucosamine, phosphate, and fatty acid residues. A structure for Lipid A has been proposed by Burton and Carter (1964).

The polysaccharide moiety of the lipopolysaccharide is composed of a non-antigenic backbone, a "core", and an antigenic side group. The backbone of the polysaccharide is composed of alternating molecules of organic phosphate and a heptose sugar. The link between the polysaccharide backbone and Lipid A is a complex 8-carbon sugar.

The core of the polysaccharide consists of two glucose residues, two galactose residues, and one N-acetylglucosamine for every heptose residue

in the backbone. The antigenic portion is composed of repeating units of galactose, rhamnose, mannose, and abequose and is attached to the N-acetylglucosamine of the core.

Thus, the lipopolysaccharide of gram-negative bacteria may contain as many as fourteen different structural components and carry several different antigenic determinants. Although quite a lot is known about the chemical composition of lipopolysaccharides, little is known about their anatomical status in the bacterial cell wall other than they are surface antigens.

Although polysaccharides are known to exist within the homogenous gram-positive cell wall, work on this aspect has been only touched upon since isolation techniques have yet to be perfected. However, work by Cummins and Harris (1959) has shown the presence of these monosaccharides: glucose, galactose, mannose, rhamnose, arabinose, and 6-Deoxytalose.

The outer electron-dense layer of the cell wall of gram-negatives is the cell wall membrane, CWM (Figure 5). Although Clarke and Lilly (1962) suggested that this outer membrane be termed a unit membrane, UM, (as is the inner membrane), Salton (1964) prefers to call this layer a compound membrane because of its distinguishing characteristics. Electron microscopy of the compound membrane suggests spherical macromolecules (Salton, 1964) which have not been seen in the underlining plasma membrane. From observations presented (Glauert and Thornley, 1969), there is reason to believe that there are differences in the construction and chemical composition of the outer and inner membranes of gram-negative bacteria. On the other hand, Clarke and Lilly (1962) have suggested that the surface layers of gram-negative bacteria consist of two unit membranes, each with the structure protein-lipid-lipid-polysaccharide, with a mucopeptide between

them. This hypothesis seems reasonable and structurally feasible. However, that this is true for all gram-negatives is obviously too generalized.

In any bacterial cell wall study there has evolved a standard sequence of events that a researcher follows. Each step in this sequence must be extensively covered since one step is used to define the preceding step.

Cell wall studies begin with autolysis, the lysis of a bacterial culture by materials within the culture. Autolytic studies generally involve the observation of cells suspended in a number of menstrua. An understanding of autolysis is needed to define heterolysis, the lysis of the cell by external materials. As heterolysis studies proceed, the researcher is able to ascertain what portion of the lysis is attributable to autolysis. Heterolysis then involves observations of the cells in known lytic materials.

The standard sequence continues with qualitative cell wall analysis. The analysis can be conducted with paper chromatography, gas chromatography, electrophoresis, or with a number of other analytical techniques.

After a thorough qualitative analysis to determine what is present in the cell wall, a quantitative analysis naturally follows. The methods of analysis differ with the material to be tested.

Finally, cell wall studies are concluded with analysis integrated with autolysis and heterolysis. After a cell has lysed, it is analyzed to determine the chemical alteration resulting from this action.

Thus, this research is the first step in a long sequence of events leading finally to the characterization of the cell walls of the Rhizobiaceae.

MATERIALS AND METHODS

Bacterial Cultures

Bacterial strains used were selected from the family Rhizobiaceae. Selection was based upon representation of all genera with particular attention given to the genus Rhizobium.

A total of six species was used. From Agrobacterium and Chronobacterium, type species were chosen. From Rhizobium, four cultures were selected representing "fast and slow growers" and light and heavy capsule production. The sources and characteristics of these cultures are discussed in Appendix A.

General

Glassware

All glassware was thoroughly washed in detergent, followed by rinsing in de-ionized water. Spectrophotometric tubes were similarly processed including a final distilled water rinse.

Sterilization

All sterilized materials were autoclaved in a Castle autoclave at 15 psi for 20 minutes.

Reagents

Reagents (Appendix B) were prepared in distilled water. Fresh reagents were prepared for each experiment. All but the degradable reagents were sterilized by autoclaving.

Centrifugation

Centrifugation was in a Sorvall Model SS-4, Manual Superspeed Centrifuge at ambient temperatures.

Gyrotory Shaker

Bacterial cultures were incubated on a New Brunswick Scientific Gyrotory Shaker (Model S-3) or in a Gyrotory Water Bath Shaker (Model G-76). All cultures were agitated at 140 RPM.

Spectrophotometry

Spectrophotometric techniques were conducted with a Bausch and Lomb Spectronic 20. An absorption spectrum was conducted using Rhizobium trifolii in DME broth against a DME blank. It was determined that 440 nm showed the greatest absorption while still remaining within the greatest sensitivity of the instrument. All spectrophotometric readings were made at this wavelength.

Standard Growth

Stock cultures were kept on DME agar slants (Appendix B) in screwcap tubes at 4-8C. Working stocks were transferred each month, with the old working stocks held in reserve.

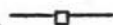
A loop full of the slant stock culture was aseptically transferred into 50 ml of DME broth (Appendix B) in a 250 ml Erlenmeyer flask. The cultures were allowed to grow until stationary phase, which was predicted initially by F. Davidson (personal communication). Fast growing cultures were predicted to reach stationary phase in 3 days and slow growers in 7 days.

Two ml of the stationary phase cultures was aseptically pipetted into a 750 ml spectrophotometric flask containing 100 ml of DME broth. The flask was incubated at 30C at 140 RPM in the Gyrotory Water Bath Shaker. Absorption at 440 nm was recorded and plotted against time to produce standard growth curves (Figure 6).

FIGURE 6

GROWTH OF THE RHIZOBIACEAE
(30C in DMYE broth)

Agrobacterium tumefaciens



Rhizobium trifolii



Chromobacterium violaceum



Rhizobium japonicum



Rhizobium phaseoli



Rhizobium lupini



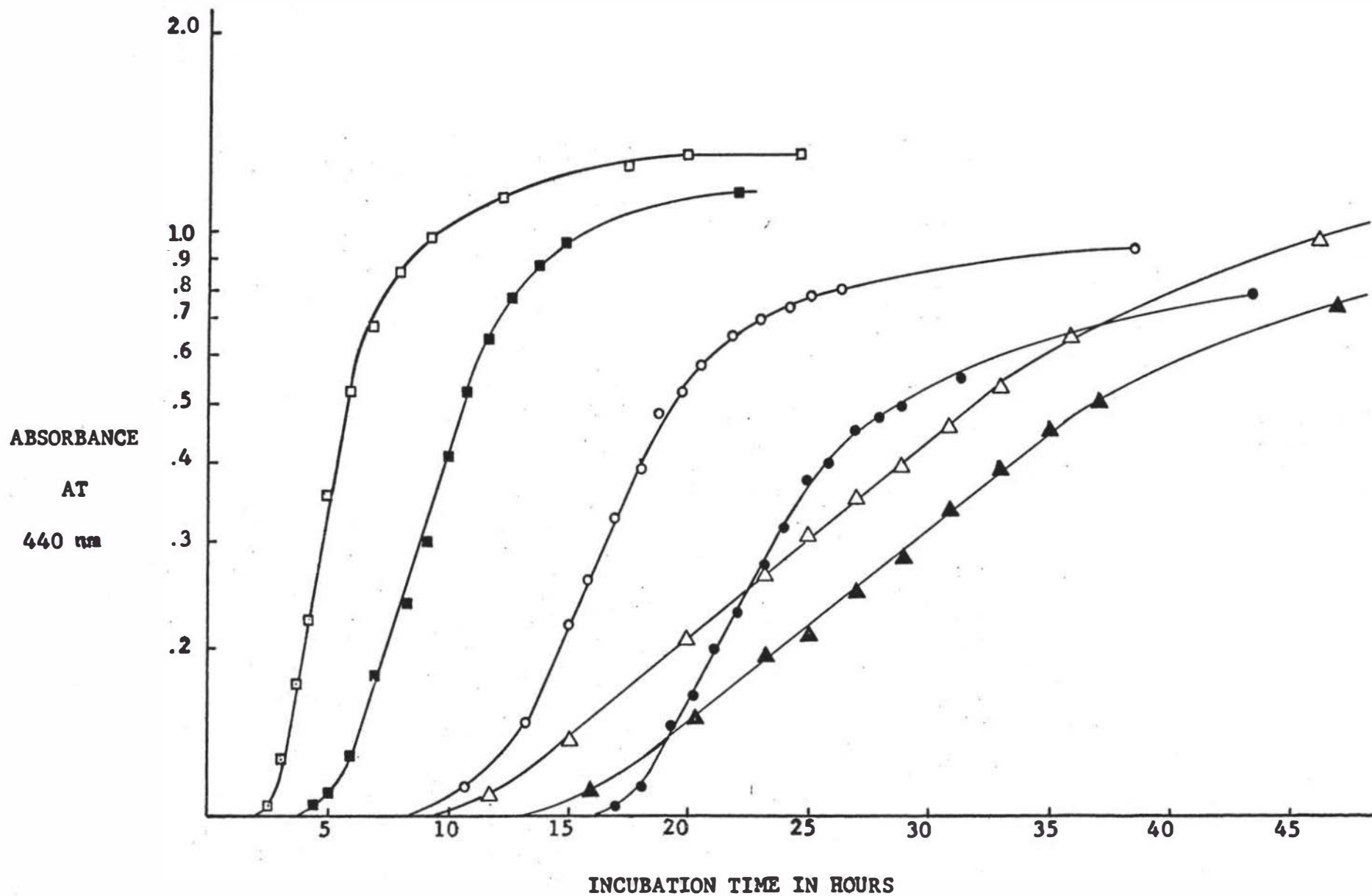


FIG. 6

GROWTH OF THE RHIZOBIACEAE

Standard Cell Preparation

Cultures were grown according to standard growth procedures. Growth phases were determined for log phase cells and stationary phase cells by reference to the standard growth curves and spectrophotometric readings. Log and stationary phases for each culture are defined in Table 2.

Thirty ml of the culture at the desired growth phase was aseptically pipetted into each of three sterile, plastic, screwcap centrifuge tubes. The centrifuge was balanced and run at 10,000 to 15,000 RPM (culture dependent) for 10 minutes. The supernatant was discarded and the pellet was resuspended in sterile distilled water (pH ca. 7.2) with the volume of sterile distilled water adjusted so that an eight-fold dilution of the cell suspension would result in an optical density of 0.4 to 0.6. Using a Vortex Junior, the cells were agitated for 10 seconds to insure a homogeneous mixture. Again the cells were centrifuged at 10,000 to 15,000 RPM for 10 minutes and the supernatant discarded. The pellet was then resuspended at the same concentration in a buffer or distilled water.

Each experiment began when 0.5 ml of the concentrated cell suspension was aseptically pipetted into 3.5 ml of the experimental solution. Spectrophotometric readings were made at 440 nm at prescribed times (varying with experiments).

Statistical Analysis

Statistical analysis was conducted with the aid of the Eastern Illinois University Computer Services using an IBM 360 (Model 50) Computer. The program was the Analysis of Variance for Factorial Designed Experiments, revised September 12, 1969, by Health Sciences Computing Facility, UCLA.

TABLE 2
LOG AND STATIONARY PHASE DEFINED

Cultures	LOG		STATIONARY	
	Absorbance	time	Absorbance	time
<u>Agrobacterium tumefaciens</u>	0.5	5 hrs.	1.4	14 hrs.
<u>Chromobacterium violaceum</u>	0.5	11 hrs.	1.2	25 hrs.
<u>Rhizobium phaseoli</u>	0.4	17 hrs.	0.9	36 hrs.
<u>Rhizobium trifolii</u>	0.4	25 hrs.	0.75	40 hrs.
<u>Rhizobium lupini</u>	0.4	25-30 hrs.	1.4	70-80 hrs.
<u>Rhizobium japonicum</u>	0.4	25-30 hrs.	1.4	70-80 hrs.

Photomicrography

Photomicrography was conducted with a Bausch and Lomb Flat Field Phase Contrast Microscope. The camera was an Exakta, 35 mm with through the lens viewing.

Kodak, Panatomic-X film (ASA-25) was used. The slow film speed called for long exposures due to the lack of strong light while using phase contrast annuli. Most cultures were photographed from stock DME agar slants. Only Chromobacterium violaceum was photographed from both broth and agar to show the drastic morphological changes characteristic of this strain.

RESULTS

Introduction

The experimental aspect of this thesis consists of six studies. Each study tested the lytic effects of agents on each of the six stock cultures. Each study is discussed separately, describing deviations from the standard methods, analysis of data, results and discussion.

Autolysis in Spent Growth Media

Cultures were grown by standard growth procedures and allowed to reach stationary phase. Four ml of each culture was aseptically pipetted into duplicate spectrophotometric tubes at each experimental temperature. The temperatures were 3, 12, 20, 30, 37, and 52C. Cultures were vortexed and read for loss in absorbance in the Spectronic 20, blanked with a sterile DME broth tube. Readings and microscopic observations were made every 12 hours for four days.

The experiment resulted in no observed autolysis for any culture at any temperature. From these results it was concluded that little or no autolysis could be attributed to the growth medium (DME broth).

Autolysis in Saline

Cultures were grown according to standard growth procedures. The desired growth phase was reached by reference to the standard growth curves. In order to statistically analyze the data for Autolysis in Saline, duplicate tubes were needed. This necessitated the elimination of one or more of the variables. Consequently the experiment was run using log phase cells

only. Cell suspensions were prepared by the standard method. The experiment began (time zero) when 0.5 ml of a concentrated cell suspension was resuspended in duplicate spectrophotometric tubes containing 3.5 ml of either distilled water, 0.5%, 1.0%, 2.0%, or 10.0% saline (NaCl), all at pH 7.2. Loss in absorbance was measured in the Spectronic 20 at 440 nm. The 3 hour and 24 hour readings were selected to represent the results of the experiment. The loss in absorbance was converted to percent autolysis in saline which is presented in Appendix C.

Percent autolysis (Appendix C) was statistically analyzed by computer and the results are given in Table 3. All treatments and most first, second, and third order treatment interactions were significant at the 5% level. In other words, most sources of variation had some affect on autolysis. However, the author will compare the magnitude of the computed F-values in order to arrive at some generalizations about the effects of the variables. Furthermore, this author will continue this practice in subsequent analyses.

All individual treatments caused significant autolysis. Temperature differences (Source 1), as expected, effected differences in autolysis. The figures in Appendix C demonstrate that higher temperature caused an increase in autolysis. This could be due to a disruption of the delicate balance between anabolic and catabolic cell wall synthetic enzymes (Strominger, in Gunsalus and Stanier, 1962). If degradative enzymes had a higher temperature optimum, they would continue to operate while the enzymes responsible for synthetic activities were inactive.

Saline concentration (Source 2) also appears to greatly affect autolysis. All cultures exhibited a marked increase in autolysis at higher saline concentrations (Appendix C). It could be speculated that the sodium ion exchanges

TABLE 3

AUTOLYSIS IN SALINE: ANALYSIS OF VARIANCE

a. SOURCE OF VARIATION:

1. TEMPERATURE
  - 20C
  - 30C
  - 40C
2. SALINE CONCENTRATION
  - 0.0% (control)
  - 0.5%
  - 1.0%
  - 2.0%
  - 10.0%
3. CULTURES (Appendix A)
  - Stock cultures
4. TIME
  - 3 hours
  - 24 hours
12. FIRST ORDER INTERACTION
  - Temperature with Saline
123. SECOND ORDER INTERACTION
  - Etc.

b. F-VALUES CALCULATED BY:

VARIATION MEAN SQUARE
REPLICATE MEAN SQUARE

ns. NOT SIGNIFICANT AT 5% LEVEL

TABLE 3.

AUTOLYSIS IN SALINE: ANALYSIS OF VARIANCE

SOURCE ^a	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARES	F ^b	TABULATED F-VALUES 5% LEVEL
1	2	3527.46	1763.73	212.24	3.07
2	4	14216.01	3554.00	427.68	2.45
3	5	20676.41	4135.28	497.63	2.29
4	1	5561.69	5561.69	669.28	3.92
12	8	1201.48	150.18	18.07	2.02
13	10	702.93	70.29	8.46	1.91
14	2	26.33	13.16	1.58 ns	3.07
23	20	8009.06	400.45	48.19	1.66
24	4	1483.62	370.91	44.63	2.45
34	5	1558.48	311.70	37.51	2.29
123	40	1552.13	38.80	4.67	1.50
124	8	265.23	33.15	3.99	2.20
134	10	1181.15	118.11	14.21	1.91
234	20	848.29	42.41	5.10	1.66
1234	40	1225.32	30.63	3.69	1.50
Replicates	180	1496.50	8.31		

with divalent cations responsible for the integrity of the cell wall, thus directly blocking salt bridges between mucopentides and/or other cell wall components. This has been demonstrated repeatedly in Escherichia coli by experiments using EDTA (a known divalent cation chelator) which causes an increase in autolysis (Noller and Hartsell, 1961). Furthermore, it is possible that the NaCl causes selective denaturation of enzymes resulting in a condition similar to those attributed to increased temperature. Still again, it might be that an increase in sodium and/or chloride ions causes a stripping away of some of the cell wall surface layers allowing greater exposure to the underlining mucopeptide, which has final control over autolysis.

As was expected, the six different cultures (Source 3) showed individualistic responses to the other variables. This undoubtedly is due to either a qualitative or quantitative difference in their cell wall structure. A thicker, more rigid cell wall would be expected to autolyze more slowly. Also, variation in composition of the cell walls of the cultures may regulate the rate of autolysis.

Time (Source 4) exhibited, as expected, greater autolysis with the greater amount of experimental exposure time.

The first order interaction, temperature vs cultures (Source 1,3) demonstrates the variation between cultures caused by temperature differences even though all cultures have the same approximate optimum growth range of 25-30C. A significant saline concentration vs culture interaction (Source 2,3) suggests still more variation between cultures.

Little autolysis was recorded for distilled water (0.0% saline, in Appendix C). As a result of this finding, distilled water was used as the cell wash solution in standard cell preparations for subsequent experiments.

Autolysis in Buffers

Cultures were grown according to standard growth procedures. Desired growth phases were reached by reference to the standard growth curves. Cell suspensions were prepared by the standard method. The experiment began (zero hour) when 0.5 ml of a concentrated cell suspension at each growth phase was resuspended in duplicate spectrophotometric tubes containing 3.5 ml of one of three buffers: McIlvane's citric acid phosphate; Sorensen's phosphate; and Gomori's tris malate (Diem, 1962). All buffers were prepared at pH 7.2 according to information in Appendix B. Duplicate spectrophotometric tubes of log and stationary phase cells in the three buffers were incubated at 20, 30, and 40C. Loss in absorbance was measured in a Spectronic 20 at 440 nm at 1, 2, 3, 4, 5, 24, and 48 hours from zero time. The 3 and 24-hour readings were selected to represent the results of the experiment. Loss in absorbance was converted to percent autolysis in buffers and is presented in Appendix C.

Percent autolysis (Appendix C) was statistically analyzed by computer and the results are given in Table 4. Almost all sources of variation were significant at the 5% level. Although growth phases (Source 1) showed significant variation, the computed F-value was the least significant when compared to the other sources of variation. Because of this, and the great number of variables in subsequent experiments, stationary phase cultures were eliminated. What little growth phase variation that was observed was probably due to a slowing of anabolic activities in stationary phase due to the presence of some inhibiting materials released by the cultures during growth. Thus, catabolic activities occurred unbalanced and caused greater autolysis.

Buffer variation (Source 2) very significantly affected autolysis. When column averages for both log and stationary phase were compiled (from Appendix C), McIlvane's buffer averaged 25% autolysis/test, followed by

TABLE 4

AUTOLYSIS IN BUFFERS: ANALYSIS OF VARIANCE

a. SOURCE OF VARIATION:

1. GROWTH PHASE
  - Log phase
  - Stationary phase
2. BUFFERS (Appendix B)
  - Sorensen's
  - McIlvaine's
  - Gomori's
3. TEMPERATURE
  - 20C
  - 30C
  - 40C
4. CULTURES (Appendix A)
  - Stock cultures
5. TIME
  - 3 hours
  - 24 hours
12. FIRST ORDER INTERACTION
  - Growth phase with buffer
123. SECOND ORDER INTERACTION
  - Etc.

b. F-VALUES CALCULATED BY:

VARIATION MEAN SQUARE
REPLICATE MEAN SQUARE

ns. NOT SIGNIFICANT AT 5% LEVEL

TABLE 4

AUTOLYSIS IN BUFFERS: ANALYSIS OF VARIANCE

SOURCE ^a	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARES	F ^b	TABULATED F-VALUES 5% LEVEL
1	1	581.00	581.00	36.20	3.84
2	2	11531.99	5765.99	359.25	3.00
3	2	2873.22	1436.86	89.52	3.00
4	5	28812.83	5762.57	359.04	2.21
5	1	10910.01	10910.01	679.75	3.84
12	2	133.58	66.79	4.16	3.00
13	2	152.30	76.15	4.74	3.00
14	5	1935.54	387.11	24.12	2.21
15	1	414.27	414.27	25.81	3.84
23	4	1151.37	287.84	17.93	2.37
24	10	1966.27	196.63	12.25	1.83
25	2	994.88	497.44	30.99	3.00
34	10	1463.71	146.37	9.11	1.83
35	2	358.23	179.11	11.15	3.00
45	5	3471.57	694.31	43.25	2.21
123	4	271.66	67.91	4.23	2.37
124	10	2549.93	254.99	15.88	1.83
125	2	47.00	23.50	1.46 ns	3.00
134	10	1092.22	109.22	6.80	1.83
135	2	92.23	46.12	2.87	3.00
145	5	1011.39	202.28	12.60	2.21
234	20	2230.38	111.52	6.94	1.57
235	4	246.58	61.64	3.84	2.37
245	10	2226.25	222.62	13.87	1.83
345	10	652.74	65.27	4.06	1.83
1234	20	2060.18	103.01	6.61	1.57
1235	4	270.35	67.58	4.21	2.37
1245	10	1515.61	151.56	9.44	1.83
1345	10	361.32	36.13	2.25	1.83
2345	20	1590.81	79.54	4.95	1.57
12345	20	1059.00	52.95	3.29	1.57
Replicates	216	3467.50	16.05		

Sorensen's buffer average of 20% autolysis/test, and finally Gomori's buffer average of 12% autolysis/test. Both McIlvane's and Sorensen's are phosphate containing buffers. Phosphate is known to readily form insoluble complexes with divalent cations. Thus, higher autolysis was expected since salt bridges could be disrupted in the cell wall. The higher average autolysis in McIlvane's could be attributed to the higher phosphate molarity and to the presence of citrate, a chelating agent. Gomori's buffer has some chelating ability (because of maleic acid); however, it is substantially less than that of citrate (Jorgensen, 1963) and the average autolysis/test reflects this fact.

Variation in temperature (Source 3) again affected autolysis. As the temperature increased, greater autolysis was observed with the exception of Rhizobium lupini and R. japonicum. These cultures showed the greatest autolysis at 30C rather than at 40C.

The temperature variable was eliminated from subsequent experiments due to its relatively low magnitude of significance and the need to eliminate some of the variables for convenience and handling during testing.

Cultures (Source 4), as expected, showed significant variation with regard to extent of autolysis. Again, a cell wall difference is indicated.

It is interesting to note the buffer vs culture interaction (Source 2,4). Although the F-value is significant, it is not very high. It is, then, an indication that all cultures respond somewhat similarly to the buffer treatments. These results seem to be misleading apparently because of averaging in the analysis of variance program. It can be seen in Appendix C that the individual cultures do vary considerably. As an example, Chromobacterium violaceum showed extremely high autolysis in McIlvane's buffer, whereas Rhizobium japonicum showed very little.

Autolysis as a Function of pH

Cultures were grown according to standard growth procedures. Log phase cells were obtained by reference to the standard growth curves. Cell suspensions were prepared by the standard method. The experiment began (zero time) when 0.5 ml of the concentrated cell suspension was aseptically resuspended in duplicate spectrophotometric tubes containing 3.5 ml of McIlvane's, Sorensen's, and Gomori's buffers (Appendix B) at each of eight pH values. The pH's tested were 5.2, 5.6, 6.0, 6.4, 6.8, 7.2, 7.6, and 8.0.

Temperature was maintained at 30C. Loss in absorbance was measured in a Spectronic 20 at 440 nm at 1, 3, and 24 hours. The 3 and 24-hour readings were selected to represent the results of the experiment. Loss in absorbance was converted to percent autolysis as a function of pH and is presented in Appendix C.

Percent autolysis was statistically analyzed by the computer and the results are given in Table 5. Once again, all sources of variation affected autolysis.

Buffers (Source 1) showed the same sort of variation that was seen in "Autolysis in Buffers." Namely, a higher autolysis in the phosphate buffers (McIlvane's and Sorensen's) than in the malate buffer (Gomori's).

pH (Source 2) also affected autolysis. As seen in Appendix C, greatest autolysis was near neutrality. The least autolysis was at more acid pH values. This might be attributed to the inactivation of enzymes that normally are responsible for autolytic processes. Lysozyme, the enzyme responsible for mucopeptide breakage has been reported to have an optimum pH of 7.6-7.8 (Wright, 1965).

The different cultures (Source 3) proved to be extremely variable under these experimental conditions. The culture vs pH interaction (Source 2.3) was one of the most significant first-order interactions. This indicates that

TABLE 5.

AUTOLYSIS AS A FUNCTION OF pH: ANALYSIS OF VARIANCE
(log phase cells at 30C)

a. SOURCE OF VARIATION:

b. F-VALUES CALCULATED BY:

1. BUFFERS (Appendix B)

Sorensen's
McIlvaine's
Gomori's

VARIATION MEAN SQUARE
REPLICATE MEAN SQUARE

2. pH

5.2
5.6
6.0
6.4
6.8
7.2
7.6
8.0

ns. NOT SIGNIFICANT AT 5% LEVEL

3. CULTURES (Appendix A)

Stock cultures

4. TIME

3 hours
24 hours

12. FIRST ORDER INTERACTION

Buffers with pH

123. SECOND ORDER INTERACTION

Etc.

TABLE 5.

AUTOLYSIS AS A FUNCTION OF pH: ANALYSIS OF VARIANCE
(log phase cells at 30C)

SOURCE ^a	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARES	F ^b	TABULATED F-VALUES 5% LEVEL
1	2	4777.41	2388.71	240.31	3.00
2	7	10535.56	1505.08	151.41	2.01
3	5	74019.66	14803.93	1489.33	2.21
4	1	1660.53	1660.53	167.05	3.84
12	14	650.44	46.46	4.69	1.67
13	10	2456.51	245.65	24.71	1.83
14	2	661.74	330.87	33.29	3.00
23	35	7136.07	203.89	20.51	1.43
24	7	753.68	107.67	10.83	2.01
34	5	5501.84	1100.37	110.70	2.21
123	70	2963.30	42.40	4.27	1.30
124	14	452.21	32.30	3.25	1.67
134	10	2079.86	207.99	20.92	1.83
234	35	1502.07	42.92	4.32	1.43
1234	70	2963.69	42.41	4.27	1.30
Replicates	288	2863.00	9.94		

the cultures exhibit different responses to variation in pH. This could be attributed to individualistic pH optima of their autolytic enzymes.

Autolysis in EDTA

Cultures were grown according to standard growth procedures. Log phase cells were obtained by reference to the standard growth curves. Cell suspensions were prepared by standard methods except that 0.05 M pH 7.2 Gomori's tris buffer (Appendix B) was substituted instead of distilled water for the concentrated cell suspension. The experiment began (zero time) when 0.5 ml of the cell suspension in Gomori's tris buffer was aseptically resuspended in duplicate spectrophotometric tubes containing 3.5 ml of 0.01, 0.005, 0.001, 0.0005, and 0.0001 M EDTA in 0.05 M pH 7.2 Gomori's tris buffer. Gomori's tris buffer was used as a control. The temperature was maintained at 30C. Loss in absorbance was measured in a Spectronic 20 at 440 nm at 0.25, 0.5, 1, 2, 3, and 24 hours from zero time. The 3 and 24-hour readings were selected to represent the results of the experiment. Loss in absorbance was converted to percent autolysis and is presented in Appendix C.

Percent autolysis was statistically analyzed by computer and the results are given in Table 6. All sources of variation had a significant effect upon autolysis.

Variation in EDTA concentration (Source 1) exhibited a significant effect on autolysis, but not at the high levels of other sources. Therefore, the concentration of EDTA, within the bounds of this experiment, was sufficient to cause some autolysis but there was little difference between concentrations. Since EDTA is a strong calcium and magnesium ion chelator, it is likely that the EDTA is removing these divalent ions from the cell wall complex, disrupting salt bridges. This makes the degradation of the cell wall by normal cellular activities that much easier and faster.

TABLE 6.

AUTOLYSIS IN EDTA: ANALYSIS OF VARIANCE

a. SOURCE OF VARIATION:

1. EDTA CONCENTRATION (Appendix B)
  - C.00 M. (control)
  - 0.01 M.
  - 0.005 M.
  - 0.001 M.
  - 0.0005 M.
  - 0.0001 M.
2. CULTURES (Appendix A)
  - Stock cultures
3. TIME
  - 3 hours
  - 24 hours
12. FIRST ORDER INTERACTION
  - EDTA with Cultures
123. SECOND ORDER INTERACTION
  - Etc.

b. F-VALUES CALCULATED BY:

VARIATION MEAN SQUARE
REPLICATE MEAN SQUARE

ns. NOT SIGNIFICANT AT 5% LEVEL

TABLE 6.

AUTOLYSIS IN EDTA: ANALYSIS OF VARIANCE

SOURCE ^a	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARES	F ^b	TABULATED F-VALUES 5% LEVEL
1	5	1610.87	322.17	26.46	2.37
2	5	15330.70	3066.14	253.40	2.37
3	1	6045.05	6045.05	499.59	4.00
12	25	1137.34	45.49	3.76	1.70
13	5	44.99	9.00	0.74 ns	2.37
23	5	2021.99	404.40	33.42	2.37
123	25	608.55	24.34	2.01 ns	4.00
Replicates	72	871.50	12.11		

The EDTA vs culture interaction (Source 1,2) was nearly non-significant, an indication that all cultures are responding similarly to the various concentrations of EDTA. Whatever is happening in one seems to be happening in all the cultures.

EDTA-Lysozyme Lysis

Cultures were grown by standard growth procedures. Log phase cells were obtained by reference to the standard growth curves. Cell suspensions were prepared by standard methods except Gomori's tris buffer at pH 7.2 (Appendix B) was substituted for distilled water in the concentrated cell suspension. The experiment began (zero time) when 0.5 ml of the cells were resuspended in duplicate spectrophotometric tubes containing 3.5 ml of Gomori's tris buffer (0.05 M pH 7.2) with various concentrations of EDTA and/or lysozyme. The experiment was conducted at 30C.

Loss in absorbance was measured in a Spectronic 20 at 440 nm at 0.25 hour intervals for 4 consecutive hours. The loss in absorbance was plotted against time.

EDTA-lysozyme lysis of Agrobacterium tumefaciens (Figure 7) shows interesting results. All (single-reagent) controls showed relatively little autolysis, with Gomori's tris buffer exhibiting the least.

Synergism between EDTA and lysozyme was observed. As was expected, the higher concentrations of EDTA (0.01 M) and lysozyme (10.0 ug/ml) exhibited the greatest lysis and 0.001 M EDTA with 1.0 ug/ml lysozyme showed the least.

It is evident that A. tumefaciens undergoes rapid and extensive lysis by EDTA-lysozyme. Similar rates have been recorded for Pseudomonas sp. (Shively and Hartsell, 1964) and Escherichia coli (Wright, 1965).

EDTA-lysozyme lysis of Chromobacterium violaceum (Figure 8) differs somewhat from that of A. tumefaciens. Except for the Gomori's tris buffer,

FIGURE 7.

EDTA-LYSOZYME LYSIS OF Agrobacterium tumefaciens

LEGEND

TRIS CONTROL	
0.01 <u>M.</u> EDTA	
0.001 <u>M.</u> EDTA	
1.0 μ g/ml. LYSOZYME	
10.0 μ g/ml. LYSOZYME	
0.01 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	
0.01 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	
0.001 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	
0.001 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	

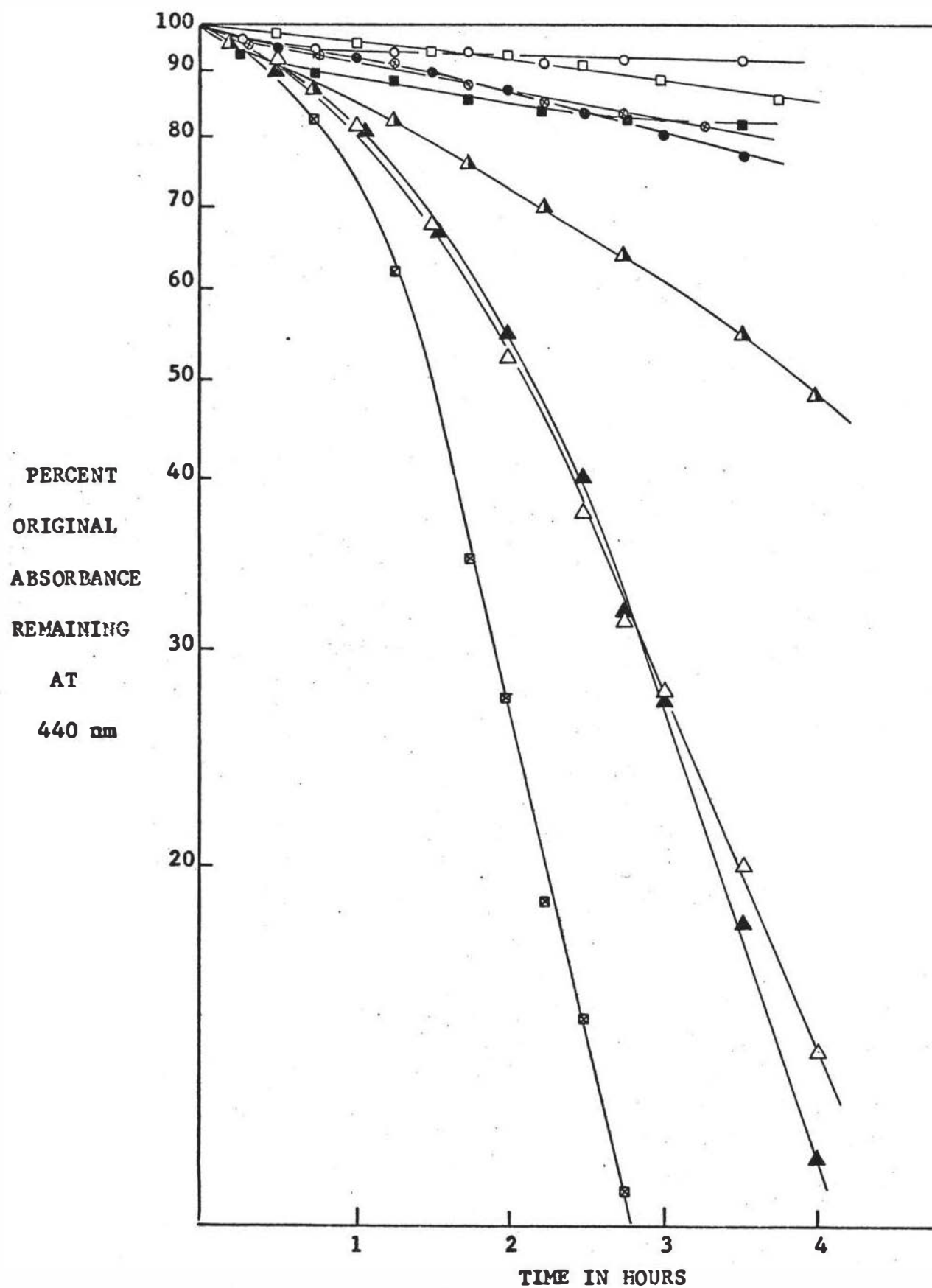


FIG. 7. EDTA-LYSOZYME LYSIS OF Agrobacterium tumefaciens

FIGURE 8.

EDTA-LYSOZYME LYSIS OF Chromobacterium violaceum

LEGEND

TRIS CONTROL	
0.01 <u>M</u> . EDTA	
0.001 <u>M</u> . EDTA	
1.0 $\mu\text{g/ml}$. LYSOZYME	
10.0 $\mu\text{g/ml}$. LYSOZYME	
0.01 <u>M</u> . EDTA + 10.0 $\mu\text{g/ml}$. LYSOZYME	
0.01 <u>M</u> . EDTA + 1.0 $\mu\text{g/ml}$. LYSOZYME	
0.001 <u>M</u> . EDTA + 1.0 $\mu\text{g/ml}$. LYSOZYME	
0.001 <u>M</u> . EDTA + 10.0 $\mu\text{g/ml}$. LYSOZYME	

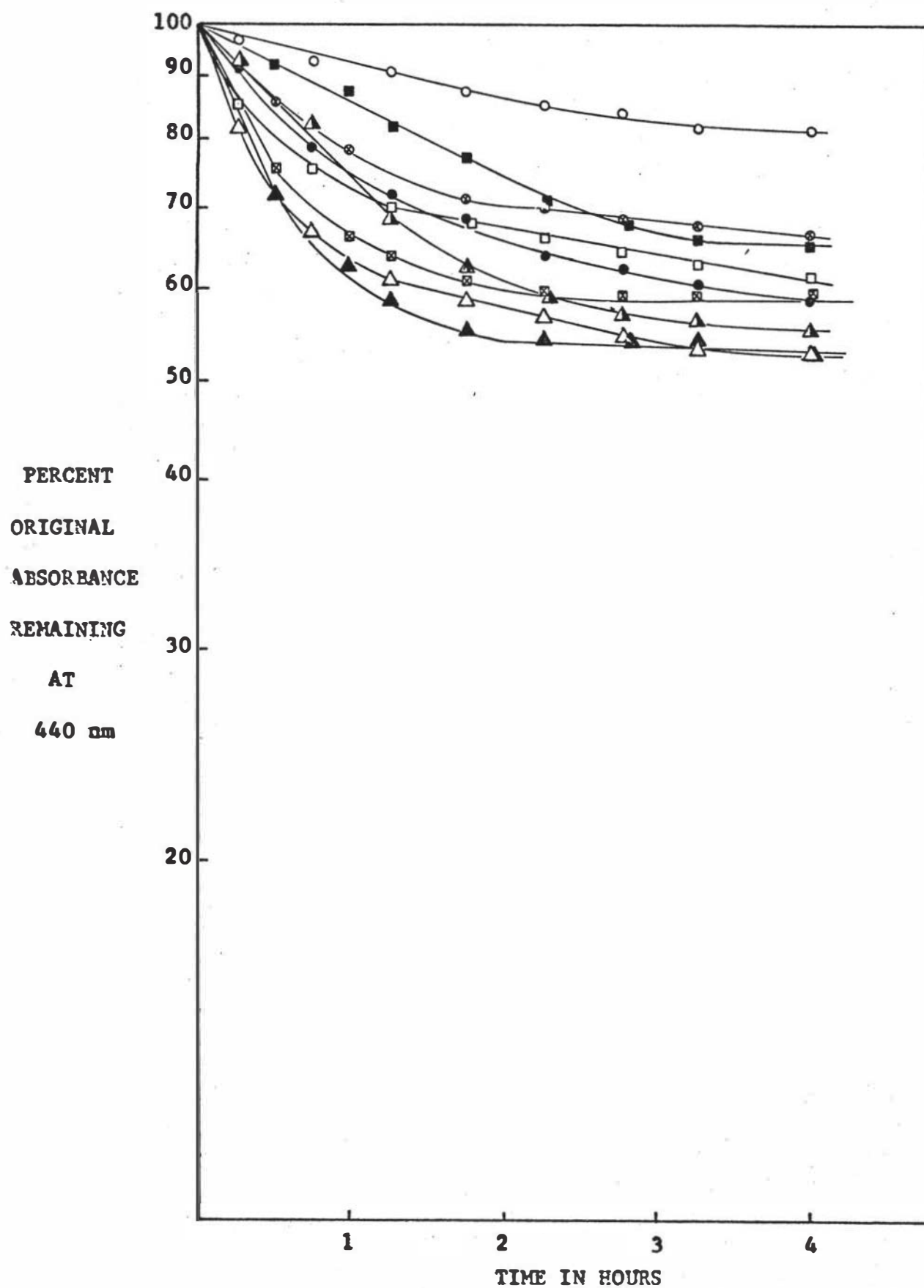


FIG. 8. EDTA-LYSOZYME LYSIS OF Chromobacterium violaceum

all (single-reagent) controls showed similar rates of lysis. The results of the experimental (double-reagent) tests seemed to be more an additive effect of the controls rather than a synergistic effect characteristic of A. tumefaciens. Variation in concentration of the reagents in the experimental (double-reagent) tests seemed to have little or no influence upon lytic action.

EDTA-lysozyme lysis of Rhizobium phaseoli (Figure 9) again showed some differences from those already discussed. Similar to the results for A. tumefaciens, the experimental (double-reagent) tests exhibited a synergistic effect but nowhere near the magnitude or rate for A. tumefaciens. All experimental (double-reagent) tests had similar plots except at the lowest concentrations of EDTA and lysozyme.

EDTA-lysozyme lysis of Rhizobium trifolii (Figure 10) was very similar to that of R. phaseoli. However, it can be seen by the results of the lysozyme controls that lysozyme had a greater affect upon this culture than did EDTA. Again, the synergistic action of EDTA + lysozyme is seen.

EDTA-lysozyme lysis of Rhizobium lupini (Figure 11) had dramatically different results from the preceding cultures. Not one of the experimental controls or tests had any affect on lysis. From these results, it can be concluded that EDTA, lysozyme, or combinations of the two do not affect the cell wall of this bacterium, or possibly that the experimental reagents were unable to penetrate the heavy capsular material found associated with this culture (Appendix A). Although Figure 11 does not show time beyond 4 hours, the author did randomly spot check the cultures at times up to 8 hours with still no observed lysis.

EDTA-lysozyme lysis of Rhizobium japonicum (Figure 12) was similar to that of R. lupini (Figure 11). Again, the results might be attributed to the heavy capsular layer of R. japonicum (Appendix A).

FIGURE 9.

EDTA-LYSOZYME LYSIS OF Rhizobium phaseoli

LEGEND

TRIS CONTROL	—○—
0.01 <u>M.</u> EDTA	—●—
0.001 <u>M.</u> EDTA	—◐—
1.0 μ g/ml. LYSOZYME	—■—
10.0 μ g/ml. LYSOZYME	—◻—
0.01 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	—◼—
0.01 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	—△—
0.001 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	—▲—
0.001 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	—▲—

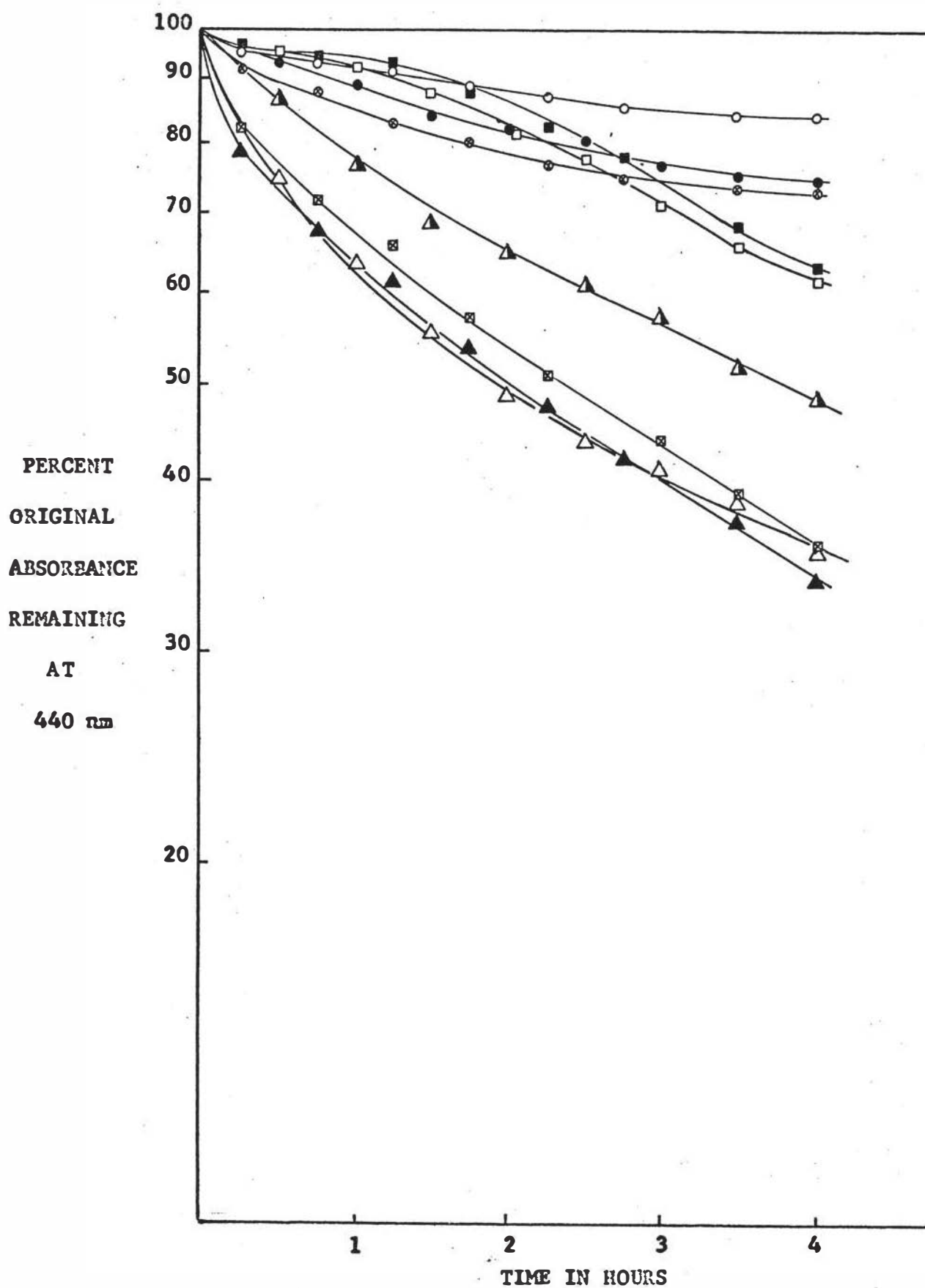


FIG. 9. EDTA-LYSOZYME LYSIS OF Rhizobium phaseoli

FIGURE 10.

EDTA-LYSOZYME LYSIS OF Rhizobium trifolii

LEGEND

TRIS CONTROL	
0.01 <u>M.</u> EDTA	
0.001 <u>M.</u> EDTA	
1.0 μ g/ml. LYSOZYME	
10.0 μ g/ml. LYSOZYME	
0.01 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	
0.01 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	
0.001 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	
0.001 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	

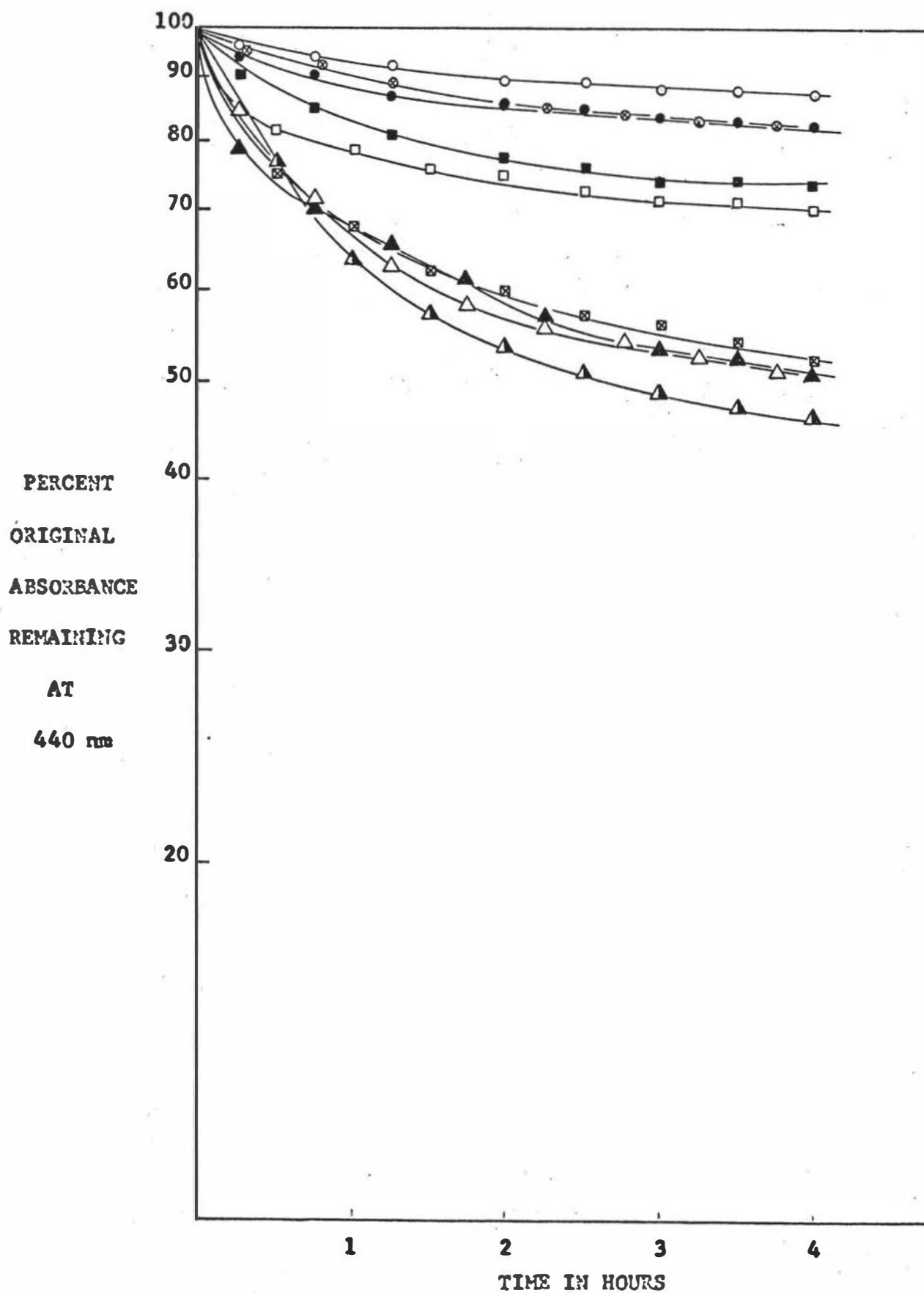


FIG. 10. EDTA-LYSOZYME LYSIS OF Rhizobium trifolii

FIGURE 11.

EDTA-LYSOZYME LYSIS OF Rhizobium lupini

LEGEND

TRIS CONTROL	—○—
0.01 <u>M.</u> EDTA	—●—
0.001 <u>M.</u> EDTA	—⊗—
1.0 μ g/ml. LYSOZYME	—■—
10.0 μ g/ml. LYSOZYME	—□—
0.01 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	—⊠—
0.01 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	—△—
0.001 <u>M.</u> EDTA + 1.0 μ g/ml. LYSOZYME	—▲—
0.001 <u>M.</u> EDTA + 10.0 μ g/ml. LYSOZYME	—▲—

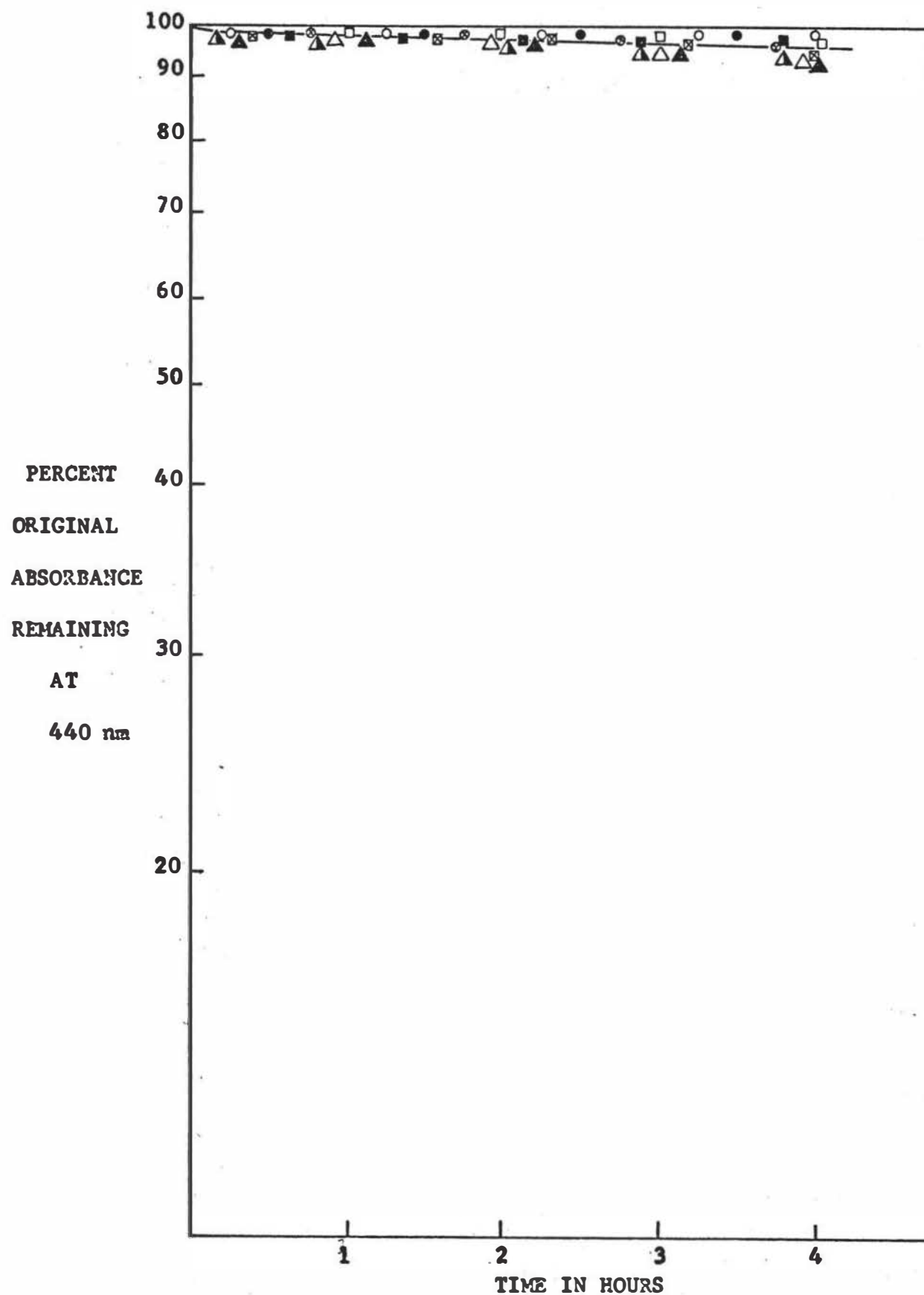


FIG. 11. EDTA-LYSOZYME LYSIS OF Rhizobium lupini

FIGURE 12

EDTA-LYSOZYME LYSIS OF Rhizobium japonicum

END

TRIS CONTROL	—○—
0.01 <u>M.</u> EDTA	—●—
0.001 <u>M.</u> EDTA	—◐—
1.0 $\mu\text{g/ml.}$ LYSOZYME	—■—
10.0 $\mu\text{g/ml.}$ LYSOZYME	—○—
0.01 <u>M.</u> EDTA + 10.0 $\mu\text{g/ml.}$ LYSOZYME	—◻—
0.01 <u>M.</u> EDTA + 1.0 $\mu\text{g/ml.}$ LYSOZYME	—△—
0.001 <u>M.</u> EDTA + 1.0 $\mu\text{g/ml.}$ LYSOZYME	—▲—
0.001 <u>M.</u> EDTA + 10.0 $\mu\text{g/ml.}$ LYSOZYME	—▲—

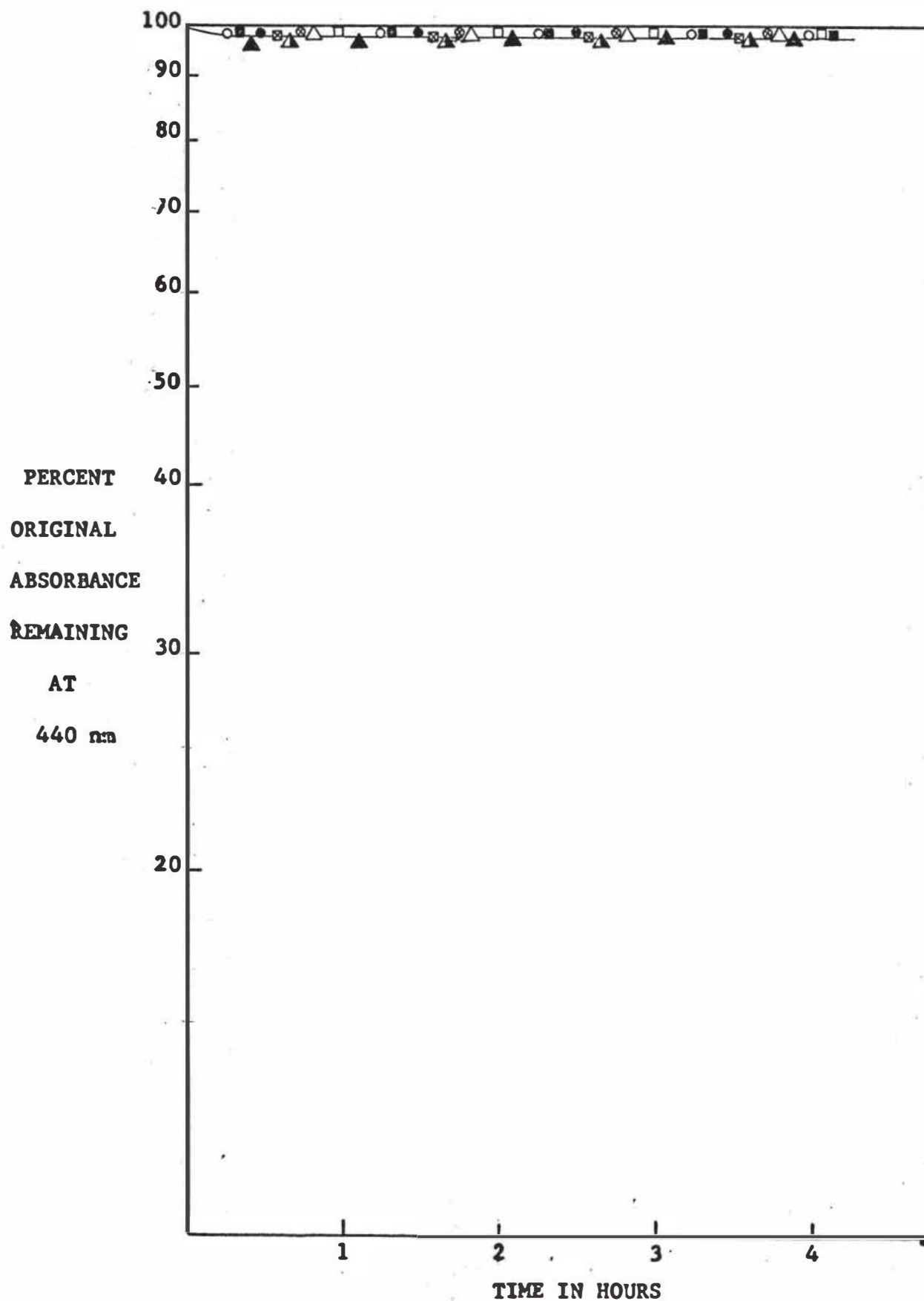


FIG. 12. EDTA-LYSOZYME LYSIS OF Rhizobium japonicum

The possibility that the capsule might protect the cell wall from heterolytic damage could be resolved by further experiments with the capsular layer of these cells totally removed. However, this approach was not attempted by this researcher due to a time limitation. The only attempt to strip the capsular layer was with a sterile distilled water wash which appeared to be not entirely effective.

DISCUSSION

Although the bacterial cell wall fulfills the same primary function (protection) in all bacterial cells, there is great diversity in cell wall structure among the different systematic groups of bacteria. Salton (1964) describes some of the variations in cell wall morphology and overall chemical composition.

The extensive work of Cummins and Harris (1955, 1956) established the chemical composition of gram-positive bacterial cell walls as valuable taxonomic characters. Using paper chromatographic analysis, they were able to distinguish genera and some species solely by biochemical differences. They regarded cell wall composition as an extension of morphology at the biochemical level. Furthermore, they reported the cell wall of gram-positive bacteria as a stable character, unaffected by variations in culture media or conditions of growth. However, Marr et al. (1964) reported some variation in the cell wall of Escherichia coli (gram-negative) by variation in cultural conditions.

The cell walls of gram-negative bacteria have a higher level of structural and chemical complexity than those of gram-positive. Thus, the variation in gram-negative bacteria would seemingly be greater.

As is the case for chemical composition, physiological responses of bacterial cell walls to external lytic agents can lend taxonomic information. Repaske (1958) listed differences between Escherichia coli and Azotobacter vinelandii by their responses to lytic agents, when these organisms were believed to be quite similar. Judith and Weinberg (1959) used lytic responses of the cell walls of the genus Bacillus to distinguish

taxonomic groups. Caldwell (1960) used cell wall differences to provide a simple, rapid, and accurate method to distinguish the species of Group D streptococci. Similarly, bacteriolysis of a large number of the Enterobacteriaceae was tested and taxonomic information was gained by Noller and Hartsell (1961).

In a similar manner the research results reported in this thesis will be applied to the present taxonomic classification in Bergey's Manual (Breed et al., 1957) and the proposed reclassification in Table 1.

Autolysis in Spent Growth Medium set the stage for subsequent experiments. Because little or no autolysis was observed, DMYE broth apparently did not effect autolysis.

Autolysis in Saline showed some general tendencies. An increase in temperature caused greater autolysis in all cultures. This corresponds with the findings of Becker and Hartsell (1954) who used heat treatment in conjunction with lytic agents to increase heterolysis. Although no lytic agents were used in this experiment, it is known that the bacterial cell itself contains lytic enzymes necessary to carry on cell wall biosynthesis (Strominger, in Gunsalus and Stanier, 1962). Possibly, the heat treatment caused a denaturation of the anabolic enzymes, and thus allowed the catabolic enzymes (lysozyme) to autolyze the cell.

Another tendency was to see greater autolysis at higher concentrations of saline. It could be that the sodium ions were exchanging with divalent cations of calcium and/or magnesium which are reported to be found in Phizobium cell walls by Humphrey (1962). Shively and Hartsell (1964) reported an inhibition of the lysis of Pseudomonas sp. with an increase in salts. This may have been due to a salt-inflicted denaturation of the catabolic enzymes. This result was not observed in the cultures of the Rhizobiaceae tested.

The cultures exhibited different responses to the various NaCl concentrations. However, upon viewing the percent autolysis (Appendix C) a general grouping of organisms was apparent. By compiling an average percent autolysis for each culture, it was noted that Agrobacterium tumefaciens, Rhizobium phaseoli, and Rhizobium trifolii had similar averages of autolysis which were 18%, 16%, and 18% respectively. Rhizobium japonicum and Rhizobium lupini exhibited average autolyses of 5% and 7%. Chromobacterium violaceum, on the other hand, showed an average of 27%, a significantly higher percent autolysis.

When these tendencies are compared with the proposed reclassification of the Rhizobiaceae, similarities are noticed. Agrobacterium tumefaciens had been proposed a member of the genus Rhizobium. Furthermore, Rhizobium phaseoli and Rhizobium trifolii have been lumped into Rhizobium leguminosarum. Also, the reclassification suggests the combining of Rhizobium japonicum and Rhizobium lupini into a single species. In addition, Chromobacterium violaceum has been proposed a member of another family (Pseudomonadaceae). The high autolysis exhibited by this species tends to favor the removal of this organism from the Rhizobiaceae.

Again, general tendencies were observed in Autolysis in Buffers. The different strengths of metal ion binders (citrate, phosphate, maleate) that the buffers contained once again suggested the need for divalent cations in the cell wall complex as discussed by Vincent (1962). As the cation-binding strength of these buffers increased, a greater amount of autolysis was observed.

Furthermore, the same autolytic responses were observed as was seen in Autolysis in Saline. Agrobacterium tumefaciens, Rhizobium phaseoli, and Rhizobium trifolii averaged 20%, 21%, and 22% autolysis respectively. Chromobacterium violaceum again showed a high average autolysis (31%), whereas

Rhizobium japonicum and Rhizobium lupini had low responses of 8% and 9% respectively. These results appear to support the suggestions to reclassify these organisms.

The results of Autolysis as a Function of pH suggest a possible pH optimum for the enzymes causing autolysis. The greatest autolysis was observed at higher pH levels. Less autolysis was observed in all cultures as the pH became more acidic. This corresponds with the autolytic response of Pseudomonas sp. as reported by Shively and Hartsell (1964). Furthermore, this pH optimum corresponds with the reported pH optimum of lysozyme (pH 7.6-8.0) as reported by Wright (1965).

In addition, the tendency of similar responses to pH by Agrobacterium tumefaciens, Rhizobium phaseoli, and Rhizobium trifolii; Rhizobium japonicum and Rhizobium lupini; and an individual response by Chromobacterium violaceum again supports the reclassification proposal.

Autolysis in EDTA resulted in some interesting results. The cultures of Agrobacterium tumefaciens, Rhizobium phaseoli, and Rhizobium trifolii once again exhibited remarkable similarity in average autolysis. The average percent autolysis was 27%, 27%, and 26% respectively. Chromobacterium violaceum showed an average of 21% autolysis in all treatments of EDTA. Finally, Rhizobium lupini and Rhizobium japonicum demonstrated a somewhat different response to EDTA with a percent autolysis of 9% and 0% respectively. Repaske (1958) and later Gray and Wilkinson (1965) speculated that the action of EDTA is to cause the removal or displacement of divalent cations from the cell wall. Furthermore, calcium and magnesium have been shown to be a requirement of the cell walls of Rhizobium trifolii and Rhizobium meliloti (Humphrey, 1962). If EDTA is, in fact, tying up or removing divalent cations from the cell wall, then the difference in response of these three groups suggests either a

quantitative difference in divalent cations in their cell walls or a more extensive protective covering (capsule or lipoprotein) in some of the strains.

It is extremely interesting to note the response of Rhizobium japonicum to EDTA. As seen in Appendix C, no autolysis occurred for any treatment. In fact, all treatments resulted in an increase in optical density. Whether this is a result of growth or some other factor was not investigated.

It is also apparent that the groups resulting from Autolysis in EDTA once again support the proposed reclassification of these organisms (Table 1).

The application of lysozyme, an enzyme known to cleave the mucopeptide backbone of bacteria (Phillips, 1966) establishes the presence of this complex (Salton, 1964). Early studies of gram-positive bacteria revealed almost uniform lysis by lysozyme. However, lysozyme treatment of gram-negative bacteria showed very little effect. Thus, early workers believed that this complex was lacking from most gram-negative bacterial cell walls.

In applying heat treatment (Becker and Hartsell, 1954) it was found that gram-negative bacteria were also susceptible to lysozymic attack provided another agent was used in conjunction with lysozyme. The addition of other agents to allow lysis has been expanded to include butanol (Becker and Hartsell, 1955), EDTA (Repaske, 1956), and sodium lauryl sulfate (Colobert, 1957).

At the same time these reagents were aiding the demonstration of the presence of the mucopeptide of gram-negative bacteria they also demonstrated an existence of another complex that was either shielding or working in conjunction with the mucopeptide. Butanol and sodium lauryl sulfate action was correlated with the lipids in the cell wall membrane of gram-negative bacteria. These agents appeared to strip away the lipid layer exposing the underlying mucopeptide to lysozyme. However, the action of EDTA (a divalent cation

chelator) is still unknown. Repaske (1958) speculated that EDTA forms a single bond with one of the positive ions found on the surface of the cell wall. Gray and Wilkinson (1965) reported the release of pentoses, phosphorus, and other materials which were detected when Pseudomonas aeruginosa was treated with EDTA. This certainly suggests cell wall or membrane damage. Lieve (1965) reported the release of 30-50% of the lipopolysaccharide from Escherichia coli when subjected to EDTA. Similar studies by Rogers et al. (1968) showed that EDTA releases a protein-lipopolysaccharide complex from Pseudomonas strains. All this research suggests that EDTA damages the cell wall membrane found in gram-negative bacteria by removing or tying up divalent cations or by breaking the link between the lipoprotein (cell wall membrane) and the inner components.

EDTA-lysozyme treatment of Agrobacterium tumefaciens yielded interesting results. The affect of EDTA and lysozyme separately was slight when compared with the tris (buffer) control. However, when the two were used together they produced a synergistic lytic response. This seems to suggest that Agrobacterium tumefaciens does in fact have a "typical" cell wall membrane covering an underlying "typical" mucopeptide.

The effect of EDTA-lysozyme upon Chromobacterium violaceum showed a somewhat different result. Chromobacterium violaceum was lysed by these two reagents, however, its rate did not appear synergistic, but rather additive. This seems to suggest that the lipoprotein and/or mucopeptide are influenced by factors other than those previously discussed.

The effect of EDTA-lysozyme upon Rhizobium phaseoli and Rhizobium trifolii were very similar: both exhibited a synergistic lytic effect to co-treatment with lysozyme and EDTA. Again, the existence of a cell wall membrane bound to the inner components plus the presence of the mucopeptide is suggested.

Finally, the results of EDTA-lysozymic lysis of Rhizobium lupini and Rhizobium japonicum were striking. Neither culture showed any signs of lysis by any treatment (EDTA and/or lysozyme). Furthermore, although the percent lysis in Appendix C does not show it, these cultures actually exhibited an increase in optical density upon treatment. This suggests the absence of the mucopeptide, a lysozyme resistant mucopentide (Brumfitt et al., 1958), a different bonding of the cell wall membrane to mucopeptide (other than by divalent cations), or further protection by another accessory layer such as the heavy capsule found associated with these organisms. It would be interesting to remove the capsular layers of these organisms and subject them to autolysis and heterolysis studies. However, this was not done due to the time needed to evaluate the voluminous data collected during this research.

Finally, EDTA-lysozyme experiments on the six strains tested produced four groups: Rhizobium lupini and Rhizobium japonicum with no observed lysis (0%); Chromobacterium violaceum with low lysis (30-40%); Rhizobium phaseoli and Rhizobium trifolii with moderate lysis (50-60%); and Agrobacterium tumefaciens with high lysis (90-90%). Again, this data lends support to the proposed reclassification scheme (Table 1). The different response of Agrobacterium tumefaciens from Rhizobium phaseoli and Rhizobium trifolii could be attributed to a quantitative difference in bonds that link the cell wall membrane and/or the mucopeptide complex. It should be noted that the new classification proposal places Agrobacterium tumefaciens in the genus Rhizobium but within the "species" Rhizobium radiobacter. Rhizobium phaseoli and Rhizobium trifolii are classified as Rhizobium leguminosarum. This could explain the somewhat different results between these two groups (Agrobacterium tumefaciens and Rhizobium phaseoli - Rhizobium trifolii) that have exhibited similar results in previous experiments.

The rather different response of these organisms to EDTA-lysozyme suggests a means of simple and rapid identification similar to the method of Caldwell (1960) on Group D streptococci. However, the small representation of this family (six species) is hardly sufficient to be conclusive. This author suggests further work along this line.

To conclude, it should be noted that the research conducted here was the first step in the elucidation of the cell walls of the Rhizobiaceae. With the information gained here, other researchers can move into qualitative and quantitative studies involving the cell wall, hopefully reaching closer to an understanding of the cytochemistry of these important bacteria.

CONCLUSIONS

1. The results of the experiments, Autolysis in Saline, Autolysis in Buffers, Autolysis as a Function of pH, and Lysis in EDTA-lysozyme, all support and lend further evidence for the need to reclassify the Rhizobiaceae according to the proposed reclassification in Table 1.

2. The EDTA-lysozyme experiment demonstrated the presence of a "typical" mucopeptide in Agrobacterium tumefaciens, Chromobacterium violaceum, Rhizobium phaseoli, and Rhizobium trifolii. Although the data does not support a similar conclusion for Rhizobium japonicum and Rhizobium lupini, this researcher suspects that the mucopeptide complex is present. However, it is probably being shielded by an additional accessory layer such as the capsule. Further data need to be compiled to further support this theory.

3. The EDTA-lysozyme experiment also demonstrated that EDTA is needed in order to expose the mucopeptide in Agrobacterium tumefaciens, Chromobacterium violaceum, Rhizobium phaseoli, and Rhizobium trifolii. Again, the negative results of Rhizobium japonicum and Rhizobium lupini suggest the presence of an EDTA resistant accessory layer other than the lipoprotein (cell wall membrane).

4. The EDTA and EDTA-lysozyme experiments suggested that the action of EDTA is on the lipoprotein (cell wall membrane) layer in the Agrobacterium tumefaciens, Chromobacterium violaceum, Rhizobium phaseoli, and Rhizobium trifolii strains used. One possible action of EDTA is that of removing divalent cations which are needed in this layer for its integrity.

5. Finally, the EDTA-lysozyme experiment seems to indicate that within the limits of the cultures tested, lytic criteria might be developed into a taxonomic key for a simple and rapid method for the identification of the species of the Rhizobiaceae.

APPENDICES

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APPENDIX A
CHARACTERIZATION OF CULTURES

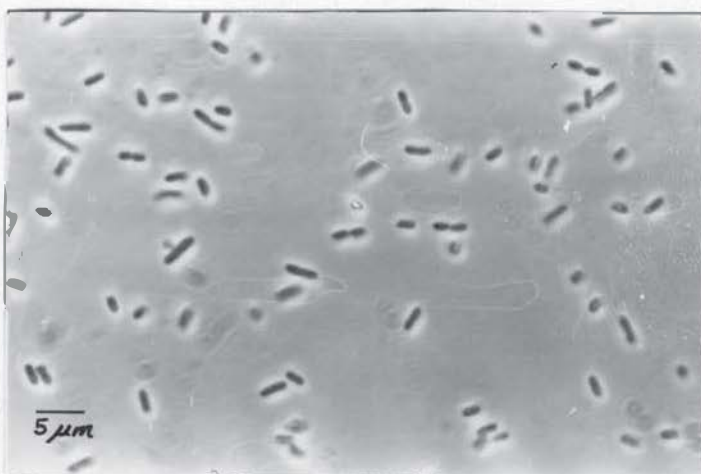
APPENDIX A

Characterization of Cultures1. Agrobacterium tumefaciens EIU-1

SOURCE: Dr. William A. Weiler
Botany Department, EIU
From Purdue University Culture Collection

CHARACTERIZATION:

Gram-negative rod
Measures: 1 x 2-5 μ m
Generation time (DMYE broth): 1.5 hours
Motility: 1-4 peritrichous flagella
Optimum growth temperature: 25-30C
Capsular production: Little or none
Found: Soil

Agrobacterium tumefaciens

2. Chromobacterium violaceum EIU-3848

SOURCE: Eastern Illinois University Culture Collection

CHARACTERIZATION:

Gram-negative rod

Measures: $.75 \times 2-5 \mu\text{m}$

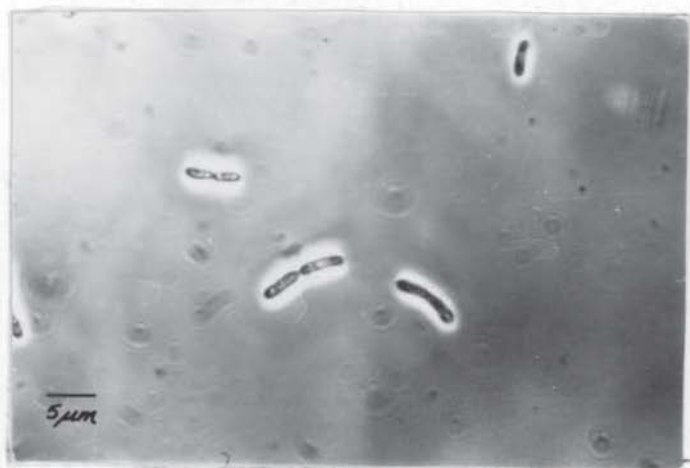
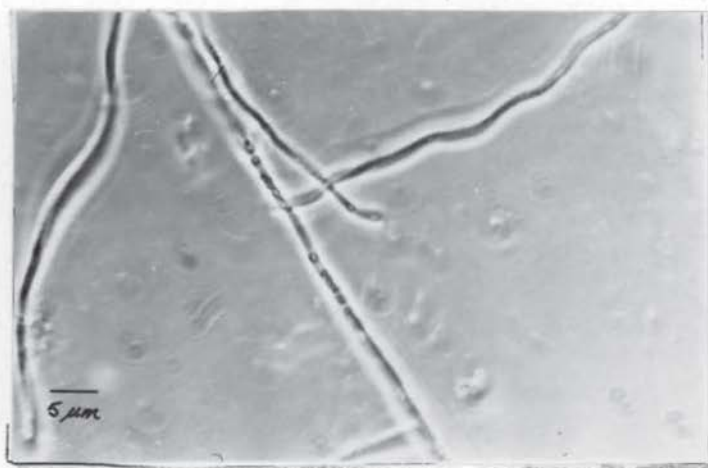
Generation time (DMYE broth): 2.4 hours

Motility: 1-4 flagella

Optimum growth temperature: 25-30C

Capsular production: Little

Found: Soil and water



Chromobacterium violaceum

Agar

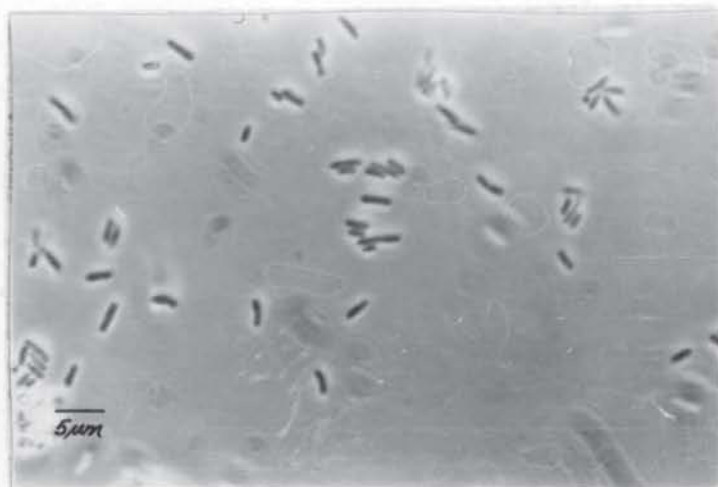
Broth

3. Rhizobium phaseoli UL-5

SOURCE: Forester Davidson
Urbana Laboratory
Urbana, Illinois

CHARACTERIZATION:

Gram-negative rod
Measures: 1 x 2-3 μ m
Generation time (DMYE broth): 3.6 hours
Motility: peritrichous flagella
Optimum growth temperature: 25C
Capsular production: Little
Found: Pathogen of Phaseolus (bean)



Rhizobium phaseoli

4. Rhizobium trifolii UL-2

SOURCE: Forester Davidson
Urbana Laboratory
Urbana, Illinois

CHARACTERIZATION:

Gram-negative rods
Measures: $1.5 \times 2-4 \mu\text{m}$
Generation time (DMYE broth): 4.6 hours
Optimum growth temperature: 25C
Motility: Peritrichous flagella
Capsular production: Small
Found: Pathogen of Trifolium (clover)



Rhizobium trifolii

5. Rhizobium lupini UL-4

SOURCE: Forester Davidson
Urbana Laboratory
Urbana, Illinois

CHARACTERIZATION:

Gram-negative rods

Measures: 1 x 2-3 μ m

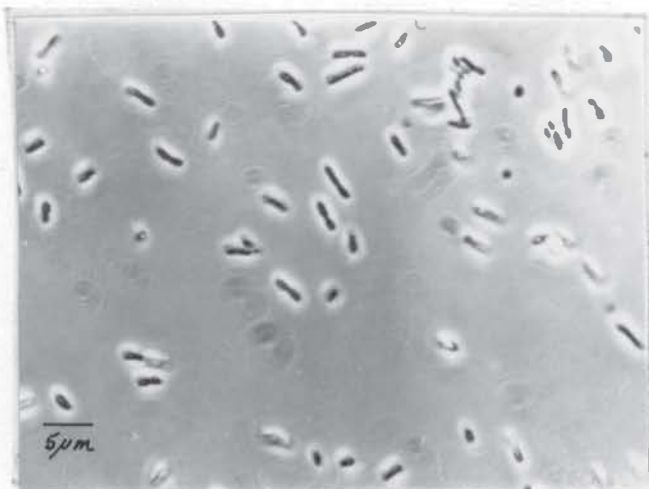
Generation time (DMYE broth): 10.0 hours

Optimum growth temperature: 25C

Motility: 1-4 flagella

Capsular production: Heavy

Found: Pathogen of Lupinus (lupine), Serradella,
and Ornithopus



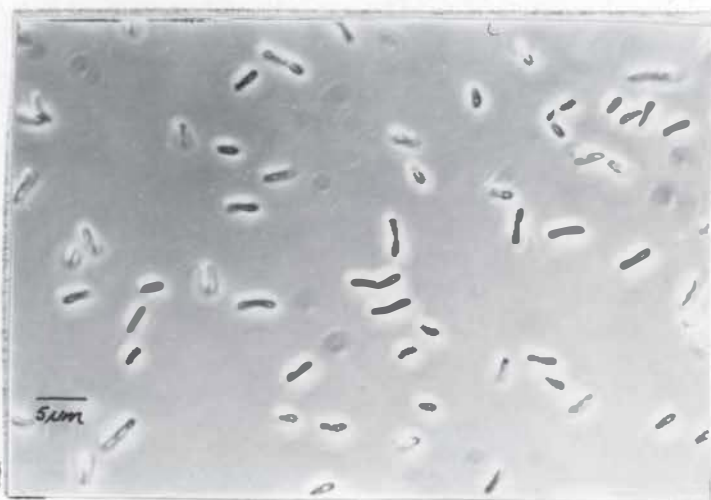
Rhizobium lupini

6. Rhizobium japonicum UL-1

SOURCE: Forester Davidson
Urbana Laboratory
Urbana, Illinois

CHARACTERIZATION:

Gram-negative rods
Measures: 1 x 2-5 μ m
Generation time (DMYE broth): 10.0 hours
Optimum growth temperature: 25C
Motility: monotrichous flagella
Capsular production: Heavy
Found: Pathogen of Soia max (soybean)



Rhizobium japonicum

APPENDIX B

MEDIA, BUFFERS, AND REAGENTS

APPENDIX B

Media, Buffers, and Reagents

Media:

1. Dextrose Mannitol Yeast Extract (DMYE broth)

Mannitol	5.0g.
Dextrose	5.0g.
Yeast Extract	1.0g.
Sodium Chloride	0.2g.
Distilled Water	1000.0 ml.

For an agar medium: Add 15.0g. (1.5%) Agar
 pH adjusted to 7.2 with NaOH
 Autoclaved for 20 minutes, 15 psi

2. Rhizobium - X *

Soil Extract	200.0 ml.
African violet soil	77.0g.
Na ₂ CO ₃	0.2g.
Distilled Water	200.0 ml.
Yeast Extract	1.0g.
Mannitol	10.0g.
Distilled Water	800.0 ml.

For an agar medium: Add 15.0g. (1.5%) Agar
 pH adjusted to 7.2 with NaOH
 Autoclaved for 20 minutes, 15 psi

- * this medium proved to be difficult to prepare due to slow filtration of the soil extract, thus it was not used in this thesis

Buffers:**1. Sorensen's Phosphate 0.066 M.**Solution A: 9.1g. KH_2PO_4 (anhyd.)/ liter distilled waterSolution B: 9.4g. Na_2HPO_4 (anhyd.)/ liter distilled waterGeneral Mix Formula: $10[X\text{ml. A} + (100-X)\text{ml. B}]$ Mix for pH:

	ml. of A	ml. of B
5.2	980	20
5.6	948	52
6.0	877	123
6.4	732	268
6.8	508	492
7.2	285	715
7.6	132	868
8.0	55	945

Sterilize: Autoclave for 20 minutes, 15 psi**2. McIlvane's Citric Acid-Phosphate 0.1 M.**Solution A: 21.0g. Citric Acid·H₂O/ liter distilled waterSolution B: 28.4g. Na_2HPO_4 (anhyd.)/ liter distilled waterGeneral Mix Formula: $10[X\text{ml. A} + (100-X)\text{ml. B}]$ Mix for pH:

	ml. of A	ml. of B
5.2	464	536
5.6	420	580
6.0	365.5	631.5
6.4	307.5	692.5
6.8	227.5	772.5
7.2	130.5	869.5
7.6	63.5	936.5
8.0	27.5	972.5

Sterilize: Autoclave for 20 minutes, 15 psi

3. Gomori's Tris-maleate

Solution A: 24.2g. Tris + 23.2g. Maleic Acid/ liter distilled water

Solution B: 8.0g. NaOH/ liter distilled water

General Mix Formula: 10[25ml. A + Xml. B up to 100 ml. with distilled water]

Mix for pH:

	ml. of A	ml. of B	distilled H ₂ O
5.2	250	35	715
5.6	250	78	672
6.0	250	130	620
6.4	250	185	565
6.8	250	225	525
7.2	250	255	495
7.6	250	290	460
8.0	250	345	405

Sterilize: Autoclave for 20 minutes, 15 psi

4. Gomori's Tris 0.05 M. pH 7.2

Solution A: 2.42g. Tris/ liter distilled water

Solution B: 0.2 N. HCl (20 ml. con. HCl/ liter distilled water)

General Mix Formula: 10[25ml. A + Xml. B up to 100 ml. with distilled water]

Mix for pH 7.2:

	ml. of A	ml. of B	distilled H ₂ O
Gives 0.2 M	250	221	529

Final Dilution: 3:1 dilution of 0.2 M. Tris buffer with distilled water

Reagents:

1. Disodium (Ethylenedinitrilo) tetracetate (EDTA)
2. Lysozyme (3X Crystalline) Globulin G₁
3. Sodium Chloride USP granular

APPENDIX C

RAW DATA

PERCENT AUTOLYSIS IN SALINE AT 20C
(by loss in absorbance)

Percent Saline		0.0	0.5	1.0	2.0	10.0
<u>Agrobacterium tumefaciens</u>	3 hours ^a	03 05	14 11	07 04	09 12	11 09
	24 hours	07 09	22 20	22 15	17 26	21 24
<u>Chromobacterium violaceum</u>	3 hours	06 00	22 19	23 21	14 15	24 21
	24 hours	00 00	34 29	31 25	41 38	38 36
<u>Rhizobium phaseoli</u>	3 hours	02 06	11 11	04 06	07 10	11 17
	24 hours	07 08	23 25	19 19	13 17	18 25
<u>Rhizobium trifolii</u>	3 hours	02 03	08 12	09 13	07 04	14 11
	24 hours	02 05	22 24	25 26	17 17	24 23
<u>Rhizobium lupini</u>	3 hours	00 02	04 04	0* 00	09 00	08 07
	24 hours	00 00	08 06	0* 00	08 00	19 11
<u>Rhizobium japonicum</u>	3 hours	0* 0*	00 0*	0* 00	04 04	07 08
	24 hours	0* 0*	0* 0*	0* 00	05 06	21 19

^a Figures represent duplicate tubes

0* Increase absorbance

PERCENT AUTOLYSIS IN SALINE AT 30C
(by loss in absorbance)

Percent Saline		0.0	0.5	1.0	2.0	10.0
<u>Agrobacterium tumefaciens</u>	3 hours ^a	03 07	15 14	10 13	14 18	11 13
	24 hours	16 15	23 22	18 16	24 33	37 31
<u>Chromobacterium violaceum</u>	3 hours	00 03	24 31	32 21	26 19	26 26
	24 hours	0* 07	24 31	42 29	61 58	44 47
<u>Rhizobium phaseoli</u>	3 hours	07 07	19 17	15 11	10 10	13 11
	24 hours	09 07	25 23	27 26	24 23	23 22
<u>Rhizobium trifolii</u>	3 hours	05 05	24 18	13 13	17 14	17 13
	24 hours	02 02	28 31	35 29	38 38	29 29
<u>Rhizobium lupini</u>	3 hours	04 04	06 08	02 00	00 02	07 10
	24 hours	09 07	08 12	00 0*	00 04	17 17
<u>Rhizobium japonicum</u>	3 hours	0* 0*	00 02	03 05	04 06	04 13
	24 hours	0* 0*	0* 0*	00 0*	07 07	23 21

^a Figures represent duplicate tubes

0* Increase absorbance

PERCENT AUTOLYSIS IN SALINE AT 40C
(by loss in absorbance)

Percent Saline		0.0	0.5	1.0	2.0	10.0
<u>Agrobacterium tumefaciens</u>	3 hours ^a	09 02	15 09	09 14	22 21	23 16
	24 hours	15 05	35 29	37 41	49 49	47 38
<u>Chromobacterium violaceum</u>	3 hours	00 00	28 33	50 48	45 47	38 43
	24 hours	0* 0*	23 33	38 50	39 39	46 49
<u>Rhizobium phaseoli</u>	3 hours	09 -7	20 19	13 15	13 14	16 13
	24 hours	09 09	25 27	26 31	27 30	34 33
<u>Rhizobium trifolii</u>	3 hours	05 02	32 17	18 17	27 24	20 23
	24 hours	02 02	39 30	28 27	38 35	35 36
<u>Rhizobium lupini</u>	3 hours	02 07	06 08	02 04	04 00	18 18
	24 hours	04 07	06 03	15 18	10 10	40 38
<u>Rhizobium japonicum</u>	3 hours	0* 0*	0* 00	03 03	04 09	20 23
	24 hours	0* 0*	0* 0*	0* 0*	00 03	21 25

^a Figures represent duplicate tubes

0* Increase absorbance

PERCENT AUTOLYSIS IN BUFFERS: LOG PHASE CELLS
(by loss in absorbance)

Buffer (pH 7.2) Temperature C		McIlvane's			Sorensen's			Gomori's		
		20	30	40	20	30	40	20	30	40
<u>Agrobacterium tumefaciens</u>	3 hours ^a	08 10	11 10	17 11	13 09	08 16	11 21	10 11	06 09	16 12
	24 hours	08 10	42 35	43 38	27 20	29 33	34 39	11 11	11 30	25 14
<u>Chromobacterium violaceum</u>	3 hours	08 24	14 15	13 25	23 25	26 28	35 37	21 22	32 28	28 36
	24 hours	14 42	62 75	71 72	40 45	55 48	72 58	25 25	34 34	12 02
<u>Rhizobium phaseoli</u>	3 hours	14 16	21 21	21 24	28 18	25 30	06 13	11 15	12 09	06 10
	24 hours	20 22	29 28	38 39	20 25	25 38	16 25	12 18	26 23	23 18
<u>Rhizobium trifolii</u>	3 hours	16 18	33 36	28 34	03 06	13 17	14 11	08 03	00 11	01 03
	24 hours	31 33	40 43	40 44	09 14	22 28	37 34	20 15	00 23	15 10
<u>Rhizobium lupini</u>	3 hours	13 19	21 20	17 15	14 08	06 03	12 06	0* 0*	0* 0*	0* 0*
	24 hours	20 24	21 17	03 03	16 06	13 09	21 16	0* 0*	00 02	0* 0*
<u>Rhizobium japonicum</u>	3 hours	13 14	11 11	14 21	09 09	02 0*	03 03	0* 0*	0* 0*	0* 0*
	24 hours	0* 03	06 0*	06 10	07 0*	0* 0*	0* 0*	0* 0*	0* 0*	0* 0*

^a Figures represent duplicate tubes
0* Increase in absorbance

PERCENT AUTOLYSIS IN BUFFERS: STATIONARY PHASE CELLS
(by loss in absorbance)

Buffer (pH 7.2) Temperature C		McIlvane's			Sorensen's			Gomori's		
		20	30	40	20	30	40	20	30	40
	3 hours ^a	13 16	18 25	36 34	04 02	03 04	16 17	02 0*	02 02	02 03
<u>Agrobacterium tumefaciens</u>	24 hours	41 42	44 49	48 50	23 23	26 33	40 43	07 05	21 21	27 32
	3 hours	18 21	21 23	23 32	23 21	19 24	19 25	17 14	17 20	24 25
<u>Chromobacterium violaceum</u>	24 hours	38 38	48 44	54 49	32 33	36 29	39 40	31 30	28 37	32 35
	3 hours	13 20	21 25	29 22	19 21	17 19	23 25	14 13	10 12	15 10
<u>Rhizobium phaseoli</u>	24 hours	23 31	38 39	42 39	26 28	23 18	27 30	24 21	10 17	20 22
	3 hours	17 22	33 29	37 34	13 15	16 24	24 23	17 15	13 17	19 13
<u>Rhizobium trifolii</u>	24 hours	37 38	47 47	40 38	17 26	31 30	34 35	15 23	08 13	27 25
	3 hours	06 06	00 00	14 21	00 00	00 02	10 05	0* 0*	10 05	00 0*
<u>Rhizobium lupini</u>	24 hours	29 28	07 04	24 28	08 04	09 09	21 23	04 08	21 23	0* 0*
	3 hours	11 07	02 14	08 13	02 16	09 08	13 14	0* 0*	13 14	0* 0*
<u>Rhizobium japonicum</u>	24 hours	05 05	18 26	08 28	18 26	25 24	30 32	0* 0*	30 32	0* 0*

^a Figures represent duplicate tubes
0* Increase in absorbance

PERCENT AUTOLYSIS AS A FUNCTION OF pH: McILVAN'S BUFFER (30C)
(by loss in absorbance)

pH		5.2	5.6	6.0	6.4	6.8	7.2	7.6	8.0
<u>Agrobacterium tumefaciens</u>	3 hours ^a	17 23	25 22	25 23	25 21	25 25	28 23	24 25	26 26
	24 hours	17 24	33 25	45 41	41 40	40 49	42 45	43 48	51 47
<u>Chromobacterium violaceum</u>	3 hours	25 28	42 30	42 38	41 35	43 31	32 34	34 42	40 42
	24 hours	22 27	34 23	31 26	35 32	44 31	45 45	50 57	64 65
<u>Rhizobium phaseoli</u>	3 hours	15 16	13 16	08 16	18 17	16 15	22 20	20 23	24 30
	24 hours	25 24	27 27	29 36	33 34	32 35	35 35	40 40	46 51
<u>Rhizobium trifolii</u>	3 hours	16 17	16 22	19 19	30 21	34 34	30 40	36 33	37 36
	24 hours	22 23	25 27	19 21	25 17	23 20	12 29	23 21	28 21
<u>Rhizobium lupini</u>	3 hours	14 17	15 12	08 11	09 11	12 13	22 16	16 18	20 20
	24 hours	05 08	06 01	0* 00	00 01	03 03	08 06	05 09	20 16
<u>Rhizobium japonicum</u>	3 hours	11 13	03 13	09 06	06 00	01 00	03 03	05 05	05 05
	24 hours	00 00	0* 0*	0* 0*	0* 0*	0* 01	0* 00	00 05	17 17

^a Figures represent duplicate tubes
0* Increase in absorbance

PERCENT AUTOLYSIS AS A FUNCTION OF pH: SORESEN'S BUFFER (30C)
(by loss in absorbance)

pH		5.2	5.6	6.0	6.4	6.8	7.2	7.6	8.0
<u>Agrobacterium tumefaciens</u>	3 hours ^a	15 16	16 12	14 14	18 16	15 23	18 24	14 18	25 20
	24 hours	17 20	20 16	22 25	27 36	31 38	22 33	31 35	40 33
<u>Chromobacterium violaceum</u>	3 hours	14 12	23 19	34 31	45 42	40 41	48 44	48 46	50 42
	24 hours	12 06	23 21	41 29	50 44	45 44	50 44	53 51	54 45
<u>Rhizobium phaseoli</u>	3 hours	08 15	11 16	15 15	11 14	17 15	17 18	20 22	26 26
	24 hours	06 17	13 18	21 23	25 25	32 30	32 39	38 39	42 39
<u>Rhizobium trifolii</u>	3 hours	19 19	13 13	13 17	15 16	21 20	18 25	34 33	38 42
	24 hours	26 26	20 18	25 26	24 28	38 35	34 38	41 39	31 34
<u>Rhizobium lupini</u>	3 hours	12 11	03 09	03 07	07 05	08 07	08 11	10 09	07 07
	24 hours	19 15	09 11	12 13	14 15	17 13	18 18	14 13	14 13
<u>Rhizobium japonicum</u>	3 hours	00 0*	0* 0*	0* 0*	0* 0*	0* 0*	0* 0*	00 00	05 00
	24 hours	0* 0*	0* 0*	0* 0*	0* 0*	0* 0*	0* 0*	0* 00	00 00

^a Figures represent duplicate tubes
0* Increase in absorbance

PERCENT AUTOLYSIS AS A FUNCTION OF pH: GOMORI'S BUFFER (30C)
(by loss in absorbance)

pH		5.2	5.6	6.0	6.4	6.8	7.2	7.6	8.0
<u>Agrobacterium tumefaciens</u>	3 hours ^a	17 15	18 17	20 18	20 18	14 23	13 08	18 13	13 12
	24 hours	15 12	17 15	24 26	33 25	24 26	30 35	37 36	36 43
<u>Chromobacterium violaceum</u>	3 hours	16 18	24 28	29 20	44 34	44 40	51 40	47 47	45 46
	24 hours	18 21	34 28	34 30	40 27	35 35	38 31	31 32	34 26
<u>Rhizobium phaseoli</u>	3 hours	07 10	10 11	12 16	15 18	16 14	14 15	22 22	20 23
	24 hours	02 08	08 20	19 20	16 25	25 26	27 24	42 43	39 41
<u>Rhizobium trifolii</u>	3 hours	13 11	15 11	14 14	15 13	15 14	22 14	24 23	32 28
	24 hours	14 03	03 00	05 00	05 03	05 00	16 11	19 17	36 28
<u>Rhizobium lupini</u>	3 hours	12 08	06 06	09 08	03 00	04 00	0* 02	00 0*	11 06
	24 hours	03 06	04 06	11 06	0* 0*	0* 0*	0* 0*	0* 0*	11 06
<u>Rhizobium japonicum</u>	3 hours	00 02	0* 00	0* 00	01 0*	03 0*	0* 00	01 02	0* 0*
	24 hours	0* 00	0* 0*	0* 0*	00 0*	03 0*	0* 0*	0* 0*	0* 0*

^a Figures represent duplicate tubes
0* Increase in absorbance

PERCENT AUTOLYSIS IN EDTA/TRIS BUFFER (30C)
(by loss in absorbance)

M EDTA		.01		.005		.001		.0005		.0001		Tris
												Control
<u>Agrobacterium</u> <u>tumefaciens</u>	3 hours ^a	29	21	21	17	15	17	18	14	17	16	05 02
	24 hours	47	43	40	36	41	60	34	30	52	33	26 18
<u>Chromobacterium</u> <u>violaceum</u>	3 hours	13	16	16	16	14	13	15	16	17	14	09 07
	24 hours	39	35	41	29	30	18	18	30	28	23	30 29
<u>Rhizobium</u> <u>phaseoli</u>	3 hours	27	22	21	19	21	25	18	20	19	18	11 08
	24 hours	37	33	37	35	36	38	36	36	37	41	27 24
<u>Rhizobium</u> <u>trifolii</u>	3 hours	23	24	20	22	16	18	14	16	20	19	08 08
	24 hours	34	37	35	41	35	41	38	38	39	41	22 23
<u>Rhizobium</u> <u>lupini</u>	3 hours	02	09	02	05	08	08	11	09	08	05	04 05
	24 hours	15	18	11	14	16	16	15	14	13	12	04 05
<u>Rhizobium</u> <u>japonicum</u>	3 hours	0*	0*	0*	0*	00	00	0*	0*	0*	0*	0* 0*
	24 hours	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0* 0*

^a Figures represent duplicate tubes

0* Increase in absorbance

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