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GRADUATE COLLEGE

# A STUDY OF AVAILABLE TECHNICS AND THEIR APPLICATION IN MICROBIAL CYTOGENETICS

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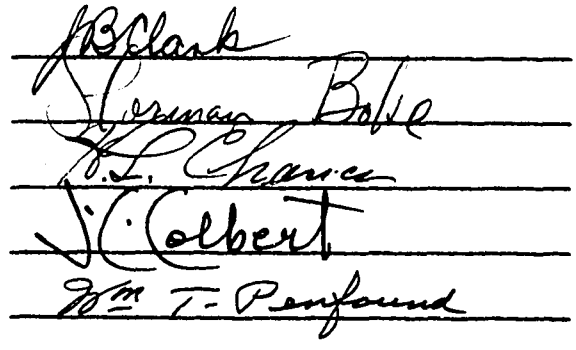
A STUDY OF AVAILABLE TECHNICS AND THEIR APPLICATION

IN MICROBIAL CYTOGENETICS

APPROVED BY



DISSERTATION COMMITTEE

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TABLE OF CONTENTS

LIST OF TABLES

## LIST OF ILLUSTRATIONS

# A STUDY OF AVAILABLE TECHNICS AND THEIR APPLICATION IN MICROBIAL CYTOGENETICS

## CHAPTER I

## INTRODUCTION

### Literature

Cytogenetics has proven to be a valuable aid in the study of higher organisms but has received only limited attention in microbiology. Microbial cytogenetics is seriously limited by the controversial atmosphere surrounding bacterial cytology which in part is due to limitations of resolution, fixation, and staining. The additional perspective obtainable by the correlation and integration of genetic findings with cytological observations should have a clarifying effect on this atmosphere.

Several recent investigations have involved some phase of microbial cytogenetics. Witkin (1951) correlated incidence of colonial sectoring with nuclear plurality of cells of Escherichia coli B at the time that lactose negative mutations were induced. These findings were in accord with the nuclear origin of the induced mutations and also with a chromosomal theory of inheritance in this organism. Unfortunately this excellent work is rendered inconclusive since the existence of cross walls between the nuclei in E. coli B has been shown to occur

(Clark, Webb and Chance, 1956). These polycelled units would be in large part indistinguishable from the multinucleated units when stained with the HCL-Giemsa technic and would give essentially the same results as described in the report. Ryan, Fried and Schwartz (1954) and Ryan and Wainwright (1954) observed a two generation delay in the onset of an increase in histidine independent mutant numbers in an histidine requiring strain of E. coli produced spontaneously and by ultraviolet induction. The results were explained by assuming that the original mutation occurred in one of four nuclei or "mutable units" observed and that the mutant nucleus was "dominant" over its sister non-mutant nuclei. Thus a delay of two generations ensued before the mutant unit segregated into the homocaryotic ancestor of a mutant clone. The workers, however, also failed to distinguish multicellular and multinuclear morphological units.

Haploid and diploid cultures of E. coli K-12 were studied cytologically by Lederberg et al. (1951) in an effort to correlate genetic heterozygosity with nuclear complexity. The comparisons revealed consistent and unequivocal differences in the nuclei of these two forms but failed to explain the ploidy of the cultures on a cytological basis.

Clark and Webb (1954) studied the large cells of Micrococcus aureus cytologically and radiobiologically and evidence was presented for the diploid and triploid nature of these forms. The findings indicated that a transient mixoploid phase occurred in M. aureus, probably due to endoploidy, which alternated with a typical haploid phase representing a type of simplified life cycle in this organism.

A cytogenetic approach has been successfully applied to yeast by

Lindegren (1949). The ploidy, life cycle, and sexual process have been substantiated by this approach although there is still some controversy in the field of yeast cytology. The success with which the limited application of cytogenetics has met in microbiology indicates that certain of the basic problems of microbial genetics may yield to some extent if attacked with the available tools of this field.

Some of the basic problems of microbial genetics include: (1) The location and nature of hereditary determinants in the microbial cell. (2) The existence of sex in microorganisms. (3) The ploidy of microorganisms. (4) The existence of life cycles in microorganisms. Although much work has been done in relation to each of the stated problems, there are as yet large areas of uncertainty. The evidence for the location of hereditary determinants within discrete nuclei is still based on a priori reasoning. Discrete structures containing desoxyribonucleic acid and having characteristics at least analogous to those of the nuclei of higher organisms have been demonstrated in a large number of bacteria, but additional evidence is necessary to unequivocally associate hereditary determinants with the discrete nucleus-like structures of bacteria.

Sexual processes such as that reported in E. coli K-12 by Lederberg (1947), transduction in Salmonella by Zinder (1952), and transformation in various species (see review by Austrian, 1953), all point to some degree of sexuality in microorganisms. Many reports of sexuality based on cytological evidence have been presented but are at best only suggestive. Convincing cell fusion has been demonstrated in Proteus vulgaris OX-19 by the direct observation of living cells in

slide cultures (Stempen and Hutchinson 1951). A cyclic process involving cell and nuclear fusion was shown to occur with a high frequency in several species of marine and fresh water spirilla by Williams and Rittenberg (1956) and Williams (1956). The literature on cytological evidence of sex in bacteria was reviewed by Lewis (1941) and Hutchinson and Stempen (1954).

Many workers have questioned the application of a ploidy concept to bacteria. However, certain findings are in opposition to this conclusion. Lea (1947) considered most bacteria to be haploid on the basis of mutation and radiation studies. Genetic evidence points to the haploid nature of the normal vegetative stage of E. coli K-12 (Lederberg 1947). No general conclusions can be drawn concerning all microorganisms.

Life cycles have been postulated for various bacteria. These reports are almost universally based on cytological evidence and have not been accepted generally by bacteriologists. Kleineberger-Nobel (1947) postulated a life cycle for Streptomyces grisseus and Bisset (1951) presented a similar idea for all the Actinomycetes and for most bacteria. Unfortunately, cytological evidence for life cycles in microorganisms should not be accepted as final proof because morphological changes are often associated with environmental differences. Since microorganisms are cultured under highly artificial conditions, any changes observed should be viewed and interpreted with caution. The concept of life cycles in higher organisms involves an alteration between a haploid and a diploid generation, thus life cycle studies and ploidy determination are necessarily associated in microorganisms.

## Procedures of Micro-cytogenetics

In the past the shortcomings of cytological technics have seriously limited the cytogenetic approach in microbiology. The HCL-Giemsa stain has received widespread use but fails to give uniformly reliable results since the stain in itself is not specific for nuclear material. The Feulgen technic is to a large extent specific for desoxyribonucleic acid but is difficult to apply and usually yields dim, variable, and non-uniform preparations. Perchloric acid hydrolysis has been advocated to replace HCL in the HCL-Giemsa technic (Cassel 1949); however, the basic problem of lack of specificity still exists. Among other nuclear stains which have been reported are the iron hemotoxylon method and various modifications, the redox nuclear stain (Chance 1950), and the "starvation-methylene blue" method (Knaysi 1942), but these technics are not widely used.

Two new staining technics show promise as tools in cytogenetic problems. The first of these, the thionin (or Asure A)-$SO_2$ technic of DeLemater (1951) is DNA specific (Clark and Webb 1955), and gives intense uniform preparations. Various fixatives may or may not be used without giving detectable variations in results. The structures revealed by the stain have been subjected to different interpretations (DeLemater 1951), (Fitz-James 1954), (Bisset 1954), but there is little reason to doubt that these differences eventually will be resolved.

The second technic is the crystal violet nuclear stain (Chance 1952). It is specific for a proteinaceous component of the nucleus (Clark and Webb 1955). The stain has been shown to reveal the same

general structure as the thionin-$SO_2$ stain and under appropriate conditions reveals a cell plate which appears to be similar to that of higher plants (Chance 1953a, b; Clark, Webb, and Chance in press). The crystal violet stain has the disadvantage of requiring modifications in order to adapt it to different organisms and also of requiring some practice in order to obtain satisfactory preparations (Bisset 1954). The stain will not resolve the nuclei of many bacilli although it will stain the cell plate.

Electron microscopy has made many fundamental contributions to the knowledge of bacterial structure; however, serious problems of interpretation exist. Osmium tetroxide, often used as a fixative for electron microscopy, functions also as a "stain" and in thin section work, osmium deposits only may be visible (Bahr 1955). Many of the structures revealed by electron microscopy may be artifacts.

Phase microscopy of bacteria has been disappointing. Many bacteria appear more or less uniform under a phase microscope and nuclear structures in general are not revealed. Excessive refraction and halo formation is a problem with most oil immersion phase objectives, but the American Optical B minus M objective seems to offer possibilities.

In a preliminary check the new AO-Baker interference microscope was not effective for demonstrating nuclear structures in bacterial cells. In general, it was found to be less satisfactory than the American Optical phase microscope for bacterial studies.

Microbial genetics has made great progress in recent years since the classical proof of bacterial mutation (Luria and Delbruck 1943). Biochemical mutations have provided additional approaches to the study

of metabolic pathways. The auxotrophic mutants of E. coli K-12 were shown to be recessive by the fortunate occurrence of persistent diploids following certain crosses (Lederberg et al. 1951). This field has been the subject of several reviews in recent years (Luria 1947; Lederberg 1949, 1951, 1952, 1955; Wyss and Haas 1953; and Stone 1956). Much of the genetic research on microorganisms has been done without consideration of cytology and, to a large extent, gene action can be studied without knowing the location of the gene involved. An overall understanding of microbial genetics, however, necessitates the integration of cytology and genetics.

Radiations frequently have been applied to genetic research. The lethal action of ultraviolet light was first discovered by Downes and Blunt (1877). The mutagenic action of ultraviolet light was noted by some early workers, but did not receive much study until the discovery by Muller (1927) that X-radiation was mutagenic. The early history of ultraviolet light research has been reviewed in considerable detail by Ellis and Wells (1941). Gates (1929a, b, 1931) noted the correspondence of the DNA absorption curve with a peak near 2600 A and the inactivation curve of the ultraviolet light which was maximal at approximately the same wave length. Various workers have ascribed the lethal action to some type of genetic damage (Gates 1931; Lea 1947); however, the poison theory of ultraviolet inactivation is still strongly advocated by some workers (Heinmets 1953). Conflicting results obtained from the work with E. coli B and E. coli B/r (Witkin 1947), photoreactivation after ultraviolet inactivation (Kelner 1949a, b), and variation of ultraviolet inactivation response shown by some bacteria have caused-

conflicting explanations of the inactivation mechanism. Recent work (Clark and Webb 1956; Webb and Clark 1956) has shown that the major inactivation effect of ultraviolet radiation on Nocardia corallina, a diploid organism, is due to hereditary damage. This lends support to the recessive lethal theory of ultraviolet inactivation.

The X-radiation situation, although less confused than the ultraviolet situation, is nevertheless subject to considerable controversy. The target theory was applied first to biological material by Crowther (1924) and later to the inactivation of bacteria by Wycoff (1930). Lea became one of its stronger proponents (Lea et al. 1937 and Lea 1947). The target theory has undergone considerable modification since X-ray action was shown to be largely indirect through the production of ions and free radicals. Recent evidence (Tobias 1952, Zirkle and Tobias 1953, Clark and Webb 1956) indicates that X-radiation affects sensitive sites within the cell which are related at least indirectly to genetic material.

The work of Latarjet and Euphrussi (1949), Zirkle (1952), Tobias (1952), Zirkle and Tobias (1953), and Clark and Webb (1955, 1956), has shown a correlation between ploidy and the radiation dose-survivor inactivation response of uninuclear-unicellular forms under carefully controlled conditions. Haploid cells show an exponential "single-action" dose-survivor response, whereas, diploid and polyploid cells show a non-exponential, sigmoid or "multi-event" type of dose-survivor response. An additional indirect, nonhereditary damage by ultraviolet light is indicated by the variations from an exponential dose-survivor response which are often obtained. Differences in ribonucleic acid content

probably account for some of this variation. X-radiation is more reliable than ultraviolet for inactivation rate studies and ploidy determinations, since Z-radiation is less affected by variation associated with secondary effects. Under carefully controlled conditions radiation studies can function as an important part of the cytogenetic approach to the study of microorganisms.

## Application of Micro-Cytogenetic Procedures

There are two important aspects to the development of micro-cytogenetics: (1) The development of procedures and (2) the application of the procedures to a suitable organism. The two parts cannot be separated readily because the validity of technics must be demonstrated on test organisms. A suitable experimental organism is not easy to obtain, since bacteria, fungi, and yeasts all have serious disadvantages. Bacteria are usually thought to be haploid (Lea 1947) but this observation is not easily proven. Diploid and polyploid stages are usually transient (Clark and Webb 1955), or unstable (Lederberg et al. 1951). Yeasts have the disadvantage of being the subject of much controversy in the field of cytology. Filamentous fungi are haploid and the spores often contain more than one nucleus (Atwood and Norman 1949) and are difficult to study cytologically.

Preliminary cytological studies on Nocardia corallina indicated that this organism possessed characteristics well adapted to the testing of micro-cytogenetic procedures (Webb and Clark 1953; Webb, Clark, and Chance 1954). The existence of a unicellular, uninuclear coccoidal stage in the growth cycle made the organism adaptable to accurate

radiation studies. Preliminary studies suggested that a life cycle might be associated with the alternation of coccoidal and hyphal stages. The possibility of the existence of a sexual process was also suggested by the preliminary cytological observations. The organism shares many characteristics with the true bacteria. N. corallina (ATCC #4273) was selected for intensive investigation into micro-cytogenetics.

The genus Nocardia is in the order Actinomycetales and is characterized by being strictly aerobic, forming hyphae which later fragment to a greater or lesser extent, and for the most part has simple nutritional requirements. N. corallina forms a red pigment and readily fragments into uniform coccoidal cells on most common media.

The order Actinomycetales was reviewed by Waksman (1950). Recent work indicates that a life cycle may exist in certain species of this order (Bisset 1951). Kleineberger-Nobel (1947) demonstrated an apparent life cycle in Streptomyces grisseus. Morris (1951) described a similar life cycle in the Actinomyces which, unfortunately, was not documented with photographs. Recent work has demonstrated recombination in Streptomyces brought about both by heterokaryon formation and by a sexual process (Szybalski and Braendel 1956). McClung (1950, 1955) studied the cytology of various species of Nocardia, but did not give critical attention to germination and fragmentation. Webb, Clark, and Chance (1954) studied the germination and fragmentation processes of N. corallina by means of phase microscopy and the crystal violet nuclear stain. No conclusion was reached as to the existence of a life cycle in this organism.

The purpose of this investigation was the study of technics

applicable to microbial cytogenetics and the application of these technics to an integrated cytogenetic study. N. corallina was studied as a basis for the valid application of these procedures to other microorganisms.

# CHAPTER II

## MATERIALS AND METHODS

Stock cultures of Nocardia corallina (ATCC#4273) were maintained on nutrient agar containing one percent fructose and incubated at 28 to 29 degrees C. This medium yields cultures of coccoidal cells after two days of growth, and there is little hyphal development. Nutrient agar was used in all cases in which hyphae were studied. Slide cultures for phase studies were prepared in nutrient agar on Shoemaker slides. The application of the slide culture technic is limited in growth cycle studies because of the strictly aerobic nature of N. corallina. Reduced oxygen tension tends to retard and inhibit fragmentation of the hyphae and has prevented a study of fragmentation by phase microscopy.

The crystal violet nuclear stain (Chance 1952) and the thionin-$SO_2$ nuclear stain (Delamater 1951) were used in the nuclear studies. The crystal violet stain was modified by using crystal violet at a pH of 6.0, mordanting in 3.0% mercuric chloride for 30 to 45 seconds and decolorizing with 5% nigrosin at a pH of 3.3 Growing hyphae required longer mordanting than did coccoids. In the thionin-$SO_2$ technic cells were either fixed in Bouin's solution, Carnoy's fixative, osmium tetroxide vapors, or were left unfixed. Each fixative produced essentially the same cytological appearance. Most preparations were fixed

five minutes in Carnoy's solution or were left unfixed. Smears were prepared on coverslips, allowed to dry, and immediately placed in the fixative. The preparations were hydrolyzed in 1N HCL at 60 degrees C. for four to six minutes; however, the time was not found to be critical. The hydrolyzed smears were transferred to a ten ml. staining jar containing 0.25 percent thionin-$SO_2$. The staining solution was prepared by adding one drop thionyl chloride to ten ml. of 0.25 percent thionin. The smears were left in the stain for ten to twelve hours. The freeze dehydration method for preparing permanent mounts was not employed. The preparations were examined and photographed as temporary water mounts, or were mounted in a medium of one part alpha-bromonaphthalene in three parts of heavy mineral oil (Minsavage 1955).

Asure A was substituted for thionin in some of the studies (DeLamater 1951). In this case two drops of thionyl chloride per ten ml. of staining solution were added fifteen minutes prior to staining. Asure A-$SO_2$ is highly specific for the desoxyribonucleic acid components of the cell and leaves the cytoplasm and cell wall completely unstained. In order to determine cell boundaries, the smears were counter stained for one minute in 0.05 percent aqueous safranin.

A modification of the tannic acid-violet cell wall stain (Webb 1954) was used in the cell wall and cross septation studies.

American Optical dark contrast and B minus contrast objectives were used in the phase microscope studies.

A Will Corporation low pressure mercury vapor lamp operating at a distance of seven inches was used in ultraviolet studies. In the ultraviolet experiments the surface of the medium in petri dishes was

seeded with a constant number of viable cells (approximately 300) or ten fold multiples of this number. The cells were spread with a sterile glass rod and placed under the ultraviolet source for varying lengths of time. Each radiation dose was represented by four or five plates and the zero point by eight to ten plates. Plates were incubated for three days at 28-29 degrees C. before the colonies were counted. Care was taken to prevent photoreactivation following ultraviolet irradiation.

The X-radiation source was a Picker Army Field X-ray unit of 30 ma and 100 kv output. The tube was operated at 96 kv and 10 ma which produced 3,000 roentgens of 3 1/3 inches and 1,500 roentgens at 4 2/3 inches. In the X-ray experiments, cell suspensions in 0.85 percent saline of approximately $3x10^3$ cells per ml. were irradiated, duplicate 0.1 ml. portions removed at intervals, appropriately diluted, and nutrient agar pour plates prepared in triplicate for each sample removed. The pour plates were incubated for four days at 28-29 degrees C. before the colonies were counted.

In order to eliminate misleading results caused by clumping, which is often extreme in N. corallina, cell suspensions were vigorously shaken with glass beads in a screw cap test tube for seven minutes on a Vibraloe, then subjected to differential centrifugation to remove the remaining clumps. The resultant suspension was next examined with dark phase contrast and the approximate percent of single celled units determined. No suspension with less than 90 percent single celled units was used in a radiation experiment except for the hyphal studies. The suspensions were also checked for cross septation, involution forms, and multinuclearity and were eliminated if such existed.

# CHAPTER III

## RESULTS AND DISCUSSION

### Growth Cycle Studies: Cytology

Nuclear structure and behavior during the growth cycle of N. corallina as revealed by the thionin-$SO_2$ nuclear stain was similar to that revealed by the crystal violet nuclear stain. The structures stained by the two technics show behavioral characteristics of the nuclei of higher organisms. The specificity of the thionin-$SO_2$ stain provides evidence for the presence of desoxyribonucleic acid in the nucleus-like structures. The typical nucleus of resting coccoids is vesicular and often has five or six spots arranged around periphery of the vesicle as revealed by the thionin-$SO_2$ stain and phase microscopy (figures 1 and 2). These figures were less completely resolved by the crystal violet technic, but the general appearance is similar (figure 3). McClung (1950) reported the persistent appearance of fat bodies in Nocardia species. The Sudan B fat stain was used to reveal the location of fat in different stages of N. corallina. Fat bodies were observed irregularly in all stages of the growth cycle and sometimes appeared to fill an entire coccoidal cell. Comparison of these fat bodies with structures revealed by the crystal violet and thionin-$SO_2$ nuclear stains showed no correlation which could lead to confusion between the two.

There is no evidence that either stain reveals fat inclusions.

The coccoids were observed to germinate by a swelling and reorganization of the nuclear material followed by an outgrowth from one and sometimes both ends (figures 4, 5, 6, and 7). The nuclear behavior during germination was suggestive of a reduction division, but classical meiotic figures were not observed and other evidence indicates that this division is not reductional. The nucleus divided twice which resulted in four nuclei in the elongated cell (figure 8). Some or all of the nuclei divided again resulting in hyphae with six to eight evenly spaced nuclei (figure 8 and 9). These nuclear divisions did not initiate cross wall formation and the hyphae remained coenocytic until just prior to fragmentation (figure 11). Stained preparations of hyphae between eight and eleven hours of growth usually failed to reveal discrete spherical nuclei with either technic (figure 12 and 13). This non-discrete stage probably occurs through the metabolic breakdown of the nuclear membrane; however, the possibility remains that the appearance of the nuclei at this stage is a result of the destruction of the nuclear membrane by the staining procedures. This does not appear likely since discrete nuclei are revealed in other stages. The nuclei at the hyphal tips and in the tips of branches were observed to remain deeply stained and spherical throughout hyphal development (figure 13). Phase studies revealed granules scattered irregularly in the hyphae (figure 14), but evidence that these granules were nuclear in nature was not found.

Fragmentation, which usually began about 15 hours after inoculation on nutrient agar at 28-30 degrees C. was clearly preceded by cross wall formation (figure 18). The first step of fragmentation appeared to

be the reorganization of the hyphal nuclei in which the stainable material contracted and assumed a deeply stained elliptical shape (figure 15 and 16). A nuclear division occurred at this stage which produced cross walls in the hyphae (figure 17 and 18). It should be noted that previous nuclear divisions did not result in cross wall formation. Figures 18, 19, and 20 show fragmenting hyphae in which cross walls may be observed clearly. Hyphal fragmentation by cytoplasmic segmentation without cross wall formation has been reported by McClung (1950) and Knaysi (1951). Cross septa, however, occurred extensively in the hyphae early in fragmentation (figure 18), thus the appearance of cytoplasmic segmentation could result easily from shrinkage of the cytoplasm away from unstained cross septa. The use of cell wall stains, such as the Dyar technic (Dyar 1949), which do not stain cross septa probably represents the basis of the theory of cytoplasmic segmentation advanced by McClung (1950).

Electron microscopy was applied to the problem of hyphal fragmentation and cytoplasmic segmentation. Preparations of fragmenting N. corallina were mounted on formvar specimen grids and studied with an RCA model EMU electron microscope operated by the Nobel Laboratory for Electron Microscopy, The University of Oklahoma. Electron dense spots of unknown nature appeared irregularly in the hyphae prior to fragmentation (figure 34). The artifact of cytoplasmic segmentation may be caused by shrinkage during dehydration (figure 33). Cross walls, associated with hyphal fragmentation, were made visible by cytoplasmic shrinkage away from both sides of cross septa (figure 36, 37, and 38). The bacillary cells later broke apart at the positions of the previously

formed cross walls (figure 39).

The fragmentation process gave rise to dinucleated bacillary cells (figure 22 and 23) which often remained attached in the form of segmented hyphae (figure 18 and 21). As the terminal nucleus was not observed to divide during fragmentation, each bacillary cell contained two nuclei (figure 22 and 23). The coccoidal forms which arose from the bacillary cells were observed to be uniformly uninucleated (figure 24, 25, 26, and 27). Cytological observations indicated that the two nuclei fused before the bacillary cell divided (figure 26 and 27). Division of the coccoidal forms was similar to that recently reported in certain bacteria (Chance 1953 a,b; Webb and Clark 1954). During division the nuclear material separated and a cell plate was formed between the divided halves (figure 28, 29, and 30). The cell plate extended to reach the cell wall and apparently differentiated into cell wall material. The two resulting cells later separated.

Fusion tubes were frequently observed connecting adjacent hyphae during the fragmentation process (figure 31, 32, and 33) which suggested that some type of a sexual process was associated with this stage. Although direct evidence is lacking, it may be postulated that an exchange of material occurs between one of the two nuclei in each of the connected bacillary cells. On the basis of this interpretation the coccoidal nucleus may arise by fusion of nuclei from the same or from different bacillary cells.

The thionin-$SO_2$ stain reveals nuclear "spots" or short bars at different stages of the growth cycle. Similar spots in another organism were called chromosomes by DeLamater (1951); however, this evidence is

considered inconclusive by the present writer. If these structures are analogous to the chromosomes of higher forms, some indication of the ploidy of the different stages can be obtained. Coccoids may show either condensed nuclei (figure 1) or vesicular nuclei (figure 24, 25, 26, and 27). Distinct spots were not observed in the condensed nuclei, whereas three, four, five, or six spots per nucleus were observed in vesicular nuclei. Young coccoids typically possessed condensed nuclei which became vesicular in the resting stage. Nuclei containing three and five spots were observed most frequently (figure 27). The haploid chromosome number appeared to be either three or two plus a nucleolus or satallite. The coccoids containing three spots may represent either haploid coccoids, or diploid forms with the homologus chromosomes closely paired. The size of the three spots in some coccoids indicates the possibility of their being double. The large number of coccoids with five spots presents a problem of interpretation. One of the spots may be double or one spot could be interpreted as a nucleolus in which case the diploid number would be four. The spots seemed to be connected by a strand somewhat below the limit of resolution, perhaps on the order of 0.1 micron. Individual spots usually could not be discerned in the nuclei of the hyphal stage. The non-discrete nuclei of the developing hyphae were observed to be composed of strands near the limit of resolution of the optical system (figure 12), but these structures could not be distinguished sufficiently to make a "chromosome count."

The individual spots became apparent in the newly formed dinucleated bacillary cells. Each nucleus was observed to possess three "chromosomes" as is shown in figure 23 and 24. The two nuclei appeared

PLATE I. Stained preparation and phase studies of *Nocardia corallina*

Figure 1. Thionin-$SO_2$ stain of five day old coccoidal cells. 3,200 X.

Figure 2. Phase study of five day old coccoidal cells. 4,000 X.

Figure 3. Crystal violet stain of five day old coccoidal cells. 3,200 X.

Figure 4. Thionin-$SO_2$ stain of 3 1/2 hr. germinating coccoidal cells. 4,000 X.

Figure 5. Thionin-$SO_2$ stain of 2 1/2 hr. germinating coccoidal cells. 4,000 X.

Figure 6. Crystal violet stain of 4 hr. germinating coccoidal cells. 3,200 X.

Figure 7. Crystal violet stain of 5 hr. germinating coccoidal cells. 3,200 X.

Figure 8. Thionin-$SO_2$ stain of 6 hr. hypha. 3,200 X.

Figure 9. Crystal violet stain of 8 hr. hyphae. 3,200 X.

Figure 10. Thionin-$SO_2$ stain of 7 hr. hyphae. 3,000 X.

Figure 11. Cell wall stain of 12 hr. hyphae. 3,200 X.

Figure 12. Thionin-$SO_2$ stain of 11 hr. hypha showing non-discrete nuclei. 3,200 X.

Figure 13. Thionin-$SO_2$ stain of 11 hr. hypha showing discrete nucleus at hyphal tip. 3,200 X.

Figure 14. Phase study of 15 hr. hyphae. 3,000 X.

Figure 15. Thionin-$SO_2$ stain of 12 hr. hypha showing condensed nuclei. 3,200 X.

PLATE I

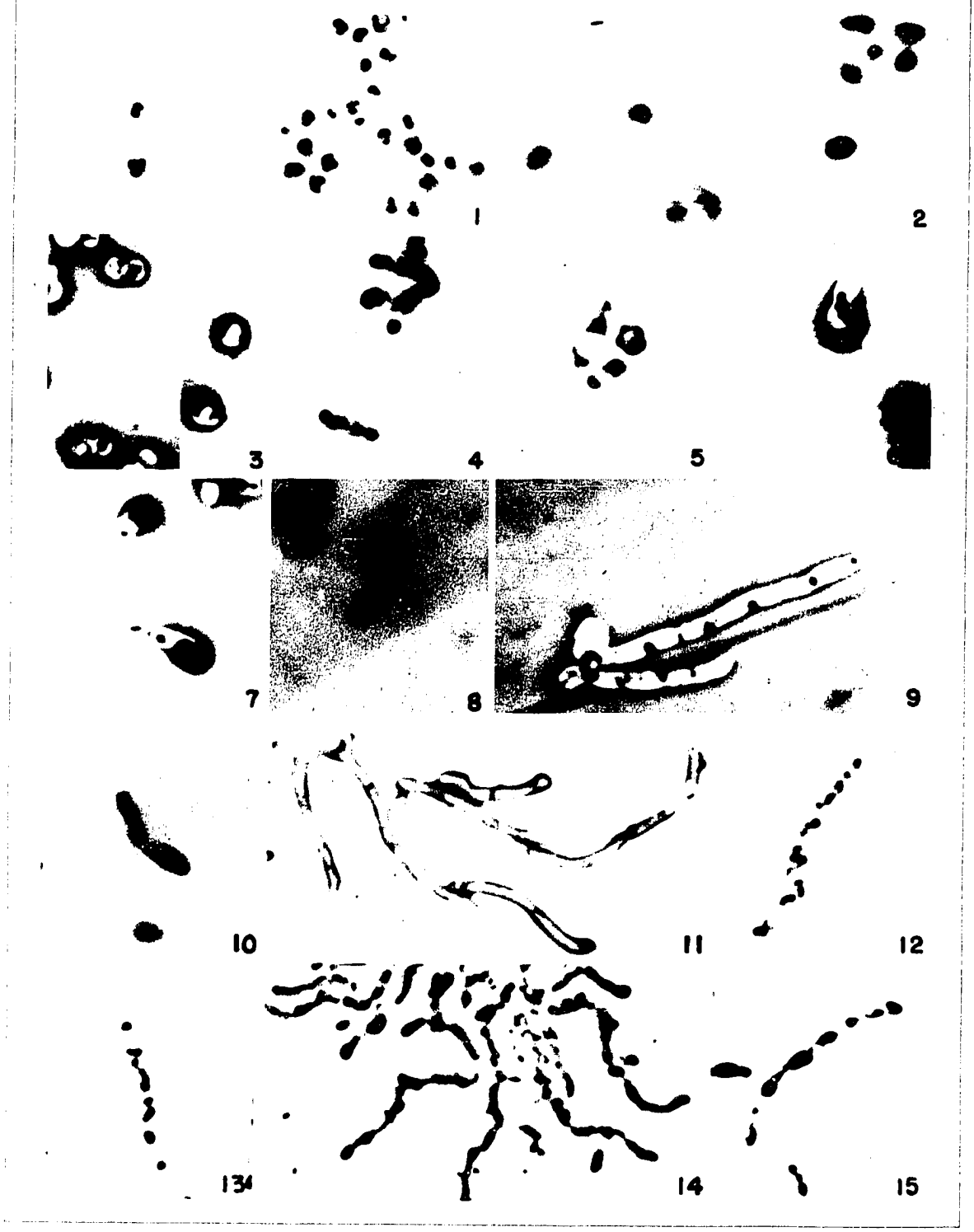


PLATE II. Stained preparations and phase studies of *Nocardia corallina*

Figure 16. Thionin-$SO_2$ stain of 13 hr. hypha. 3,200 X.

Figure 17. Crystal violet stain of 13 1/2 hr. hyphae showing nuclear division preceding fragmentation. 3,200 X.

Figure 18. Cell wall stain of 14 hr. hyphae showing cross wall formation early in fragmentation. 3,200 X.

Figure 19. Cell wall stain of 16 hr. hyphae showing late stage of fragmentation. 3,200 X.

Figure 20. Cell wall stain of 17 hr. hyphae showing late stage of fragmentation. 3,200 X.

Figure 21. Crystal violet stain of 17 hr. hypha showing chain of bacillary cells. 3,200 X.

Figure 22. Thionin-$SO_2$ stain of dinucleated bacillary cells after completion of fragmentation. 3,200 X.

Figure 23. Thionin-$SO_2$ stain of dinucleated bacillary cells. 4,000 X.

Figures 24 - 27. Thionin-$SO_2$ stain of uninucleated coccoidal cells and dinucleated bacillary cells. 4,000 X.

Figures 28 - 30. Crystal violet stain of dividing coccoidal cells showing cell plate. 3,200 X.

Figures 31 - 32. Phase studies of 14 hr. hyphae showing hyphal fusion. 2,500 X.

Figure 33. Crystal violet stain of 14 hr. hyphae showing hyphal fusion. 3,200 X.

PLATE II

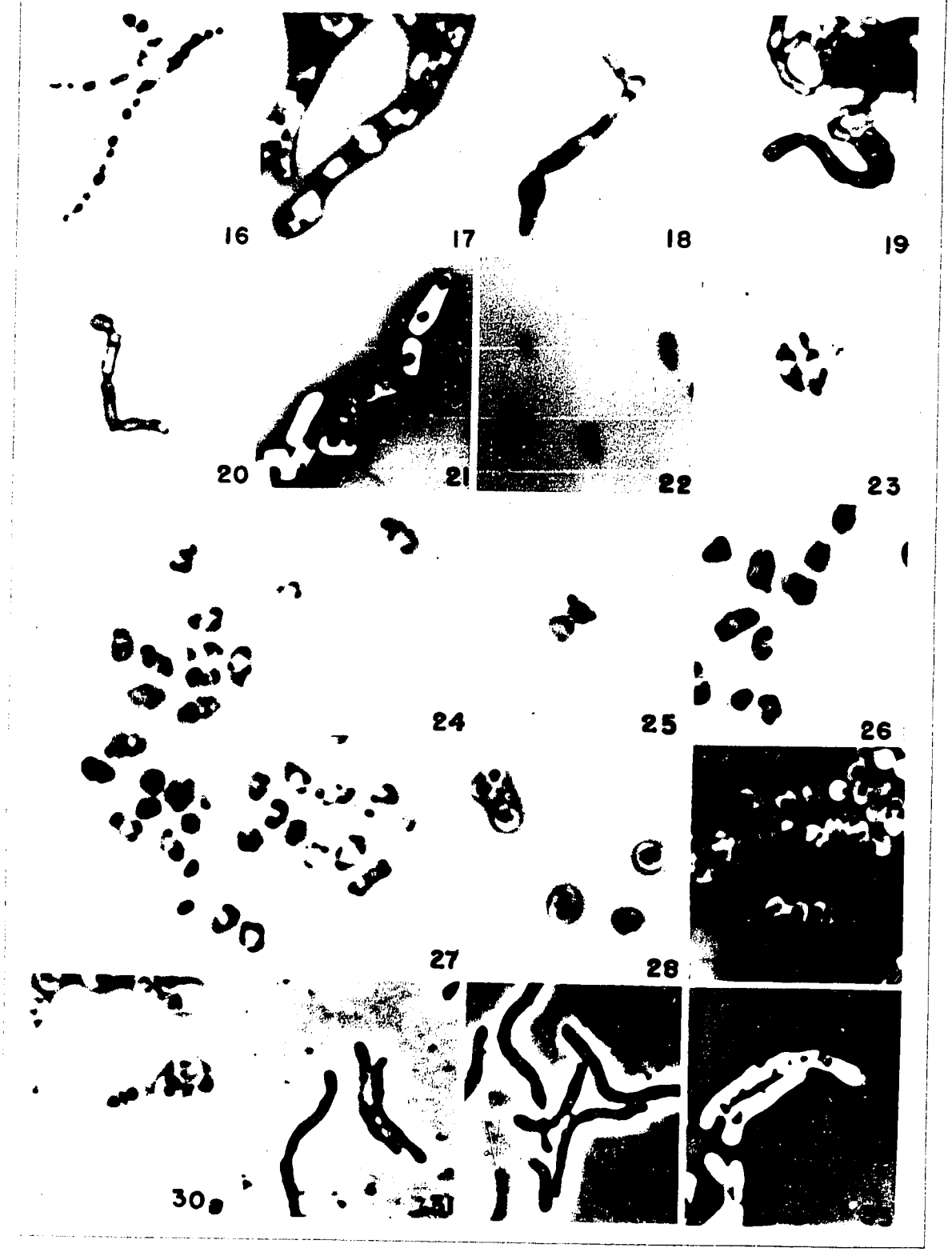


PLATE III. Electron microscope studies of Nocardia corallina

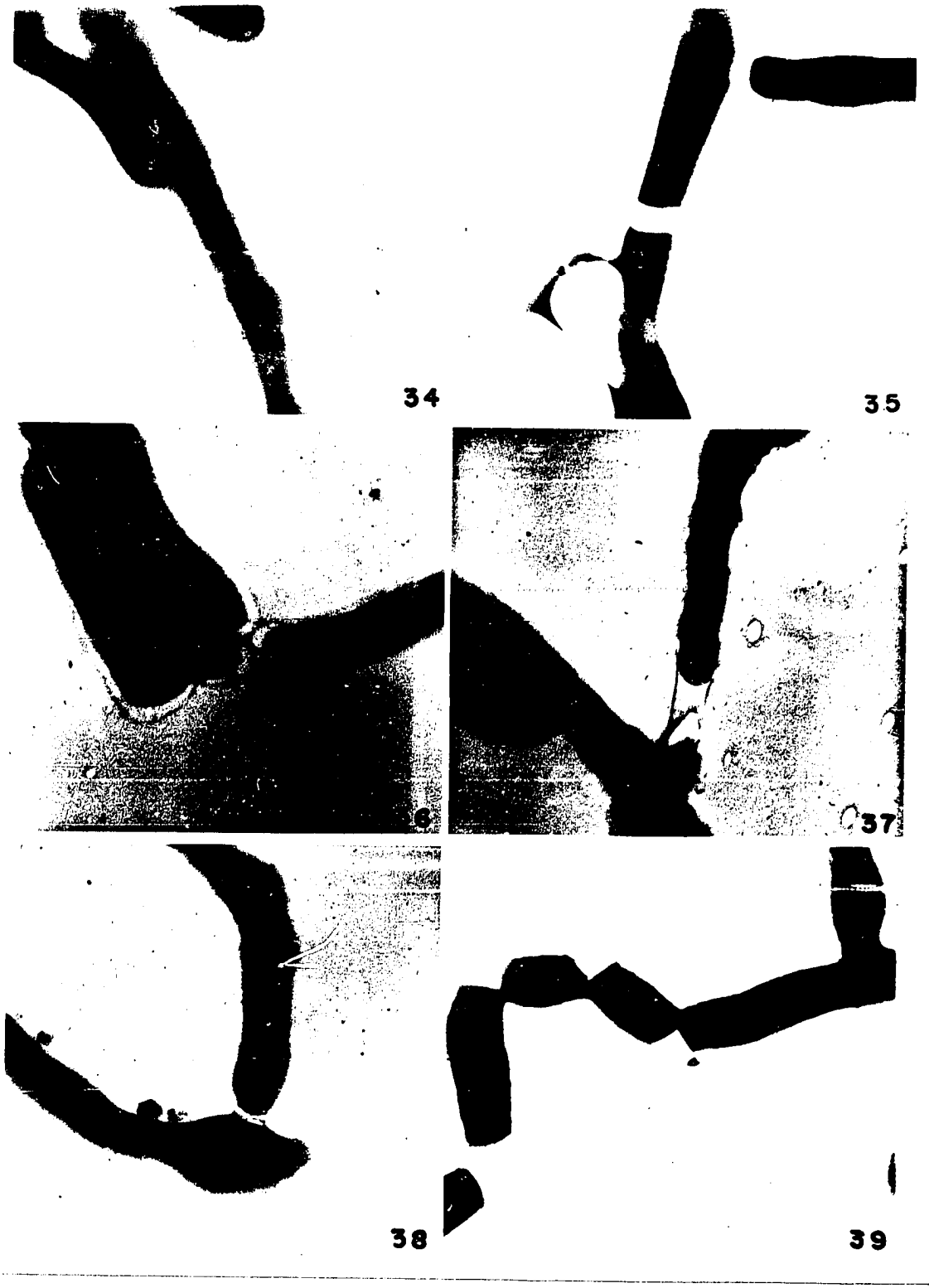
Figure 34. Twelve hr. hyphae showing electron dense spots of unknown nature. 8,000 X.

Figure 35. Fourteen hr. fragmenting hypha showing "cytoplasmic segmentation" produced by shrinkage during dehydration. 8,000 X.

Figure 36 - 38. Fourteen hr. hyphae showing cytoplasmic shrinkage away from a cross septum. 8,000 X.

Figure 39. Eighteen hr. hypha showing a late stage of fragmentation. 8,000 X.

PLATE III



to fuse, forming a single nucleus with five or six spots (figure 25, 26, 27, and 28).

The position of a reduction division to account for haploid bacillary nuclei is undertain on the basis of cytological evidence. However, the nuclear condensation preceding fragmentation is suggestive of a meiotic prophase. On this basis, and on evidence to be presented later, it is suggested that the fragmentation division is reductional and the dinucleated bacillary cells represent the haploid phase of the life cycle.

The cytological evidence, although not conclusive, is suggestive that the coccoidal and hyphal stages may be diploid and the dinucleated bacillary cells represent the haploid stage. With the possible exception of the "three spot coccoids," the dinucleated bacillary cells appear to be the only haploid phase of the life cycle.

## Growth Cycle Studies: Radiobiology

The cytological evidence for the life cycle in N. corallina is at best only suggestive. In carrying out the cytogenetic approach, radiation studies were made in conjunction with cytological observations for the purpose of further elucidating the basic problem.

Some authors consider that the shape of the dose-survivor curve is characteristic of the radiation rather than the organism. The ultraviolet survival curve is considered to be typically sigmoid by some workers (Lederberg 1952). Although the ultraviolet survival curve is often not completely exponential, the typical survival curve of the haploid, unicellular, uninuclear forms is more or less curved; but under

appropriate conditions it is essentially exponential. The shape of the non-exponential survival surve has been found to vary with the same organism under different conditions, but it is ordinarily not of the "hit multiplicity" type. This variation may be associated with the amount of ribonucleic acid in the cell which can protect the sensitive sites of the cell from the effects of ultraviolet irradiation. No such disagreement exists with X-radiation, which in general gives more consistent results.

In spite of factors which influence the response of cells to inactivation by radiations and the contradictory results current in the literature, workers have shown that the ploidy of organisms does affect the response to radiations. In yeast cells there is a close correspondence between the ploidy of uninuclear, unicellular cells and the shape of the survival curve (Latarjet and Ephrussi 1949, Latarjet 1952, Zirkle 1952, Tobias 1952, Zirkle and Tobias 1953). It must be recognized that the form of the radiation dose-survivor curve as such means very little. The shape may correspond to the number of cells per morphological unit, the number of nuclei per cell, or the ploidy of the cell. Thus morphological units and clumps must be determined and accounted for in the interpretation of radiation inactivation results (Clark and Webb 1955, Atwood and Norman 1949).

X-ray experiments with N. corallina demonstrated that the unicellular, uninuclear coccoidal cells showed a sigmoid or multihit dose-survivor response (figure 40). These findings are in harmony with the cytological observations suggesting that the coccoids are diploid.

According to the modification of the target theory of Lea (1936,

1947) by Zirkle (1952) and Tobias (1952), inactivation by ultraviolet and ionizing radiations occurs by damaging sensitive sites within the cell. This damage was not considered necessarily to occur through a direct "hit" of a "target," but rather from free radicles produced by the radiation, thus, the mechanism was construed to be indirect. In haploid yeast cells, studies showed that a single event, "hit" or damaged sensitive site was sufficient to inactivate the cell, whereas diploid cells required several events or hits for inactivation (Tobias 1952). On the basis of the inactivation results, calculations suggested that the haploid form had about 20 sensitive sites of which damage to any one would result in inactivation of the cell or cell progeny. In the diploid cell a pair of corresponding sites must be damaged to bring about inactivation, thus damage to any pair of the twenty sensitive sites will inactivate the cell but unpaired damage will not (Tobias 1952). It was postulated that these sensitive sites include certain genes and areas associated with the reactions involved in cell division. Additional work demonstrated that the diploid yeast cells which survived the X-ray treatment were more sensitive to subsequent X-ray treatments and the dose-survivor curve became less sigmoidal. The results were explained on the basis of the production of unpaired defects in the surviving cells.

N. corallina was subjected to successive X-ray treatments in order to test the unpaired defect theory of Tobias (1952). A suspension of N. corallina coccoids from a four day old culture incubated on one percent fructose agar at 29 degrees C., was subjected to an X-ray inactivation experiment to begin the series. The plates were incubated

for four days at 29 degrees C. and massive inoculations were made from twenty or more colonies developing from coccoidal cells which had received 18,000 roentgens of X-ray. This X-ray dose inactivated 25 percent of the normal coccoidal cells. The culture was incubated for four days and the X-ray dose-survivor response of the culture was determined. This procedure was continued through four successive X-ray treatments. The results from this series of experiments are given in figure 40. It may be observed that the culture which was prepared from coccoidal cells surviving the initial irradiation showed a dose-survivor response curve which differed from normal (figure 40, curve 2). A large portion of the population was more sensitive than the parent culture to X-rays as evidenced by the greater inactivation effect from moderate doses. The culture was inactivated at a rate approaching exponential. The second and third successive treatments resulted in survivors which showed even more sensitivity. The cultures which had received two and three previous treatments showed an exponential dose-survivor response (figure 40, curves 3 and 4).

The sharp break or "tail" in the survivor curves of the third and fourth successive treatments probably represents naturally resistant forms in the population. Recent work (Frady and Clark 1956) indicates that a small percent of the normal population is resistant to radiation inactivation. This break cannot be explained by clumping since cytological observations on each of the experimental suspensions revealed less than six percent clumping. The fact that the treatment should have been strongly selective for the resistant part of the population makes the findings even more striking.

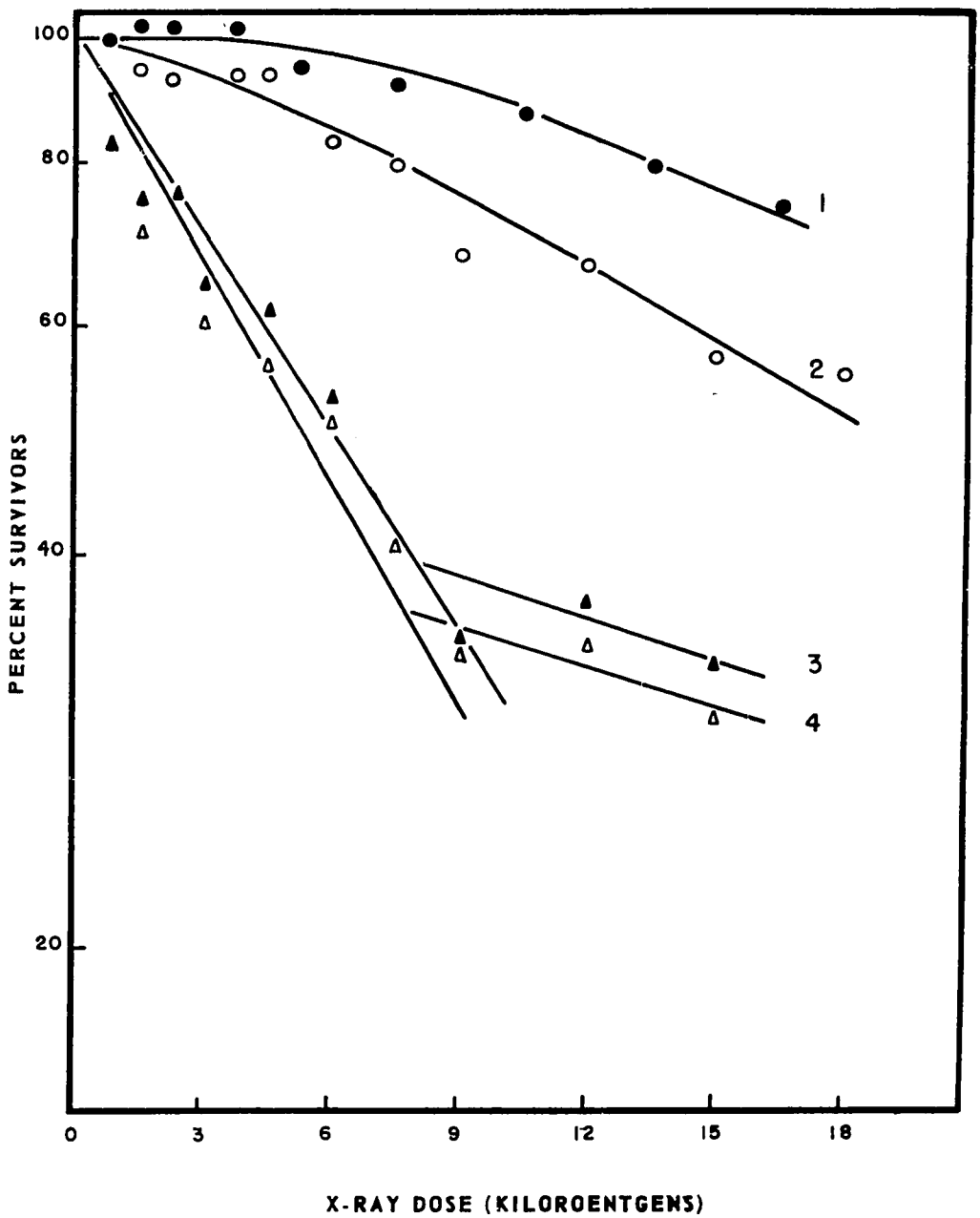



Figure 40. The Effect of Successive X-ray Treatments on Nocardia corallina Coccoids.

These results are in accord with the cytological suggestion that the coccoidal stage of the growth cycle is diploid. The increased sensitivity can be explained on the basis of the production of unpaired defects in the surviving fraction of the irradiated population. These unpaired defects are hereditary and are considered analogous to recessive lethal mutations.

Ultraviolet experiments on coccoids were carried out in parallel with those of X-ray. The results were similar to those from X-ray, which lends evidence for a basic similarity in the site of action of X-ray and ultraviolet radiations. Upon successive radiation treatments the population became more sensitive and the inactivation rate became less sigmoid but not completely exponential. The failure of the ultraviolet survival curves to become completely exponential is probably due to the production of secondary effects such as RNA interference.

Successive X-ray treatments of a haploid organism should not result in increased sensitivity if the recessive lethal mutation theory is correct. Micrococcus aureus, a haploid organism, was investigated following the same general procedure as for N. corallina. The dose per treatment was 12,000 roentgens which produced 35 percent survivors. The results showed that no change occurred in the X-ray dose-survivor response after five successive treatments.

Different stages of the growth cycle of N. corallina were studied by means of X-ray dose-survivor experiments. In this series a single well-aerated broth growth culture was used; however, tests showed that growth on solid medium produced similar results. Aeration and agitation was obtained by placing a 500 ml. erlenmeyer flask containing

100 ml. of medium, on a Burrel Wrist-Action Shaker adjusted to produce a 3/4 inch oscillation arc measured at flask bottom at a frequency of 300 cycles per minute. The standard X-ray irradiation procedure was used. The culture was sampled and the X-ray dose survivor response checked at culture ages of $3\frac{1}{4}$, $7\frac{1}{4}$, 12, 16, 20, $25\frac{1}{4}$, 28, and 36 hours. The results of the series are given in figure 41. The resistance increased through $7\frac{1}{4}$ hours after which the culture became more sensitive. The percent survival of stages during the life cycle at a constant X-ray dose of 30,000 roentgens is given in figure 42. The cytological appearances of the culture at each growth cycle stage of figure 42 is given in figures 43 to 50. The $3\frac{1}{4}$ hr. culture was composed of germinating coccoidal cells containing, for the most part, two nuclei per unit (figure 44). The $7\frac{1}{4}$ hr. culture was composed of hyphae from 8 to 14 microns in length and containing from 6 to 8 indistinct nuclei (figure 45). Note that this stage showed the greatest resistance to X-radiation. The culture at 12 and 16 hrs. was composed of hyphae from 12 to 16 microns in length and 10 to 12 deeply staining spherical "condensed" nuclei (figure 46 and 47). These stages were more sensitive than the $7\frac{1}{4}$ hr. non-discrete nucleus stage. X-ray studies of these stages of growth on nutrient agar showed even greater sensitivity, very nearly equal to the coccoidal cells. Nuclear condensation first appeared at 10 hrs., point a, and fragmentation of the hyphae was first in evidence at 15 hrs., point b. The 16 and 21 hr. cultures showed about the same X-radiation response as the 12 hr. condensed nucleus hyphae. Figure 48 reveals extensive fragmentation at this stage. The culture was composed almost entirely of dinucleated bacillary cells at $25\frac{1}{2}$ hrs. (figure 49). A comparison of

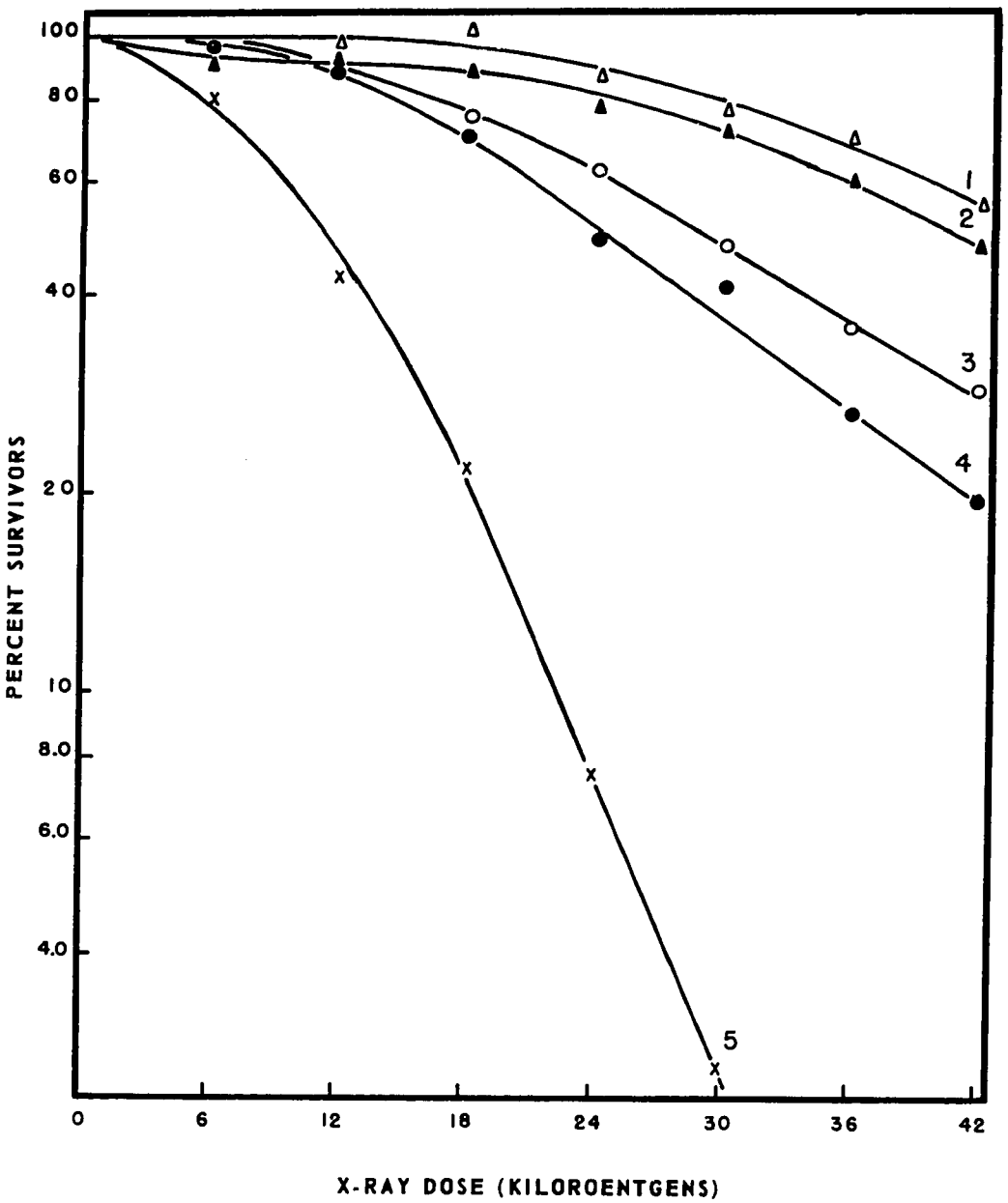



Figure 41. X-ray Dose-Survival Curves of Growth Cycle Stages of Nocardia corallina.

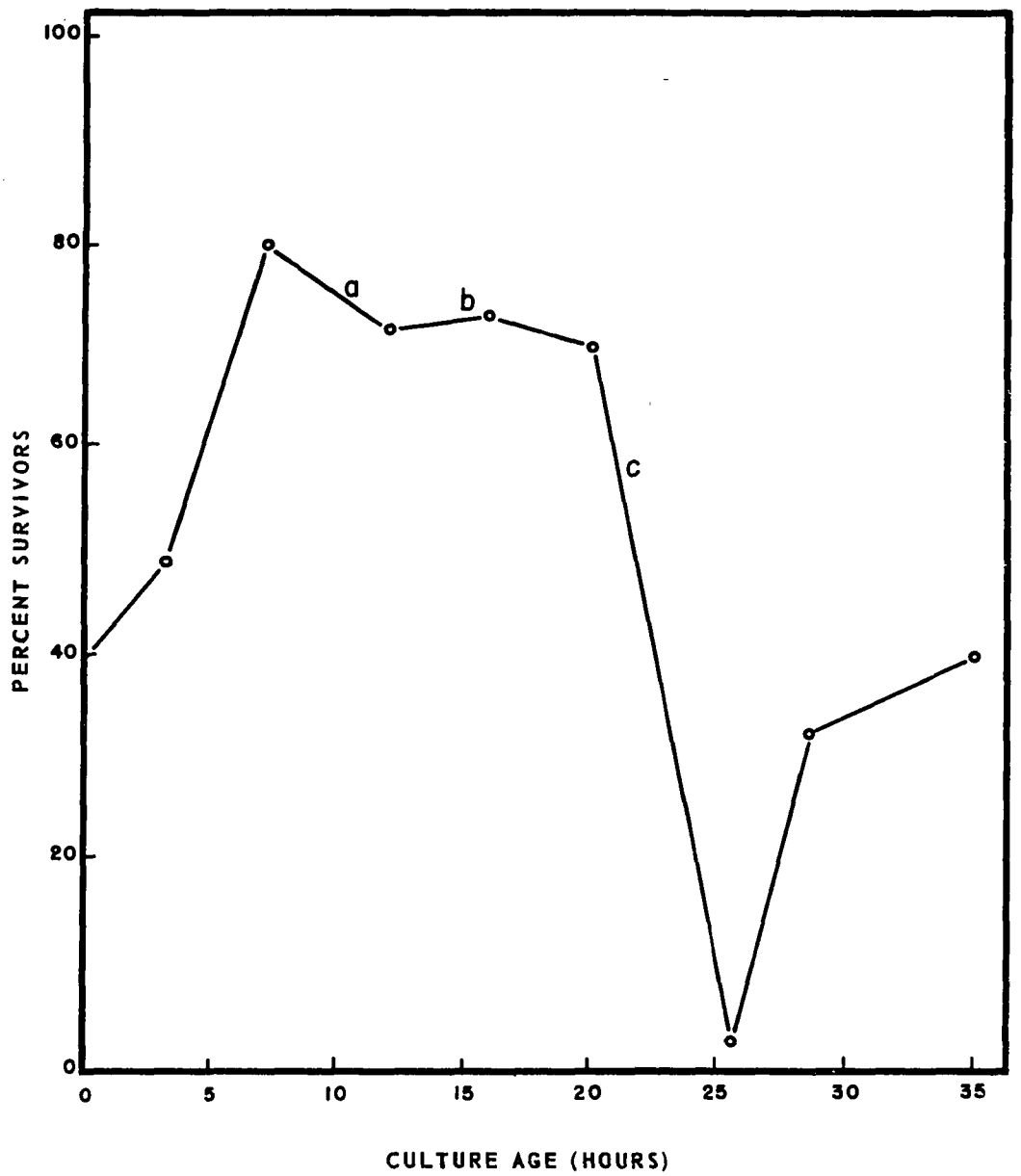



Figure 42. Percent Survival of Nocardia corallina Growth Cycle Stages after a Dose of 30,000 Roentgens X-radiation.

PLATE IV

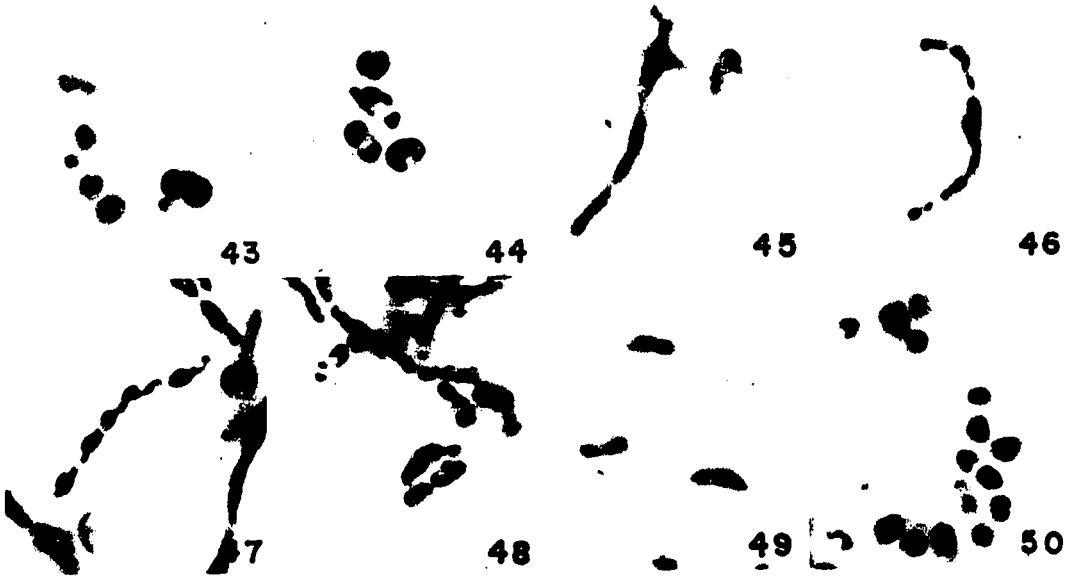



PLATE IV. Thionin-$SO_2$ nuclear stain preparations of the growth stages of Nocardia corallina used in the radiation experiment recorded in figure 42. All figures 3,200 X.

Figure 43. Five day old coccoids.

Figure 44. Three and one-fourth hr. germinating coccoids.

Figure 45. Seven and one-fourth hr. hypha.

Figure 46. Eleven hr. hypha.

Figure 47. Sixteen hr. hypha.

Figure 48. Twenty hr. hyphae.

Figure 49. Twenty-five and one-half hr. bacillary cells.

Figure 50. Thirty-six hr. coccoids.

the X-radiation response of the bacillary stage with the 3¼ hr. dinucleated germinating coccoids reveals a striking difference in that at a dose of 30,000 roentgens the dinucleated germinating coccoids showed 48 percent survivors and the dinucleated bacillary cells only 2.7 percent.

The culture was composed largely of coccoidal cells at 28½ hrs. but was more sensitive than the normal resting coccoids. At 35 hrs. the X-ray dose-survival response was essentially the same as that of the five day old coccoids and the cytological appearance was much the same (figure 50).

The increase in X-radiation resistance during germination is evidence against a reduction division at this stage. A cell containing two haploid nuclei should be considerably more sensitive than a similar cell containing a single diploid nucleus. The former has a hit multiplicity of two (Atwood and Norman 1949) and the latter a hit multiplicity considerably greater (Tobias 1952). Thus the hyphal nuclei remain diploid until the fragmentation process. Radiation studies provide evidence that the nuclear division initiating fragmentation is reductional. Partial reduction division explains the increase in sensitivity at 12 hrs. of the hyphae with discrete condensed nuclei. The X-ray sensitivity of the dinucleated bacillary stage is explained by the presence of haploid "three-spot" nuclei. This dose-survival curve is very nearly of the two event type and thus corresponds to the two haploid nuclei (figure 41, curve 5).

The increased radiation resistance of the culture precisely paralleled the change in the composition of the culture from dinucleated bacillary cells to uninucleated coccoidal cells. The evidence is

strongly suggestive that nuclei within the bacillary cells may either fuse to form a uninucleated coccoidal cell or else an exchange of nuclei occurs between adjacent bacillary cells through hyphal fusion and fusion tubes.

The integration of cytology and radiobiology into the cytogenetic approach provides evidence for the following sequence of events. Resting diploid coccoids germinate, forming diploid multinucleate coenocytic hyphae. A nuclear division, cytologically suggestive of meiosis, initiates hyphal fragmentation and gives rise to dinucleated haploid bacillary cells. Two nuclei of the same or of adjacent bacillary cells fuse and form a single diploid nucleus typical of the coccoidal stage.

## Cultural Characteristics and Environmental Effects

Growth cycle variations. Cultural conditions greatly influence the morphology and duration of phases of the growth cycle. The influence of carbohydrate in reducing the hyphal stage is shown in Table 1. The data presented in this table represent a standard size inoculum, since fragmentation is delayed and hyphae are much longer if very small inocula are used. Broth cultures especially, show differences in fragmentation time in response to differences in inoculum size. Fragmentation in fructose broth may vary from 12 hours if a very large inoculum is used to 48 hours if a very small inoculum is used. The amount of aeration is also important in liquid cultures. The fragmentation process may be much delayed or absent under reduced oxygen tension. The addition of fructose or glucose to the medium reduces the hyphal stage both by shortening the average length of the hyphae and by decreasing

TABLE 1

THE EFFECT OF MEDIUM ON THE AMOUNT OF GROWTH, TIME OF FRAGMENTATION AND PIGMENT INTENSITY

| Medium | Maximum Av. Hyphal l. | Time of Fragmentation | Relative Growth | Involution Forms | Pigment Intensity |
|---|---|---|---|---|---|
| Nutrient agar | 15 u | 14 hrs. | ++ | - | ++ |
| Glucose agar | 9 u | 10 hrs. | +++ | - | +++ |
| Fructose agar | 8 u | 10 hrs. | ++++ | - | +++ |
| Glycerol agar | 12 u | 14 hrs. | ++ | ++ | +++++ |
| McLoed's Med. broth: aerated | 15 u | 20 hrs. | + | + | + |
| Nutrient broth: aerated | 30 u | 30 hrs. | ++ | - | + |
| Glucose broth: aerated | 20 u | 20 hrs. | +++ | - | +++ |
| Fructose broth: aerated | 20 u | 18 hrs. | ++++ | - | +++ |

the duration of the stage. Since the total volume of growth is increased upon the addition of glucose or fructose, the increased growth must occur in the coccoidal stage. Initial growth in broth culture occurs more slowly in the presence of glucose or fructose, but the total growth is greater in the presence of a carbohydrate. Concentrations of glucose or fructose above 2 percent in nutrient broth were found to be

inhibitory. The optimum concentration of glucose or fructose in respect to total growth was found to be about 0.75 percent.

The addition of glycerol to the medium greatly increased pigment production and fat formation. The fat soluble and water insoluble pigment seemed to be located principally in the fat inclusions. The presence of glycerol in the medium resulted in decreased total growth and the stimulation of various types of involution forms.

Although the optimum temperature for maximum growth of N. corallina is 29 to 31 degrees C., growth can occur at temperatures of from 5 degrees C. to 45 degrees C. Incubation temperatures above 35 degrees C. produced extreme alterations in the growth cycle. At 37 degrees C. involution forms developed slowly from germinating coccoids (figure 54). The fragmentation process, which occurred at 20 to 24 hrs., produced extensive cross septation in the involution forms (figure 55 and 56). The multicellular units later fragmentated forming a culture of large multicellular bacillary cells and coccoids (figure 57 and 58).

Colonial morphology and growth. Surface colonial morphology of the normal stain of N. corallina on nutrient agar is shown in figure 59,a. The ridged rough surface showed little variation on various solid media. However, the moisture content of a medium influenced colonial morphology to a considerable extent.

Germination of coccoids on nutrient agar approached 100 percent, even from cultures several weeks old. The resultant hyphae developed to considerable lengths, often exceeding 50 microns, before fragmentation began. The behavior of a single isolated coccoid in forming a colony

PLATE V. Phase studies of colonial growth and stained preparations of involution forms of Nocardia corallina

Figure 51. Phase study of 15 hr. micro-colony growing on nutrient agar. 1,000 X.

Figure 52. Phase study of 24 hr. micro-colony growing on nutrient agar. 1,000 X.

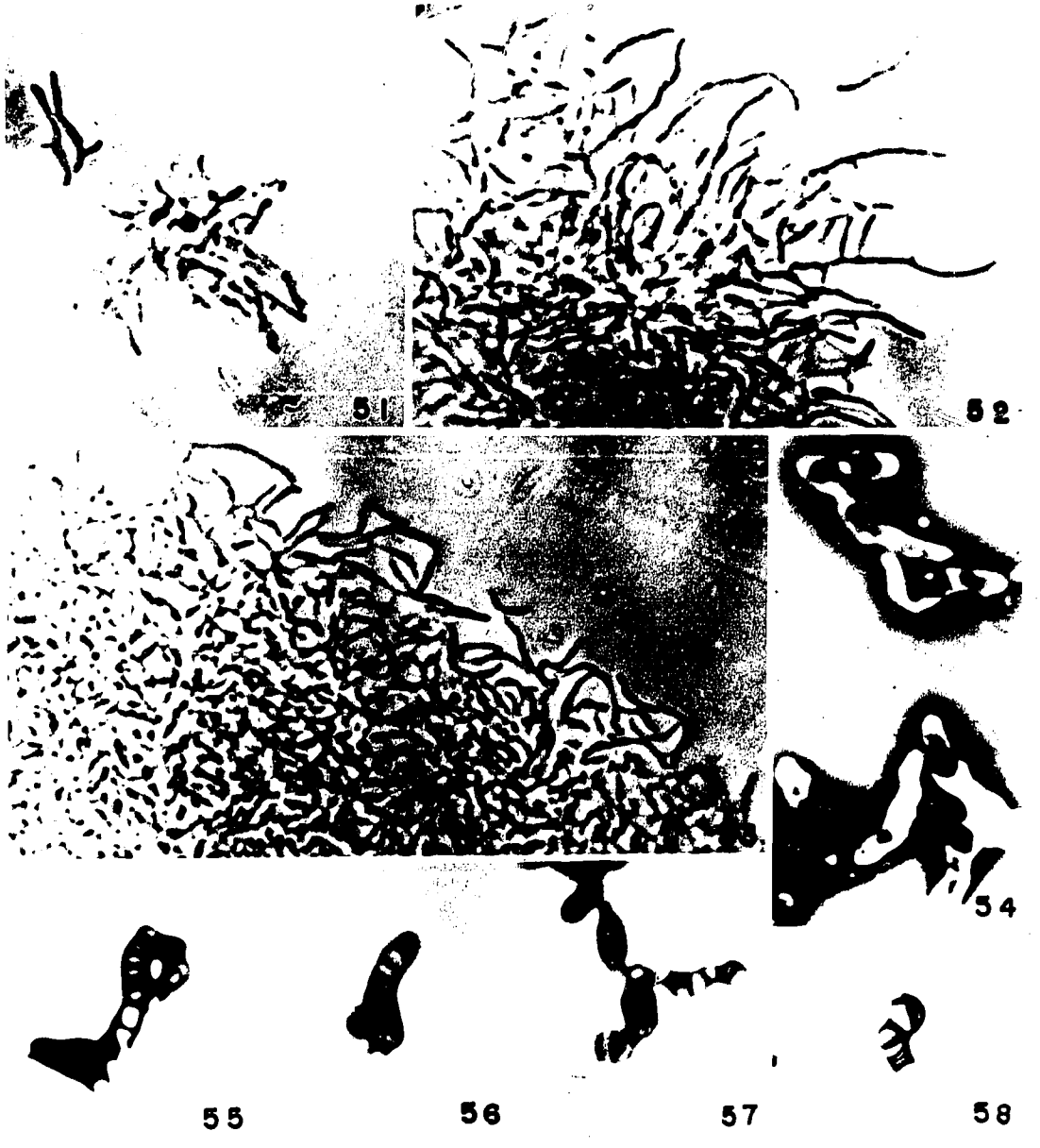
Figure 53. Phase study of 40 hr. micro-colony growing on nutrient agar. 1,000 X.

Figure 54. Crystal violet stain of large forms after 20 hr. at 37 degrees C. 3,000 X.

Figures 55 - 56. Cell wall stain of large multicellular forms after 24 hr. at 37 degrees C. 3,000 X.

Figures 57 - 58. Cell wall stain of large multicellular forms after 30 hr. at 37 degrees C. 3,000 X.

PLATE V



differed from that previously described in that the inoculum size is different. The influence of inoculum size on hyphal development and time of onset of fragmentation has been previously noted. The colony expanded by hyphal growth at the periphery until 59 to 55 hours (figure 51, 52, and 53) after which most growth both in thickness and in circumference occurred by coccoidal division. Long hyphae continued to grow out from the margin. These hyphae were followed by a margin of dividing coccoids which produced most of the size increase of the colony. The method of colony growth reported by Waksman (1950) in which the increase in colony size was accomplished by waves of hyphal growth and gragmentation was not observed in N. corallina except in the 24 to 36 hour microcolony (figure 52 and 53). In older colonies the major increase in size was through coccoidal division, whereas, hyphal growth and fragmentation played a minor role.

Subsurface colonial growth differed from surface growth in that growth was much slower and remained in the hyphal stage until the colony was 8 to 10 days old. The hyphae remained in the 8 to 15 micron length range, undergoing single divisions as the length of individual hyphae increased beyond this point.

Mechanism of fragmentation and germination. On nutrient agar containing fructose the major cultural growth occurs in the coccoidal stage; the coccoids dividing by a process similar to the true bacteria (figure 28, 29, and 30). Upon transfer of actively dividing coccoids to fresh medium or even distilled water, germination occurred and hypha were produced. Table 2 shows the results of inoculating actively

TABLE 2

THE EFFECT OF THE RATIO OF STALE TO FRESH MEDIUM ON GERMINATION, HYPHAL LENGTH AND TIME OF FRAGMENTATION

| Stale medium/Fresh medium | Hyphal length | Time of fragmentation | Germination |
|---|---|---|---|
| 1 / 100 | 10 - 20 μ | 18 - 20 hrs. | + |
| 1 / 50 | 12 - 16 μ | 18 - 20 hrs. | + |
| 1 / 10 | 10 - 12 μ | 12 - 15 hrs. | + |
| 1 / 5 | 8 - 10 μ | 10 - 12 hrs. | + |
| 1 / 3 | 6 - 8 μ | 8 - 10 hrs. | + |
| 1 / 2 | 4 - 6 μ | 6 - 8 hrs. | + |
| 1 / 1 | 2 - 4 μ | 4 - 6 hrs. | + |
| 2 / 1 | - | - | - |

dividing coccoids into media containing varying amounts of filtered stale medium. The inoculum size was constant in these tests. It may be observed that stale medium caused a reduction in both hyphal growth and time of fragmentation. The simplest explanation for this phenomenon is the production of some metabolic product which initiates fragmentation when a certain critical concentration is reached. Thus the greater amount of stale medium added, the quicker the fragmenting concentration is reached.

Two parts stale medium to one part fresh medium completely inhibited coccoid germination. Since coccoids germinated in distilled water this failure to germinate in stale medium is evidence for a

germination inhibition factor. The fragmentation and germination inhibition factors may be the same or different metabolic products. Apparently a metabolic product which builds up in the medium and hyphae initiates fragmentation and inhibits the germination of the resultant coccoids. However, it permits the coccoids to continue dividing under appropriate conditions.

There is evidence that the material builds up inside the hyphae, since rapid repeated transfer does not entirely prevent fragmentation. Upon transfer to fresh solid medium at 8 hour intervals the hyphae reached a maximum average length of about 12 microns for the first six transfers then shortened to a length of 6 to 10 microns, dividing apparently as the latter length was exceeded. Transferring the culture just after fragmentation had begun resulted in the formation of some involution forms, but usually fragmentation, once initiated continued to completion.

No attempt was made to isolate a "fragmentation factor" from the stale medium.

Nutrition Studies. The growth of N. corallina was tested on several synthetic media. A medium containing asparagine 0.05 percent, glucose 1.0 percent, potassium monohydrogen phosphate 0.05 percent, at pH 7 produced slow and scanty growth and was not considered suitable for general use. McLoed's medium (McLoed 1940), developed for the growth of E. coli, was tested for N. corallina. This medium contained: 0.472 percent ammonium sulfate, 0.200 percent asparagin, 0.50 percent sodium chloride, 0.20 percent glucose, 0.261 percent potassium dihydrogen

phosphate, 1.64 percent sodium hydroxide, and trace amounts of magnesium chloride, ferric chloride, and calcium chloride. The pH was adjusted to 7.6. The growth rate on this medium was rather slow and a heavy viscous sediment was formed. Omission of the asparagin resulted in faster growth and reduction of the amount of sediment.

One percent glucose in McLoed's medium was found to be optimum for N. corallina. One percent fructose was found to be at least as effective as glucose in most uses and was superior for the production of isolated coccoids.

McLoed's synthetic medium was found to be effective for the growth of N. corallina if modified by increasing the glucose content to 1.0 percent, omitting asparagine and adjusting to a pH of 7.4. Thiamine may be added to increase growth. For a solid medium, 1.5 percent agar may be added.

The ability of N. corallina to utilize various organic compounds as the sole source of carbon was investigated. In the study, each compound was added to a test tube containing 5 ml. McLoed's synthetic medium minus glucose and asparagin and at pH 7.4. Growth was determined visually by the direct comparison of the culture in question with nutrient broth and no carbon cource controls and recorded as relative growth. A standard inoculum of resting coccoids was added to all tubes which were then incubated at 29 degrees C. The results of this experiment are given in Table 3. The findings indicated that glucose, galactose and fructose were effective in producing growth. Of the disaccharides tested, trehalose was most effective. The pentoses tested showed no growth effect. The alcohols, sorbitol, and mannitol produced

TABLE 3

GROWTH OF *NOCARDIA CORALLINA* UPON A SYNTHETIC MEDIUM CONTAINING VARIOUS COMPOUNDS AS THE SOLE CARBON SOURCE

| Carbon source | Growth in days | | | Carbon source | Growth in days | | |
|---|---|---|---|---|---|---|---|
| | 2 | 6 | 8 | | 2 | 6 | 8 |
| D Glucose | + | ++ | ++ | Arginine | - | - | - |
| D Galactose | + | ++ | ++ | Aspartic acid | - | - | - |
| D Fructose | + | +++ | +++ | Glutamic acid | + | + | ++ |
| Sucrose | - | + | ++ | Tryptophane | - | - | - |
| Lactose | - | + | + | Sodium oleate | + | + | + |
| Maltose | - | - | - | Sodium butyrate | + | ++ | ++ |
| Trehalose | + | ++ | ++ | Sodium caproate | + | ++ | ++ |
| D Xylose | - | - | - | Sodium lactate | + | ++ | ++ |
| D Ribose | - | - | - | Sodium acetate | + | ++ | ++ |
| L Rhamnose | - | - | - | Sodium pyruvate | + | +++ | +++ |
| Raffinose | - | - | - | Sodium citrate | - | - | - |
| D Sorbitol | + | ++ | ++ | Sodium cis-aconitate | - | + | ++ |
| D Mannitol | + | + | ++ | Sodium isocitrate | + | + | ++ |
| Inositol | - | - | + | Sodium alpha-keto | | | |
| Inulin | + | ++ | ++ | glutarate | + | ++ | ++ |
| Cystine | - | - | - | Sodium oxalo- | | | |
| Cysteine | - | - | - | succinate | + | +++ | +++ |
| Asparagine | + | + | + | Sodium succinate | + | +++ | ++++ |
| Glycine | - | - | - | Sodium fumarate | + | ++ | +++ |
| Alpha alanine | - | - | - | Sodium malate | + | +++ | +++ |
| Beta alanine | - | - | - | Sodium oxalacetate | + | + | ++ |
| Isoleucine | + | + | + | Phenol (0.1%) | + | ++ | ++ |
| Phenylanine | - | - | - | Phenol (0.5%) | - | - | - |
| Tyrosine | - | - | - | Control | - | - | - |
| Hydroxyproline | - | - | + | Nutrient broth | | | |
| Methionine | - | - | + | with/0.75% fructose | + | +++ | ++++ |

moderate growth.

The animo acids as a group showed from slight to no growth effect, whereas the fatty acids; acetic acid, oleic acid, butyric acid, and caproic acid produced moderate growth.

Phenol was found to function effectively as the carbon source at a concentration of 0.1 percent; however, a concentration of 0.5 percent was found to be toxic.

Compounds of the citric acid cycle were found to function effectively as the carbon source with the conspicuous exception of citrate. The fact that pyruvate, cis-aconitate, isocitrate, alpha-ketoglutatate oxalosuccinate, succinate, fumarate, malate, and oxalacetate were effective in producing growth provided evidence for the presence of the Krebs cycle as a major metabolic pathway in this organism. But, the failure of N. corallina to utilize citrate is difficult to explain. The amount of enzyme may be insufficient for the utilization of the compound. Delwiche and Carson (1953) reported that Proprionibacterium pentosaceum could oxidize intermediates of the citric acid cycle with the exception of citrate. Cell-free extracts, however, quantitatively degraded citrate to alpha-ketoglutarate and acetone dried cells synthesized citrate from pyruvate and oxalacetate. Thus the failure to utilize citrate was due to an impermeability of the cell membrane. A similar situation may exist in N. corallina.

## Genetic Studies in N. corallina

Morphological and Color Mutants. Spontaneous mutants, isolated from the normal population of N. corallina, were studied as a basis for

further correlation between cytology and genetics. Some thirty mutant strains were isolated and studied; and, in certain cases, mutant frequencies were determined. The cytological evidence for a sexual process involving hyphal fusion and fusion tubes during fragmentation provided a basis for the study of possible recombination of color and morphological characteristics in "crosses" between appropriate strains. Recombination recently has been reported in the genus Streptomyces (Szybalski and Braendle 1956); however, the genetics of the genus Nocardia has not received much study.

Some of the mutant strains isolated and studied are listed in Table 4. These mutants involved changes in colony color, colony morphology, fragmentation time, and average maximum hyphal length. A mixture of normal and mutant strains of N. corallina plated on nutrient agar is shown in figure 59. In this figure, colonies representative of normal, a; red smooth, b; white rough, c; and sectored, d and e are shown. The normal colonial morphology on most common laboratory media is rough and somewhat wrinkled as is shown in figure 59, a. The normal colony color is coral red, but variation in both shade and intensity has been found on different media (Table 11).

The smooth variants differed from the normal strain in that the hyphal stage was shorter (Table 4) and colonial growth was mostly by coccoidal division. The edge of the smooth colony was entire with a few protruding long hyphae. The plane of coccoidal division seemed to be more at random and the coccoids broke apart more readily after division. Colonial morphology was found to be related to moisture in the medium

TABLE 4

CHARACTERISTICS OF VARIOUS MUTANT STRAINS ISOLATED FROM NOCARDIA CORALLINA

| Strain designation | UV treated | Colony color | Colony morphology | Fragmentation time | Hyphal length |
|---|---|---|---|---|---|
| S-1 | + | Red | Smooth | 8-10 hrs. | 7- 9μ |
| S-2 | + | " | " | 10-12 " | 8-10μ |
| S-3 | - | " | " | 8-10 " | " |
| S-4 | - | " | " | " | " |
| S-5 | - | " | " | " | " |
| S-6 | - | " | " | " | " |
| W-1 | + | White | Rough | 12-14 hrs. | " |
| W-2 | + | " | " | " | 10-15μ |
| W-3 | + | Pale Yellow | " | " | " |
| W-4 | - | Pale red | " | " | " |
| W-5 | - | Very pale red | " | " | " |
| W-6 | - | " | " | " | " |
| Y-1 | + | Yellow | " | " | " |
| Y-2 | - | " | " | " | " |
| Y-3 | - | " | " | " | " |
| SY-1 | - | " | Smooth | 8-10 hrs. | " |
| R-1 | - | White | " | " | " |
| R-3 | - | " | " | " | " |
| DR-1 | + | Dark red | " | 7-9 hrs. | 6- 8μ |
| Normal | | Red | Rough | 12-14 " | 10-15μ |


Figure 59. Colonies of normal and variant forms of N. corallina.

a. Normal N. corallina colony.

b. Red smooth variant.

c. White rough variant.

d. White rough colony with red smooth sector.

e. Normal colony with white sector.

and, on three percent agar or partially dehydrated plates, the smooth variants became somewhat rough and ridged.

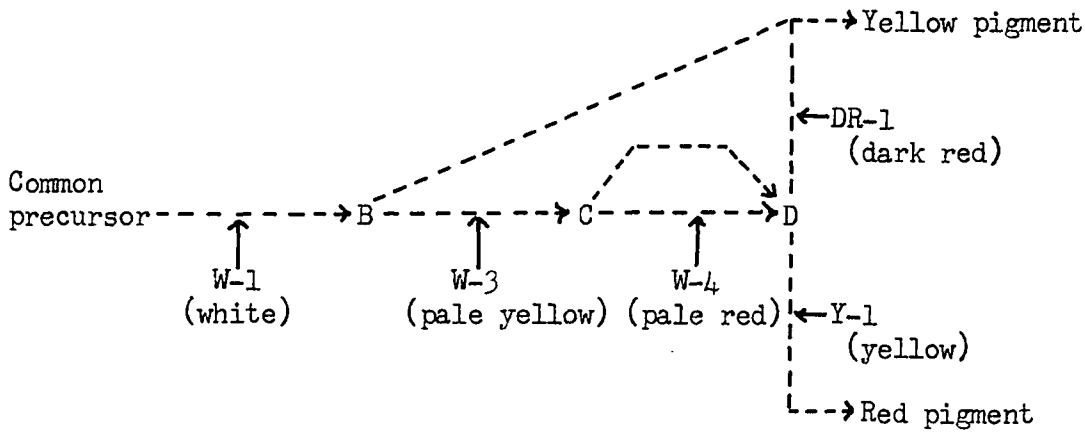
Smooth variants appeared consistently in the normal strains used throughout the study, at a frequency of 0.01 to 0.3 percent. A transplant of the normal strain of N. corallina (ATCC #4273) recently obtained from ATCC, however, produced smooth colonies at a frequency of 16.5 percent. Twenty single celled rough colony isolates were made from this strain and the frequency of smooth colony variant was determined for each. Eleven of the twenty isolates produced smooth colonies at a frequency of 0.05 to 3.0 percent. Ten smooth colony variants were isolated from the same strain and the frequency of rough colony forms determined for each. None of the smooth strains produced rough colony forms detectable by the experimental procedure. Further studies demonstrated that smooth colony forms were greatly reduced, but not completely eliminated, by the successive selection of only rough colonies for the inoculation of new cultures.

The effect of successive ultraviolet irradiation on the frequency of smooth variants in a normal strain of N. corallina was studied. Coccoids were plated on nutrient agar and ultraviolet irradiated at a dose sufficient to inactivate 30 percent of the coccoid population. Inocula were prepared from twenty rough colonies and streaked on nutrient agar slants containing one percent fructose. After four days incubation at 29 degrees C., the culture was again plated and ultraviolet irradiated with the same dose previously used and the procedure repeated. The series was continued through eighteen successive ultraviolet

treatments. The frequency of smooth colony variants varied between 0.05 and 0.3 percent; however, this variation did not follow a pattern. Successive transfer of rough colony isolates, without intervening ultraviolet irradiation, resulted in the reduction of the frequency of smooth colony variants to a level of about $1 \times 10^{-8}$. It is therefore concluded that the induced mutation rate was large enough to counteract the effect of selecting only normal rough colonies for inoculation. A study of ultraviolet induced smooth colony mutations confirmed this conclusion. The percent frequency of the mutant in the population was found to increase linearly from a background frequency of 0.02 percent to 2.1 percent after a radiation dose sufficient to inactivate 99.5 percent of the population.

The color mutants were found to represent varying degrees of the absence of two pigments present in the normal coral red strain. The yellow mutants occurred through a metabolic block involving synthesis of dark red pigment. The dark red mutants occurred through a metabolic block preventing the synthesis of a yellow pigment. White mutants were the result of the low concentration or absence of both the yellow and red pigments. The mutants W-3 and W-4 (Table 4) turned slightly yellow and slightly red respectively upon aging. Thus, the W-3 strain involves a mutation which completely blocks the formation of the dark red pigment and partially blocks the yellow pigment synthesis. The W-4 strain involves a mutation which partially blocks both the red and yellow pigments. Strain W-1 and W-2 remain colorless, which indicates that the formation of both pigments is completely blocked. These findings

provide evidence that the two pigments have a common precursor which, if blocked, will prevent pigment formation. Biochemical evidence to be discussed later also is consistent with a structural similarity existing between the two pigments. Although the data are too preliminary to permit definite conclusions as to the specific genes involved, a hypothetical scheme is given which indicates some possible relationships between color mutants and the synthesis of the two pigments. The common




precursor illustrated as A, undergoes a sequence of reactions leading to compound B, which normally gives rise to compounds C and D through n steps. Compound D through a series of n steps forms the two pigments. The W-1 strain is completely white and is thus blocked between A and B and has no shunt pathway leading to pigment production. The metabolic blocks of strain W-3 are illustrated by a block between B and C and results in some B, following a shunt pathway through n steps to the formation of the yellow pigment. The metabolic change of strain W-4 is illustrated by a partial block between the steps C and D which still

allows a reduced amount of D to be produced so that both pigments are equally reduced. The DR-1 strain results from a block of the D to yellow pathway and the Y-1 strain from a block of the D to red pathway. Even though the chemical nature of the pigments is unknown, the color mutants provide definite information about pigment synthesis and the genetic makeup of the organism.

Preliminary study of the color mutants revealed that the mutant frequencies in the normal population were much lower than for the smooth colony mutants. The white and yellow mutants were found to occur in the normal population at a frequency of about $1 \times 10^{-8}$. The color variants sometimes appeared as sectors in otherwise normal red rough colonies (figure 59,e).

The behavior of the smooth mutation is characteristic of a recessive character in a diploid organism. As a consequence, normal colonies heterozygous for the smooth colony type could, by nuclear segregation and rearrangement or sexual recombination during hyphal fragmentation, become homozygous in a certain percent of the situations. A coccoid heterozygous for smooth, theoretically should give rise to a diploid, heterozygous, multinucleate hypha. At hyphal fragmentation, a reduction division occurs in which the smooth and rough alleles would segregate into different bacillary cells. If the orientation of the nuclei is at random, the resultant coccoidal cells would approximate a 1:2:1 ratio of homozygous rough, heterozygous rough, and homozygous smooth. As a consequence, one-third of the progeny of a coccoidal cell, heterozygous for the smooth character, should show a smooth colony

morphology. Instead, observations reveal that coccoidal cells, apparently heterozygous rough, show a frequency of 1 to 5 percent smooth colonies in the clone. This observation can be explained on the basis that the diploid hyphal nuclei do not segregate at random; but rather, there may be a tendency for non-random distribution which results in most of the coccoidal cells becoming again heterozygous.

N. corallina was found to be stable to metabolic mutations. The Davis (1949) penicillin technic for the isolation of nutritional deficient mutants was applied without success. This stability is probably due to the normally recessive nature of these mutants, since the expression of a recessive mutant in a diploid population would be expressed at a frequency much lower than that of the gene itself.

Nature of Pigments. The chemical nature of the pigments was studied to provide a better basis for genetic and cytogenetic interpretations. Reader (1926) studied the pigments of an organism, later classified as N. corallina, and showed that the pigment had the general properties of the lipochrome group of fat soluble compounds. However, he made no effort to separate the components of the pigment.

Cultures of N. corallina coccoids were harvested after 14 days incubation at 28 degrees C. on nutrient agar containing one percent fructose. A heavy suspension of coccoids was washed once and dried at 60 degrees C. The dried cells were extracted with chloroform in a Soxhlet extractor. Qualitative solubility tests were run by placing a small amount of pentane precipitated material in a test tube containing the solvent. Descending paper chromatography was carried out using

three solvent systems. The solvent systems were 15 percent acetic acid, 60 percent acetic acid and butanol-acetic acid-water (6:2:1). Complete separation of the pigments was achieved on paper with 60 percent acetic acid. Absorption spectra were run on the separated pigments with the Beckman model DU spectrophotometer.

Solubility characteristics of the pentane precipitated crude extract are given in Table 5. The pigment solubilities were determined qualitatively by visual observation. The characteristics of the two pigments in the solvents tested were similar except in 60 percent acetic acid. These data fail to suggest any particular group of pigments.

Two chemical tests gave positive reactions on chloroform extracts of the pigments. Both pigments turned deep blue-green upon the addition of concentrated sulphuric acid and formed a heavy light gray flocculent precipitate upon the addition of basic lead acetate. Ferric chloride, neutral lead acetate, hydrochloric acid and ammonium hydroxide produced no color effect on chloroform solutions of either pigment. These data suggest a possible carotinoid relationship for both pigments; however, the reactions are not considered as final proof (Goodwin 1955).

The results of the paper chromatograph studies are given in Table 6. The $R_f$ value for both pigments was 0 in 15 percent acetic acid and 1 in the butanol-acetic acid-water solvent systems. A decided difference was obtained in the 60 percent acetic acid solvent system, in which the red pigment had an $R_f$ value of about 0.9 and the yellow pigment a value of 0. Separation of the pigment components was achieved by streaking the chloroform extract on large sheets of Whatman number 3

TABLE 5

SOLUBILITY CHARACTERISTICS OF THE PIGMENTS OF *NOCARDIA CORALLINA*

| Solvent | Solubility | |
|---|---|---|
| | Red pigment | Yellow pigment |
| Anhydrous isopropanol | ± | ± |
| 85% Isopropanol | ± | ± |
| Water | - | - |
| Ethanol | - | - |
| Methanol | - | - |
| Pentane | - | - |
| Isoamyl alcohol | ± | ± |
| Acetone | ± | ± |
| Chloroform | ++++ | ++++ |
| Ether | ± | ± |
| Butanol-acetic acid-water | +++ | +++ |
| 15% Acetic acid | - | - |
| 60% Acetic acid | +++ | - |

TABLE 6

$R_f$ VALUES FOR THE TWO PIGMENTS OF NOCARDIA CORALLINA IN THREE SOLVENT SYSTEMS

| Solvent System | $R_f$ Value | |
|---|---|---|
| | Red pigment | Yellow pigment |
| 15% Acetic acid | 0 | 0 |
| 60% Acetic acid | 0.9 | 0 |
| Butanol-acetic acid-water (6:2:1) | 1 | 1 |

chromatographic paper, running the material in 60 percent acetic acid, cutting out the separate bands, and extracting the pigments from the paper with chloroform.

The absorption spectra of the two pigments in chloroform indicated a close similarity between the two compounds, but the paper contaminant obscured much detail of the absorption curves and prevented an accurate comparison with known carotinoid compounds.

The data are in general agreement with the findings of Reader (1926) on the non-separated pigment mixture. The evidence as to the chemical nature of the pigments is inconclusive. However, the possible relationship of the pigments to the carotenoid is indicated.

Genetic Recombination in N. corallina. The mutant strains showing color and morphological variations, listed in Table 4, offered an opportunity to test for the existence of a sexual process in N. corallina.

Each of the six red smooth strains were mixed with each of the white rough strains in all possible combinations.

The "crossing" of mutant strains was accomplished by placing equal amounts of each of the two strains into a 0.85 percent saline blank and thoroughly mixed with glass beads on a Vibraloe shaker. The resultant suspension was then inoculated on the surface of a nutrient agar slant containing one percent glucose and incubated at 23 degrees C. for five days at which time each culture was composed of coccoids. The culture was then harvested, mechanically shaken with glass beads, appropriately diluted, and surface plated on nutrient agar.

The results from thirty "crosses" involving six red smooth and five white rough mutant strains are given in Table 7 which records the percent frequency of the total recombinant forms and the total sectored colonies. A total of about 1500 colonies were examined and tabulated for each cross; thus the procedure was not sensitive to recombinant frequencies below 0.2 percent. The lower frequencies found represent only one or two colonies and thus have no quantitative meaning. Each of the eleven mutant strains was plated separately and examined for the occurrence of mutants. None of the eleven strains was found to produce mutants within the sensitivity range of the experimental procedure. Fourteen of the thirty crosses yielded recombinant types at a frequency of 0.02 percent to 7.5 percent. Two recombination phenotypes were theoretically possible if a sexual process occurred. These phenotypes were white smooth and red rough (normal type). Four additional types would be possible if recombination could occur between the dark red and

TABLE 7

PERCENT FREQUENCY OF RECOMBINANT FORMS AND SECTORED COLONIES OBTAINED FROM "CROSSES" BETWEEN MUTANT STRAINS OF *NOCARDIA CORALLINA*

| Mutant strain | W-1 | W-2 | W-3 | W-4 | W-5 |
|---|---|---|---|---|---|
| S-1 | | | | | |
| Recombinant forms | 0.13 | 0.0 | 0.0 | 0.0 | 0.5 |
| Sectored colonies | 11.0 | 1.0 | 7.8 | 3.6 | 7.7 |
| S-2 | | | | | |
| Recombinant forms | 2.0 | 2.0 | 1.2 | 0.2 | 2.7 |
| Sectored colonies | 3.2 | 3.8 | 9.4 | 3.3 | 10.5 |
| S-3 | | | | | |
| Recombinant forms | 0.0 | 0.0 | 1.2 | 0.0 | 0.02 |
| Sectored colonies | 5.1 | 0.5 | 5.7 | 1.0 | 8.5 |
| S-4 | | | | | |
| Recombinant forms | 0.67 | 0.0 | 0.0 | 1.6 | 0.23 |
| Sectored colonies | 20.0 | 0.0 | 14.0 | 6.5 | 16.0 |
| S-5 | | | | | |
| Recombinant forms | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 |
| Sectored colonies | 2.0 | 0.37 | 0.5 | 0.2 | 2.0 |
| S-6 | | | | | |
| Recombinant forms | 0.0 | 0.0 | 0.0 | 0.0 | 7.5 |
| Sectored colonies | 0.0 | 0.02 | 0.2 | 0.1 | 2.0 |

yellow loci. These would show in an $F_2$ cross in which one or both parent cells were heterozygous for the two color loci; dark red and yellow. Four recombinant types were observed in the progeny of the red smooth and white rough mixtures. These were: white smooth, red rough, yellow smooth, and yellow rough. The yellow smooth and yellow rough recombinants were observed in five of the fourteen fertile crosses. White smooth was observed more frequently than red rough, yellow smooth, or yellow rough which as yet remains unexplained.

Table 7 presents evidence for mating types in N. corallina. A system is postulated in which + and - show a capacity for mating whereas + and + and - and - do not. On this basis the S-2 strain is arbitrarily designated as - and the white rough strains which show recombination with this strain, + (Table 8). Thus W-1, W-2, W-3, W-4, and W-5 are all +. The S-1, S-3, S-5, and S-6 strains are +, whereas S-2 and S-4 strains are -. If W-5 is designated as $\pm$, then the only crosses which do not fit the postulated mating types are W-1 x S-1, S-3 x W-3, S-4 x W-2, and S-4 x W-3. Since the latter two crosses show an absence of recombination forms, there is a strong likelihood that the frequency was not high enough to be detected by the technic used. Thus W-1 x S-1 and S-3 x W-3 are the only two definite exceptions. Mating type mutations in the cultures could explain the aberrant results; however, no conclusions can be drawn on the basis of the experimental results.

Percent frequency of sectored colonies for the thirty crosses is given in Table 7. Only two of the thirty crosses showed no sectoring; however, fourteen were below 3.0 percent. As 5 to 10 percent clumping

TABLE 8

MATING TYPE DESIGNATIONS BASED ON RESULTS FROM MUTANT STRAIN "CROSSES"

| Strain | Mating type | Strain | Mating type | Strain | Mating type |
|---|---|---|---|---|---|
| S-1 | + | W-1 | + | Y-1 | - |
| S-2 | - | W-2 | + | Y-3 | - |
| S-3 | + | W-3 | + | R-1 | ? |
| S-4 | - | W-4 | + | R-3 | ? |
| S-5 | + | W-5 | ± | R-5 | ? |
| S-6 | + | W-6 | + | Normal | + & - |

was present in the plating suspensions, most of the sectored colonies could be explained on this basis; however, the same frequency clumping was present in the cultures showing 0 to 0.2 percent sectored colonies. Also clumps of coccoids are usually formed from the progeny of a single hypha that did not completely separate following fragmentation. It is thus postulated that much of the sectoring represents segregation of mutant types from heterozygous or heterocaryotic hyphae. The radiobiological evidence does not support the existence of reduction division at germination, which would result in a heterocaryonic hypha from a diploid heterozygous coccoid. If reduction division does not occur at germination, the hyphal nuclei are genetically identical to the coccoidal nucleus. Thus a heterocaryon would be produced only if a new mutation occurred. The hyphal stage is considered to be diploid on the basis of cytological and radiobiological evidence and sectored colonies largely

occur by segregation and recombination of heterozygous hyphal nuclei during fragmentation. The sectors frequently appeared near the edge of the colony which suggests delayed segregation. The sectors were usually of the parental types; however, frequently the sector was a recombinant type. Some correlation exists between the occurrence of recombinant forms and sectoring. Of the sixteen crosses showing a sectoring frequency above 3.0 percent, twelve also show recombinant forms. Two fertile crosses produced sectored colonies at less than 3.0 percent. The correlation is not absolute but the results are suggestive that the sectoring tendency is in some way related to the sexual mechanism.

Additional crosses are listed in Table 9. The S-2 x P-1 and S-2 x P-3 crosses produced no recombinant types and little sectoring. Thus tentatively P-1 and P-3 can be considered of the + mating type. Three "back crosses" were made between white smooth recombinant strains; R-1, R-3, and R-5; and the normal parent strains (Table 9). The recombinant forms, white rough and red smooth were produced in each case. The R-1 strain was observed to be a mixed population containing about 1.5 percent white rough colony forming variants. Thus the 3.9 percent frequency does not represent conclusive results. The R-3 normal back cross produced recombination forms at a frequency of 0.3 percent. Controls failed to detect any mutants in the parent strains. The S-6 x W-6 cross produced a small number of recombinants and 4.5 percent sectored colonies.

Cytological evidence indicated that the sexual process, demonstrated by recombination of genetic factors, occurs through hyphal fusion

TABLE 9

FREQUENCY OF RECOMBINANT FORMS AND SECTORED COLONIES OBTAINED FROM "CROSSES" BETWEEN MUTANT STRAINS OF *NOCARDIA CORALLINA*

| Cross | Percent recombinant forms | Percent sectored colonies |
|---|---|---|
| S-2 x Y-1 | 0.0 | 0.5 |
| S-2 x Y-3 | 0.0 | 0.0 |
| Normal X R-1 | 3.9 | 4.9 |
| Normal X R-3 | 0.3 | 4.8 |
| Normal X R-5 | 0.25 | 0.2 |
| S-6 x W-6 | 0.52 | 4.5 |

and exchange of nuclei. The possibility that the observed recombination occurred by means of a filterable factor such as a symbiotic bacteriophage was also investigated. A modified Davis U tube experiment (Davis 1950) was performed in which mutant strains S-2 and W-5 were grown on either side of a fritted glass filter in aerated nutrient broth containing one percent fructose. One half of the liquid was passed back and forth through the filter twice each day during the six days of growth. After six days incubation at 29 degrees C. coccoids from each side were plated on the surface of nutrient agar. No recombinant forms were observed among the colonies examined, which indicates that at least for this cross and under the condition of the experiment a filterable agent is not involved in the recombination process.

The impossibility of following precisely the events in a

population while two different strains are growing together make genetic interpretation rather uncertain. The growth of the strains on an agar slant usually involves more than a single life cycle. Progeny coccoids in a culture may also go through a second life cycle so that the coccoid population from the crosses may represent not one generation, but two.

The results from genetic studies do not give a clear solution to the dominance problem in the variants of N. corallina. The findings give some suggestion that the genes producing smooth colonies and color variations are recessive. The findings were complicated by the fact that some of the white smooth recombinants were found to segregate into white smooth and white rough forms. A reversal in dominance seems to have occurred in some of the white smooth variants; however, no overall conclusions can be drawn on the basis of the present findings.

The genetic findings substantiate the diploid nature of the coccoidal cells. Difficulty would be encountered in explaining the genetic results on the basis of a haploid state since uninucleated coccoidal cells could not be considered as heterozygous.

# CHAPTER IV

## CONCLUSIONS

It has been demonstrated in this investigation that the cytogenetic approach to the study of microorganisms, using suitable cytological technics, radiobiological methods and genetic studies, is valid. It is proposed that these procedures are applicable to microorganisms in general if properly controlled and interpreted with some caution. The radiobiological findings were consistent with the recessive lethal theory of radiation damage. It is proposed that the ploidy of cytologically demonstrated unicellular, uninuclear, microorganisms can be determined by the use of appropriate radiobiological procedures.

A life cycle in which diploid coccoidal cells and coenocytic hyphae alternate with haploid bacillary cells was demonstrated in N. corallina. The diploid nature of the coccoidal cells was confirmed by a study of color and morphological mutants and by radiobiological studies.

Sexual recombination was demonstrated between morphological and color mutants which confirmed the cytological evidence for a sexual process in this organism.

During the course of the investigation various side problems arose which were given only preliminary study since their solution was not considered an essential part of this study.

# CHAPTER V

## SUMMARY

Cytogenetic procedures, applicable to microbiology, were selected and tested on a suitable organism as a basis for the valid application of these procedures to other microorganisms. Nocardia corallina was chosen as a test organism on the basis of preliminary cytological studies.

The crystal violet nuclear stain, the thionin-$SO_2$ nuclear stain, the crystal violet-tannic acid-congo red cell wall stain, phase microscopy, and electronmicroscopy were found to be valid tools of microbial cytology if interpreted with restraint.

Properly controlled X-radiation and ultraviolet irradiation technics were shown to be valid as aids to ploidy and genetic investigations. Evidence from successive irradiation of the diploid coccoidal cells strongly supported the unpaired defect theory of Tobias and the recessive lethal theory of radiation damage.

The correlation of cytological and radiobiological findings demonstrated that, in N. corallina, a diploid coccoidal stage gives rise to a coenocytic diploid hyphal stage which fragments through a nuclear reduction division to form haploid dinucleated bacillary cells. The bacillary cell nuclei fuse and the cell divides to form diploid coccoids.

The haploid "chromosome" number for this organism is suggested as three.

Environmental effects, nutrition requirements, colonial growth and morphology and the chemistry of the pigments were studied in N. corallina to aid in various parts of the overall investigation.

Study of morphological and color mutants confirmed the diploid nature of the coccoidal cells. Recombination between appropriate mutant strains substantiated the cytological evidence for a sexual process in this organism. Genetic evidence was obtained for the existence of two mating types in the mutant strains studied.

It has been demonstrated that a microbial cytogenetic approach involving the correlation and integration of cytological procedures with genetic and radiobiological methods can aid in solving basic problems of microbial cytology and genetics.

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